Survivin in breast cancer–derived exosomes activates fibroblasts by up-regulating SOD1, whose feedback promotes cancer proliferation and metastasis

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Cancer-associated fibroblasts (CAFs) play a critical role in the coevolution of breast tumor cells and their microenvironment by modifying cellular compartments and regulating cancer cell functions via stromal-epithelial dialogue. However, the relationship and interaction between stromal and epithelial cells is still poorly understood. Herein, we revealed that breast cancer cells have a stronger ability to activate fibroblasts and transform them into myofibroblasts (CAF-like) than normal breast epithelial cells, and this stronger ability occurs through paracrine signaling. In turn, myofibroblasts promote the proliferation, epithelial-to-mesenchymal transition (EMT), and stemness of breast cancer cells. Detailed regulatory mechanisms showed that, compared with normal cells, Survivin is overexpressed in breast cancer cells and secreted extracellularly in the form of exosomes, which are then internalized by fibroblasts. Breast cancer cell–derived survivin up-regulates SOD1 expression in fibroblasts and then converts them into myofibroblasts, conversely inducing breast cancer progression in vitro and in vivo. Thus, our results indicate that survivin acts as an activator of the tumor microenvironment and that SOD1 up-regulation in fibroblasts can promote breast cancer progression. These results suggest that targeting survivin and SOD1 may be a potential therapeutic strategy for breast cancer.

Breast cancer is a serious threat to the health and lives of women worldwide because its global incidence and mortality rate are 11.6 and 6.6%, respectively, according to 2018 cancer statistics (1). Although many strategies have been developed for breast cancer treatment, patients still suffer from a low 5-year survival rate, which is caused mainly by recurrence and metastasis. Recently, accumulating evidence has suggested that the tumor microenvironment (TME) plays a crucial role in the initiation, treatment, mobility, and relapse of breast cancer (2). The TME is a complex and dynamically changing group of cells created during tumor progression that is composed mainly of stromal cell components, such as fibroblasts, macrophages, adipocytes, endothelial cells, inflammatory cells, and extracellular matrix (3, 4). Previous reports indicate that it is derived from paracrine signaling, mediated mainly by extracellular cytokines and exosomes, which contribute to the communication between various components of the TME and maintain the malignant states of breast cancer cells (5–7). Specifically, the paracrine signaling of stromal cells is important in cancer progression because it regulates cancer cell proliferation, epithelial-to-mesenchymal transition (EMT), stemness and chemoresistance, which result in the synergistic effect of cancer cells and their microenvironment (8).

According to the special anatomical structure of the human mammary gland, cancer-associated fibroblasts (CAFs) are the most abundant components in the breast tumor microenvironment and are pivotal to the proliferation, invasion, angiogenesis, and drug resistance of breast cancer cells (9–11). CAFs, prepared directly from invasive human mammary carcinomas, contain substantial numbers of myofibroblasts that acquire stronger capacities for growth, migration, and extracellular matrix modification and can secrete more tumor-promoting cytokines, such as MMP-2, TGF-β, SDF-1, and IL-6 (12). Recent studies have revealed that some gene mutations in fibroblasts regulate their transformation into CAFs in the breast tumor microenvironment. For example, p62 in fibroblasts promotes tumorigenesis by regulating its own metabolism (13). TIMP gene family deficiencies are sufficient for fibroblast activation (14). Recently, we also revealed that the aberrant low expression of p85α in fibroblasts facilitates breast cancer cell metastasis by paracrine WNT10B (15).

Genetic mutations or abnormal expression of specific genes are still considered to be the fundamental causes that alter multiple cellular regulatory pathways and drive breast cancer initiation and progression, although the underlying molecular model of these cancer boosters remains elusive; up to 25% of breast cancer cases are due to one of the few identified rare but highly penetrant genes. Survivin is a member of the inhibitor of apoptosis (IAP) family, also known as BIRC5, or baculoviral inhibitor of apoptosis repeat-containing 5, which is highly expressed in breast cancer cells. It is widely considered a potential marker of cancer and a target for developing anticancer drugs (16–18). An increasing number of studies have demonstrated that survivin promotes the progression of cancer by participating in multiple cellular regulatory pathways and drive breast cancer initiation and progression, although the underlying molecular model of these cancer boosters remains elusive; up to 25% of breast cancer cases are due to one of the few identified rare but highly penetrant genes. Survivin is a member of the inhibitor of apoptosis (IAP) family, also known as BIRC5, or baculoviral inhibitor of apoptosis repeat-containing 5, which is highly expressed in breast cancer cells. It is widely considered a potential marker of cancer and a target for developing anticancer drugs (16–18). An increasing number of studies have demonstrated that survivin promotes the progression of cancer by participating in cell division, angiogenesis, and drug resistance (19–21). However, the effects and functional roles of extracellular survivin in the breast tumor microenvironment remain poorly understood.

In this study, we demonstrate that the exosome-mediated paracrine signaling of survivin in breast cancer cells promotes fibroblast activation into myofibroblasts by up-regulating SOD1, and this feedback promotes the proliferation, metastasis, and stemness of breast cancer. Specifically, compared with normal breast epithelial cells, breast cancer cells express more...
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Survivin, which can be released extracellularly through exosomes. The activation of fibroblasts is mediated by SOD1 up-regulation after the internalization of breast cancer–derived exosomal survivin. Furthermore, activated fibroblasts promote the proliferation, migration, EMT, and stemness of breast cancer cells in vitro and in vivo. The results of this study demonstrate the considerable role of breast cancer–derived survivin in activating fibroblasts and reveal combined survivin and SOD1 targeting as a new strategy for the treatment of breast cancer.
Results

Breast cancer cells promote the conversion of fibroblasts to myofibroblasts (CAF-like)

High ACTA2 and vimentin expression is the most distinguishable feature of myofibroblasts (22). Based on an analysis of microarrays from the Oncomine and GEO databases, we found that ACTA2 and vimentin expression was significantly increased in breast tumor stroma compared with normal breast stroma (Fig. 1A). Specifically, ACTA2 and vimentin expression levels were higher in breast tumor fibroblasts than in normal fibroblasts, which suggests that a large number of fibroblasts are converted into myofibroblasts in breast cancer tissue (Fig. 1B). To investigate whether breast cancer cells affect fibroblasts during this transition, we treated human normal fibroblast HFF-1 cells with conditioned medium (CM) collected from the normal breast cell line HBL-100 and the breast cancer cell lines BT-549 and MDA-MB-231. Compared with that from normal breast cells, CM from malignant transformed breast cancer cells significantly increased the ACTA2 and vimentin levels (Fig. 1C and Fig. S1A), which indicated that breast cancer cells have the potential to promote fibroblast conversion into myofibroblasts. Other characteristics of myofibroblasts included enhanced capabilities of contractility, proliferation, and motility. To further confirm our observation, collagen gel contraction assays indicated that CM from the breast cancer cell lines BT-549 and MDA-MB-231 exhibited an increased ability to induce HFF-1 fibroblast gel contraction (Fig. 1D). Moreover, we found that CM from BT-549 and MDA-MB-231 cells significantly promoted HFF-1 colony formation and proliferation (Fig. 1E and F) as well as enhanced cell migration (Fig. 1G and Fig. S1B). In addition, the results showed that HFF-1 cells produced and secreted higher levels of cytokines after treatment with CM from BT-549 or MDA-MB-231 than after treatment with CM from HBL-100 cells (Fig. 1H), which means that HFF-1 fibroblasts are greatly activated by treatment with CM of breast cancer. To confirm the results in the cell line, we isolated breast fibroblasts from eight breast cancer patients, including one hyperplasia, two TNM stage I, four TNM stage II, and one TNM stage III, and performed a series of in vitro experiments. Interestingly, breast fibroblasts from the TNM stage II and III patients exhibited more properties of myofibroblasts than those from the hyperplasia and TNM stage I patients (Fig. 1J and F) and Fig. S1 (C–E)). Collectively, these data indicate that breast cancer cells promote stromal fibroblast activation and transformation into myofibroblasts.

The feedback of activated fibroblasts promotes breast cancer progression

Because the most critical action of myofibroblasts is promoting cancer development, to investigate the role of activated fibroblasts in breast cancer progression, we treated MDA-MB-231 and MCF7 cells with CM from breast cancer–educated HFF-1 cells (Fig. S2A). As shown in Fig. 2 (A and B), HFF-1 cells educated by cancer cells increased the proliferation and colony formation of MDA-MB-231 and MCF7 cells compared with those educated by normal breast HBL-100 cells. Similarly, HFF-1 cells educated by cancer cells exhibited stronger induction of cancer cell migration than those educated by normal cells (Fig. 2 (C and D) and Fig. S2B). EMT, which enables stationary epithelial cells to gain enhanced motility and invasiveness, is known to play an important role in breast cancer progression and drug resistance. As shown in Fig. 2E and Fig. S2C, the mesenchymal markers vimentin and Snail were up-regulated, and the epithelial marker E-cadherin was down-regulated when MDA-MB-231 and MCF7 cells were treated with breast cancer–educated HFF-1 CM compared with HBL-100–educated HFF-1 CM. Moreover, flow cytometry analysis of CD44<sup>high</sup>/CD24<sup>low</sup> indicated that compared with HBL-100–educated HFF-1 cells, BT-549– or MDA-MB-231–educated HFF-1 cells also promoted the stemness of MDA-MB-231 and MCF7 cells (Fig. 2F). Further spheroid formation cell assays also confirmed this result (Fig. 2G). Therefore, we concluded that compared with normal breast cells, breast cancer cells contribute to promoting fibroblast activation and in turn facilitate their own progression.

Breast cancer–secreted exosomes contribute to fibroblast activation

Exosome secretion has been recently implicated as an important method of cell communication in the TME (23, 24) and rationally leads to the question of whether breast cancer cell–derived exosomes are involved in fibroblast activation. To investigate this hypothesis, we separated the exosomes from the CM of normal breast cells or breast cancer cells and used them to treat HFF-1 cells. EM observations indicated that there were more exosome–like structures located close to the cell membrane in HBL-100 cells than in BT-549 and MDA-MB-231 cells (Fig. 3A). Exosomes isolated from CM

Figure 1. Breast cancer cells convert fibroblasts into myofibroblasts (CAF-like). A, ACTA2 and vimentin expression levels in normal and breast cancer stroma from the Oncomine (Finak Breast and Karmoub Breast) and GEO databases (GSE83591). B, ACTA2 expression levels in normal and breast cancer fibroblasts from the GEO database (GSE20086). C, Western blotting analysis of ACTA2 and vimentin protein levels in HFF-1 cells treated with the indicated conditioned medium or blank control for 48 h. GAPDH was used as a loading control. D, collagen contraction assay of the blank control and indicated CM–treated HFF-1 cells. The area change of the gels was recorded at 0, 24, 48, 72, and 96 h. Representative images of three replicates of each group at 96 h are shown. Scale bar, 1 cm. E, plate colony formation assay of HFF-1 cells treated with the indicated CM or blank control for 5 days. Scale bar, 1 cm. F, the proliferation of HFF-1 cells treated with the indicated CM was assessed by counting the number of cells. G, transwell assays of HFF-1 cells treated with the indicated CM or blank control for 20 h. Representative images are shown, and the migrated cells were counted. Scale bar, 200 μm. H, quantitative PCR analysis of TGF–β1, MMP2, SDF-1, and IL-6 expression in HFF-1 cells treated with the indicated CM or blank control for 48 h. I, Western blotting analysis of ACTA2 and vimentin protein levels in fibroblasts from breast cancer patient samples. Hyperplasia, patients with hyperplasia of the breast; I, patients with TNM stage I breast cancer; II, patients with TNM stage II breast cancer; III, patients with TNM stage III breast cancer. GAPDH was used as a loading control. J, collagen contraction assay of fibroblasts from breast cancer patient samples. The area change of the gels was recorded at 0, 12, 24, and 36 h. Representative images of three replicates of each group at 36 h are shown. Scale bar, 1 cm. The experiments were performed at least in triplicate, and the results are presented as the mean ± S.D. (error bars). The data were analyzed by Student’s t test (*, p < 0.05; **, p < 0.01; ***, p < 0.001).
Figure 2. The feedback of activated fibroblasts promotes breast cancer progression. A, the proliferation of MDA-MB-231 and MCF7 cells treated with the indicated CM was assessed by counting the number of cells. B, plate colony formation assay of MDA-MB-231 and MCF7 cells treated with the indicated CM for 7 days. Scale bar, 1 cm. C, scratch assays of MDA-MB-231 and MCF7 cells treated with the indicated CM for 20 and 36 h, respectively. Statistical data are shown. D, transwell assays of MDA-MB-231 and MCF7 cells treated with the indicated CM for 20 and 36 h, respectively. Representative images are shown, and the migrated cells were counted. Scale bar, 200 μm. E, Western blotting analysis of EMT marker protein levels in MDA-MB-231 and MCF7 cells treated with the indicated CM for 60 h. GAPDH was used as a loading control. F, after the indicated CM treatment for 60 h, flow cytometry analysis of MDA-MB-231 and MCF7 cells stained with antibodies against CD44 and CD24 was performed. G, spheroid formation assay of MDA-MB-231 and MCF7 cells treated with the indicated CM for 5 days. Representative images are shown, and the spheroids were counted. Scale bar, 500 μm. The experiments were performed at least in triplicate, and the results are presented as the mean ± S.D. (error bars). The data were analyzed by Student’s t test (*, p < 0.05; **, p < 0.01; ***, p < 0.001).
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(A) HBL-100, BT-549, and MDA-MB-231

(B) Exosome

(C) NTA Particle Count

(D) Whole-cell lysates and Exosome

(E) HFF-1

(F) HBL-100 exosome, BT-549 exosome, MDA-MB-231 exosome

(G) ACTA2, Vimentin, GAPDH

(H) BLK, HBL-100, BT-549, MDA-MB-231

(I) Relative width of scratch

(J) Relative migration rate
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by the classical isolation method of ultracentrifugation exhibited typical cup-shaped structures and corresponding particle sizes (Fig. 3, B and C). In addition, the detection of the exosome marker TSG101, CD9, and the Golgi membrane protein GM130 further verified that we successfully isolated exosomes without cellular contamination (Fig. 3D). Quantitative analysis results showed that the total levels of secreted exosomes were significantly higher in HBL-100 cells than in BT-549 and MDA-MB-231 cells (Fig. 3E), which is consistent with previous observations (Fig. 3A). To identify exosome internalization by HFF-1 cells, we labeled exosomes with the lipophilic dye DiD and incubated them with HFF-1 cells for 24 h. The fluorescence microscopy results showed the presence of DiD spots in living recipient fibroblasts, which indicated that the exosomes collected from various breast epithelial cells were transferred into living fibroblasts via active transport (Fig. 3F). Next, to evaluate whether the exosomes derived from breast cancer cells can convert fibroblasts to myofibroblasts, we treated HFF-1 cells with different isolated exosomes. As shown in Fig. 3G and Fig. S2D, the levels of ACTA2 and vimentin in HFF-1 cells were remarkably increased by BT-549- or MDA-MB-231-secreted exosomes. Furthermore, the contraction and migration abilities of HFF-1 cells were markedly enhanced after treatment with BT-549- or MDA-MB-231-derived exosomes compared with HBL-100-derived exosomes (Fig. 3 (H–J) and Fig. S2E). These results demonstrate that exosomes from breast cancer contribute to fibroblast activation.

Inhibition of breast cancer–derived exosomes attenuates the activation of fibroblasts

To further confirm the effect of breast cancer cell–derived exosomes in the activation of fibroblasts, the exosome secretion of BT-549 and MDA-MB-231 was inhibited by treatment with GW4869 or knockdown of RAB27A (Fig. 4 (A and B) and Fig. S2F). Then conditioned medium was collected to treat HFF-1 cells. As anticipated, the decrease of exosomes in conditioned medium eliminated the up-regulation of ACTA2 and vimentin expression (Fig. 4C and Fig. S2G). Moreover, the collagen contraction ability of HFF-1 was attenuated with the reduction of exosomes (Fig. 4D). In addition, scratch and transwell assays were performed to prove that exosome-reduced conditioned medium loses the ability to promote migration of fibroblasts compared with the control group (Fig. 4 (E and F) and Fig. S3A). Altogether, the above results demonstrate that breast cancer–derived exosomes convert fibroblasts into myofibroblasts.

SOD1 is involved in breast cancer–mediated fibroblast activation

A recent discovery revealed that reactive oxygen species (ROS) are a critical regulator of myofibroblast function (13). Thus, we investigated the intracellular ROS levels of HFF-1 cells after treatment with breast cancer cell–derived exosomes to assess the role of ROS in breast fibroblast activation. As shown in Fig. 5A, exosomes from BT-549 or MDA-MB-231 cells decreased intracellular ROS levels in HFF-1 cells more than exosomes from HBL-100 cells. Moreover, after intracellular ROS were eliminated by NAC, ACTA2 and vimentin expression and migration ability in HFF-1 cells were increased significantly (Fig. 5B and Fig. S3 (B and C)). Furthermore, we examined the expression of several genes that participate in classic oxidative stress after treating HFF-1 cells with the indicated exosomes. The mRNA and protein levels of SOD1 were increased significantly upon breast cancer–derived exosome treatment (Fig. 5 (C and D) and Fig. S3D). In addition, breast fibroblasts from TNM stage II and III patients expressed more SOD1 (Fig. S3E), and analysis of microarrays from GEO databases indicated that SOD1 expression was significantly increased in CAFs compared with their breast counterpart fibroblasts (Fig. S3F). To further explore the role of SOD1 in fibroblast activation, we knocked down SOD1 in HFF-1 cells (Fig. S3G), and SOD1 knockdown did not change the uptake of exosomes (Fig. S3H). Compared with control conditions, SOD1 knockdown prevented the activation of HFF-1 cells by breast cancer exosomes (Fig. 5 (E and F) and Fig. S4 (A and B)). However, SOD1 overexpression increased ACTA2 and vimentin protein expression and enhanced contraction and migration abilities in HFF-1 cells (Fig. 5 (G and H) and Fig. S4 (C–E)), which indicates that SOD1 activated fibroblasts to become myofibroblasts.

We next examined the role of SOD1 in HFF-1 cells in regulating the progression of breast cancer cells. The numbers of MDA-MB-231 cells and MCF7 cells were significantly increased by treatment with CM from SOD1-overexpressing HFF-1 cells compared with treatment with CM from control HFF-1 cells (Fig. 5I). Moreover, scratch and transwell migration assays indicated that the migration ability of MDA-MB-231 and MCF7 cells was remarkably improved by...
Figure 4. Inhibition of breast cancer–derived exosomes attenuates the activation of fibroblasts. A, NTA analysis on the average concentration of exosomes in CM from BT-549 and MDA-MB-231 cells after treatment with GW4869 (10 μM) or DMSO for 2 days. B, left, Western blotting analysis of RAB27A protein in shRAB27A-expressing BT-549 and MDA-MB-231 cells and control cells. GAPDH was used as a loading control. Right, NTA analysis on the average concentration of exosomes in CM from shRAB27A-expressing BT-549 and MDA-MB-231 cells. C, Western blotting analysis of the ACTA2 and vimentin protein levels in HFF-1 cells treated with the indicated CM for 48 h. GAPDH was used as a loading control. D, collagen contraction assay of HFF-1 treated with the indicated CM. The area change of the gels was recorded at 0, 24, 48, 72, and 96 h. Representative images of three replicates of each group at 96 h are shown. Scale bar, 1 cm. E, scratch assays of HFF-1 cells treated with the indicated CM for 20 h. Statistical data are shown. F, transwell assays of HFF-1 cells treated with the indicated CM for 20 h. Representative images are shown, and the migrated cells were counted. Scale bar, 200 μm. The experiments were performed at least in triplicate, and the results are presented as the mean ± S.D. (error bars). The data were analyzed by Student’s t test (*, p < 0.05; **, p < 0.01; ***, p < 0.001).
SOD1-overexpressing HFF-1 cells (Fig. 5J and Fig. S4F). In addition, SOD1-overexpressing HFF-1 cells promoted the EMT and stemness of breast cancer cells (Fig. 5 (K–M) and Fig. S5A). These results collectively suggest that breast cancer cells convert fibroblasts to myofibroblasts by increasing the SOD1 expression level in fibroblasts.
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Survivin in breast cancer–derived exosomes promotes conversion to myofibroblasts by up-regulating SOD1 in fibroblasts

Next, we sought to determine how SOD1 in fibroblasts was up-regulated by exosomes from breast cancer cells. After analyzing the gene expression profiles of breast tumor epithelium from GSE83591 using bioinformatics tools, we identified 48 significantly up-regulated genes in breast tumor epithelium compared with normal breast epithelium (Fig. S5B) and constructed a PPI network to screen the hubs of these 48 genes. After ordering these genes by PPI degree, the core gene survivin, which was reported to be released from cancer cells via exosomes and involved in ROS regulation (25, 26), was screened out (Fig. 6A). In addition, RNA-Seq data from TCGA indicated that there is a linear correlation between the mRNA levels of survivin and SOD1 in human breast cancer tissue, which is composed of tumor epithelial cells and stroma (Fig. 6B). Further Western blotting results and GEO database analysis also showed that breast cancer cells expressed higher survivin levels than normal breast cells and fibroblasts (Fig. 6C and Fig. S5 (C and D)). Interestingly, exosomes of BT-549 and MDA-MB-231 also contain more survivin than that of HBL-100 cells (Fig. 6D and Fig. S5E). Moreover, breast cancer cell–derived survivin can be transported into fibroblasts through exosomes (Fig. 6E). To determine the function of breast cancer–derived survivin in fibroblast activation, we knocked down survivin expression with shRNA in BT-549 and MDA-MB-231 cells (Fig. S5F). Survivin knockdown did not change the quantity of exosomes secreted by BT-549 or MDA-MB-231 cells (Fig. S5G). As shown in Fig. 6F and Fig. S5H, upon survivin knockdown, CM of MDA-MB-231 and BT-549 cells lost the ability to increase the expression levels of SOD1, ACTA2, and vimentin in HFF-1 cells. Similar results were obtained in gel contraction and migration experiments (Fig. 6 (G–I) and Fig. S5I). These results suggest that survivin in breast cancer cells up-regulates SOD1 expression in fibroblasts, thereby mediating fibroblast activation.

SOD1-overexpressing fibroblasts facilitate the malignant proliferation and metastasis of breast cancer cells in vivo

First, to confirm the role of exosome and survivin on fibroblasts in vivo, we injected BT-549 Ctrl, BT-549 shRAB27A, and BT-549 shSurvivin cells subcutaneously into the fourth right mammary fat pad of BALB/c-NU female mice and then isolated fibroblasts from this mammary to identify characteristics of myofibroblasts. As shown in Fig. 7 (A and B) and Fig. S6 (A–C), after reducing the secretion of exosomes and the expression of survivin, BT-549 cells lose the ability to convert mouse breast fibroblasts into myofibroblasts. These results also demonstrated that the expression level of SOD1 in mouse mammary fibroblasts is regulated by survivin in the exosomes of breast cancer cells.

To follow up on our findings that SOD1 levels in fibroblasts were increased by breast cancer cells, we investigated SOD1 expression in the stroma of different breast cancer patient samples by immunohistochemical analysis and scored the staining intensity. As shown in Fig. 7 (C and D), strong SOD1 staining was more frequently present in TNM stage II, III, and IV than in TNM stage I. We next aimed to further verify that SOD1 overexpression is important for myofibroblast function in vivo, which includes promoting tumor growth. We mixed SOD1-overexpressing HFF-1 cells or control cells with a poorly aggressive, noninvasive breast cancer cell line, MCF7 cells, and injected them subcutaneously into female BALB/c-NU mice. HFF-1 cells and SOD1-overexpressing HFF-1 cells alone failed to form tumors in mice (data not shown). Consistent with the in vitro experiments, SOD1-overexpressing HFF-1 cells increased the volume and weight of the tumors (Fig. 7 (E and F) and Fig. S6D). To examine the roles of SOD1-overexpressing HFF-1 cells in breast cancer metastasis, luciferase-labeled MDA-MB-231 cells either alone or together with SOD1-overexpressing HFF-1 cells or control HFF-1 cells were injected intravenously into female BALB/c-NU mice through their tail vein, and their body weight was measured every 2 days (Fig. S6E). As shown in Fig. 7 (G–J) and Fig. S6F, SOD1 up-regulation in HFF-1 cells increased the ability of MDA-MB-231 cells to metastasize. Together, these results suggest that survivin in breast cancer–derived exosomes activates fibroblasts by up-regulating SOD1, whose feedback promotes cancer proliferation and metastasis in vivo (Fig. 8).

Discussion

The communication between stroma and cancer cells plays a critical role in driving tumor progression (27), but how normal
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fibroblasts are activated to become myofibroblasts remains unclear. Here, our study explains the origin of myofibroblasts in the breast tumor microenvironment. First, we analyzed the effects of normal breast epithelial cells and breast cancer epithelial cells on fibroblast activation and found that breast cancer cell–educated fibroblasts possess the characteristics of...
myofibroblasts and can significantly promote the proliferation, metastasis and stemness of breast cancer cells. The bilateral interaction between breast cancer cells and fibroblasts further illustrates the mechanism of high metastasis rates and strong drug resistance in breast cancer.

Intracellular ROS are a group of oxygen derivatives resulting from the distinct oxidation status of $O_2$, which is widely recognized as crucial for cell fate (28). In addition to directly acting on cancer cells, ROS can also affect breast cancer progression by altering the metabolism of stromal cells. For instance, a high level of ROS induces the loss of Cav-1 in stromal cells and is sufficient to generate a pseudohypoxic state that further promotes tumor growth (29). Specifically, in breast cancer, ROS promote fibroblast conversion into myofibroblasts through the accumulation of HIF-1α and the chemokine CXCL12 (30). However, here, we found that a decrease in intracellular ROS levels contributes to fibroblast activation, which further illustrates the versatility of ROS in cells. Overall, the cellular regulation of ROS by antioxidant enzymes, including SOD1, SOD2, CAT, ANTI1, and GPX1, demonstrates the importance of the antioxidant defense system in maintaining normal cellular physiology. SOD1, one of the most abundant metal-containing enzymes, has been shown to play a vital antioxidant role in human health (31).

Recent studies also demonstrated that SOD1 affects the function of fibroblasts. For example, SOD1 knockdown induces senescence in human fibroblasts (32), and attenuated SOD1 expression and subsequent ROS dysregulation leads to diminished capacity to regenerate wounds and express ACTA2 in aged fibroblasts (33). Consistent with these studies, our findings further elucidate that SOD1 overexpression converts fibroblasts into myofibroblasts.

Survivin is highly expressed in 70.7–90.2% of breast cancer patients and can be released from cancer cells via exosomes involved in ROS regulation (25, 26, 34). In addition, previous work clearly demonstrated an exosome-mediated mechanism of ROS detoxification and chemoresistance in PC cells (35). However, different from these studies, which just separately illustrated the regulatory function of survivin or exosomes in the same type of cells, we explored the function of exosomal survivin based on tumor microenvironment, including breast cancer cells and stromal fibroblasts.

An exosome is one type of transporter that mediates intercellular communication and is identified as a cup-shaped microvesicle ranging from 30 to 100 nm in diameter that is released by almost all types of cells (36). Recent studies have indicated that exosomes are involved in promoting the growth, invasiveness, drug resistance, and metastasis of cancer by transporting protein, RNA, lipids and so on (11, 37–39). Here, we found that even though breast cancer cells secrete fewer exosomes than normal breast cells (Fig. 3E), their exosomes have a more significant effect on the TME, which further indicates that breast cancer exosomes contain more active contents. In addition, several members of the RAB GTPase family, such as RAB27A, RAB27b, and RAB35, participate in vesicle transport and regulate the release of exosomes (40, 41). Hence, RAB27A knockdown in breast cancer cells failed to convert fibroblasts into myofibroblasts. The above studies demonstrate that exosomes from breast cancer cells transport the survivin protein to fibroblasts and activate SOD1, which is involved in fibroblast activation. Due to the complexity of gene regulation and signal transduction, whether exosomal survivin directly or indirectly up-regulates SOD1 needs to be further studied. In addition, although we have found that the basal survivin level of normal fibroblast is low (Fig. 6C and Fig. S5C), we are still interested in whether the exosome up-regulated SOD1 can mediate the expression of survivin in fibroblasts or breast cancer cells to form a vicious feedback loop. Different CAF populations can secrete distinct cytokine profiles in a variety of cancers (42, 43); therefore, we also need to determine the signal transduction pathways involved in the cancer-promoting function of SOD1–up-regulated myofibroblasts. Even so, based on the single anti-survivin therapeutics currently available (44, 45), our research indicated that combination therapy targeting survivin and SOD1 is a new option for cancer treatment strategies.

In conclusion, our results demonstrate that breast cancer–derived exosomal survivin converts fibroblasts into myofibroblasts by up-regulating SOD1. In addition, SOD1–up-regulated fibroblasts promote the proliferation, EMT, and stemness of breast cancer. Furthermore, up-regulation of stromal SOD1 is associated with a poor prognosis in breast cancer. Thus, the identification of SOD1–up-regulated myofibroblasts has a profound impact not only on developing novel anticancer strategies but also on using SOD1 as a promising clinical biomarker to predict cancer patient outcomes. Our study elucidates the new molecular mechanisms of communication between breast cancer cells and stromal fibroblasts and contributes to effective prevention and treatment strategies for breast cancer.

Figure 6. Survivin in breast cancer–derived exosomes promotes myofibroblast conversion by up-regulating SOD1 in fibroblasts. A, PPI network of 48 significantly up-regulated genes in breast tumor epithelium from GSE83591. B, left, heatmap of the relationship between the expression levels of survivin and SOD1 according to the RNA-Seq data from TCGA breast cancer data. Right, correlation analysis between SOD1 and survivin according to the RNA-Seq data from TCGA breast cancer data. C, Western blotting analysis of the survivin protein level in indicated cells. GAPDH was used as loading controls. D, Western blotting analysis of the survivin protein level in exosomes from HBL-100, BT-549, and MDA-MB-231 cells. TSG101 was used as loading control. E, confocal imaging showed the delivery of EGFP-labeled survivin (green) and DIL-labeled exosomes (red) to DAPI-labeled HFF-1 cells (blue). Representative images are presented. Scale bar, 15 μm. F, Western blotting analysis of SOD1, ACTA2, and vimentin protein levels in HFF-1 cells treated with the indicated CM for 48 h. GAPDH was used as a loading control. G, collagen contraction assay of HFF-1 treated with the indicated CM. The area change of the gels was recorded at 0, 24, 48, 72, and 96 h. Representative images of three replicates of each group at 96 h are shown. Scale bar, 1 cm. H, scratch assays of HFF-1 cells treated with the indicated CM for 20 h. Statistical data are shown. I, transwell assays of HFF-1 cells treated with the indicated CM for 20 h. Representative images are shown, and the migrated cells were counted. Scale bar, 200 μm. The experiments were performed at least in triplicate, and the results are presented as the mean ± S.D. (*error bars). The data were analyzed by Student’s t-test (*, p < 0.05; **, p < 0.01; ***p < 0.001).
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Experimental procedures

Cell lines and cell culture

The human normal breast epithelial cell line HBL-100, the human foreskin fibroblast cell line HFF-1, and the HEK-293T cell line were purchased from the China Center for Type Culture Collection (Wuhan, China). The human breast cancer cell lines MDA-MB-231 and MCF-7 were purchased from the ATCC (Manassas, VA, USA). The BT-549 cell line was a kind gift from Professor Cheguo Cai of Wuhan University. The method of cell culture is shown in the supporting Experimental procedures.
**Conditioned medium preparation**
Various cell lines were cultured with complete medium for 2 days until the cell numbers were the same. Then the supernatants were collected and centrifuged at 4 °C and 300 × g for 10 min to remove the cells, at 2000 × g for 10 min to remove the dead cells, and at 10,000 × g for 30 min to remove the cell debris. Next, the samples were filtered through a 0.22-μm filter and mixed with complete medium at a 1:1 ratio.

**Isolation of fibroblasts from patient breast cancer samples**
Isolation of fibroblasts from patient breast cancer sample was performed as described before (46). Informed consent was obtained from all of the examined subjects, and the related studies were approved by the ethics committees of the participating hospitals and institute.

**Isolation and analysis of exosomes**
Exosome isolation and EM observations were performed according to the guidelines provided by MISEV2018 (47). Briefly, an equal number of cells were cultured in exosome-depleted complete medium for 2 days, and exosomes were isolated from an equal volume of supernatant. After the same centrifugation and filter steps detailed in the CM preparation method, the supernatant was harvested and centrifuged at 120,000 × g for 90 min to collect the exosome pellet. Then, the exosome pellet was washed with PBS to remove any contaminating proteins and centrifuged again at 120,000 × g for 90 min. For cell treatment, the exosome pellets were resuspended in exosome-free complete medium.

**Figure 7. SOD1-overexpressing fibroblasts facilitate the malignant proliferation and metastasis of breast cancer cells in vivo.**
A, Western blotting analysis of SOD1, ACTA2, and vimentin protein levels in fibroblasts isolated from a mouse xenograft model of BT-549 Ctrl cells, BT-549 shRAB27A cells, and BT-549 shSurvivin cells. BLK, WT mice. n = 3 mice/group. 1#, 2#, and 3#, an independent mouse of each group. GAPDH was used as a loading control. B, collagen contraction assay of fibroblasts isolated from mouse xenograft model of BT-549 Ctrl cells, BT-549 shRAB27A cells, and BT-549 shSurvivin cells. BLK, WT mice. n = 3 mice/group. 1#, 2#, and 3#, an independent mouse of each group. The area change of the gels was recorded at 0, 12, 24, and 36 h. Representative images of three replicates of each group at 36 h are shown. Scale bar, 1 cm. C, representative sections of tissue from different stage breast cancer patients immunohistochemically (IHC) stained with SOD1. Scale bar, 850 μm (top) and 100 μm (bottom). D, scatterplots show the score of SOD1 staining intensity in the stroma among different stages of breast cancer. E, mean tumor volume measured every other day. F, scatterplots of the individual weights of tumors from MCF7-bearing mice (n = 6). G, bioluminescence imaging was used to observe metastatic colonization at 25 days after intravenous injection of the three groups; representative images are shown. H, representative hematoxylin and eosin–stained sections of lungs from mice with MDA-MB-231 intravenous injection. Metastases are indicated by the arrows. Scale bar, 500 μm. I, quantification of metastatic colonization in the lungs. The experiments were performed at least in triplicate, and the results are presented as the mean ± S.D. (error bars). The data were analyzed by Student’s t test (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

**Figure 8. A proposed working model.**
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h at room temperature and stained with uranyl acetate (GZ02625, EMCN, Beijing, China). Then the cells were dehydrated with an ethanol gradient and embedded in epoxy resin. Finally, the cells were sliced and observed with a transmission electron microscope (JEM-1400plus, JEOL, Japan). For nanoparticle analysis of exosomes, exosomes isolated from equal volumes of CM were resuspended in 0.22 μm-filtered PBS and measured by NanoSight (NS-300, Malvern, UK).

The internalization of exosomes was performed by incubating HFF-1 cells with DiD (V-22887, Invitrogen)-labeled exosomes. As a control, another group of HFF-1 cells was fixed in 4% paraformaldehyde (80096618, Sinoreagent) before incubation. After 24 h, HFF-1 cells were stained with DAPI (D9542, Sigma), and images were taken by confocal microscopy (SP8, Leica, Wetzlar, Germany). After incubating HFF-1 cells with DiD dye-labeled exosomes for 24 h, cells were collected for flow cytometry for internalization of exosomes.

Exosomal survivin distribution assay

DiD-labeled BT-549 cells, which were cultured in the 0.4-μm Transwell chamber were transiently transfected with survivin-EGFP–expressing plasmid, and the medium was replaced after 6 h to remove free plasmid, and then cells were co-cultured with HFF-1 cells, which were seeded on coverslips in a 12-well plate for 24 h. After co-culture, HFF-1 cells were stained with DAPI (D9542, Sigma). The distribution of exosomal survivin in HFF-1 cells was determined by confocal microscopy (SP8, Leica).

Bioinformatics analysis

RNA-Seq data were extracted from the Oncomine database (Karnoub Breast and Finak Breast) and GEO database (GSE83591, GSE20086, and GSE29270) based on the following strategies: containing normal breast stromal or fibroblasts collected from breast cancer patients who have never undergone any radiotherapy, chemotherapy, or targeted therapies. For the indicated genes, a scatter plot was generated by GraphPad Prism 6. A heatmap and volcano plot showing the differential expression between breast cancer epithelium and normal breast epithelium (GSE83591) were generated by the “ggplot2” R package consistent with a cut-off value of [log2FC] ≥ 1 and a false discovery rate of <0.01. The top 48 up-regulated genes with the most significant differential expression were selected and analyzed by STRING (https://string-db.org/), and the PPI network was drawn by Cytoscape to sequence these genes by PPI degree and screen for the hub gene. A heatmap that shows the relationship between survivin and SOD1 in breast cancer was generated by the online exploration tool XENA (https://xena.ucsc.edu/) based on TCGA (BRCA) data (https://cancergenome.nih.gov/).

Animal experiments

All of the mouse studies were approved by the Animal Care and Use Committee of the Laboratory Animal Center of Wuhan University and conducted in accordance with the guidelines established by the National Academy of Sciences.

For isolation of fibroblasts from mice, 1 × 10⁶ BT-549 shRAB27A cells, BT-549 shSurvivin cells, or control BT-549 cells were injected subcutaneously into the mammary fat pads of female BALB/c-NU mice. Each group had three mice. Mice were sacrificed when tumor burden reached the size limit, and then mammary glands and fibroblasts were dissected from them as described (46).

For xenograft assays, 3 × 10⁶ SOD1-overexpressing HFF-1 cells or control HFF-1 cells were mixed with 1 × 10⁶ MCF7 cells and suspended in 200 μl of Matrigel (356230, Biocoat)/PBS mixture. These cell mixtures or MCF7 cells alone were injected subcutaneously into the mammary fat pads of female BALB/c-NU mice. Each group had six mice. Once tumors became palpable, the tumor volumes were measured every other day and calculated by the formula, V = 0.5 × length × width². 32 days later, the mice were euthanized, and their tumors were isolated by dissection.

For metastasis models, 1 × 10⁶ luciferase-labeled MDA-MB-231 cells alone or mixed with 3 × 10⁶ SOD1-overexpressing HFF-1 cells or control HFF-1 cells were suspended in 200 μl of PBS and injected into the tail veins of female BALB/c-NU mice. Each group had five mice. 25 days after injection, a bioluminescent imaging system (Lumina II, Caliper Life Science, Hopkinton, MA, USA) and luciferin (40902ES01, Yeasen) were used to detect cancer cell metastasis.

Statistical analysis

All data were repeated in at least three independent experiments. Data analysis was performed using Microsoft Excel and GraphPad Prism 7. All quantitative data are presented as the mean ± S.D. Quantitative significance between two independent groups was determined by Student’s t test, unless otherwise stated. p < 0.05 was considered statistically significant.

Data availability

All data are contained within the article.


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Abbreviations—The abbreviations used are: TME, tumor microenvironment; EMT, epithelial-to-mesenchymal transition; CAF, cancer-associated fibroblast; CM, conditioned medium; ROS, reactive oxygen species; PPI, protein-protein interaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NTA, nanoparticle
tracking analysis; DAPI, 4',6-diamidino-2-phenylindole; EGFP, enhanced GFP.

References

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