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ORIGINAL ARTICLE

MED27 plays a tumor-promoting role in breast cancer progression by targeting KLF4

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Abstract

The mediator complex usually cooperates with transcription factors to be involved in RNA polymerase II-mediated gene transcription. As one component of this complex, MED27 has been reported in our previous studies to promote thyroid cancer and melanoma progression. However, the precise function of MED27 in breast cancer development remains poorly understood. Here, we found that MED27 was more highly expressed in breast cancer samples than in normal tissues, especially in triple-negative breast cancer, and its expression level was elevated with the increase in pathological stage. MED27 knockdown in triple-negative breast cancer cells inhibited cancer cell metastasis and stemness maintenance, which was accompanied by downregulation of the expression of EMT- and stem traits-associated proteins, and vice versa in non-triple-negative breast cancer. Furthermore, MED27 knockdown sensitized breast cancer cells to epirubicin treatment by inducing cellular apoptosis and reducing tumorsphere-forming ability. Based on RNA-seq, we identified KLF4 as the possible downstream target of MED27. KLF4 overexpression reversed the MED27 silencing-mediated arrest of cellular metastasis and stemness maintenance capacity in breast cancer *in vitro* and *in vivo*. Mechanistically, MED27 transcriptionally regulated KLF4 by binding to its promoter region at positions −156 to +177. Collectively, our study not only demonstrated the tumor-promoting role of MED27 in breast cancer progression by transcriptionally targeting KLF4, but also suggested the possibility of developing the MED27/KLF4 signaling axis as a potential therapeutic target in breast cancer.

KEYWORDS
breast cancer, KLF4, MED27, metastasis, stemness

Abbreviations: CD44, CD44 antigen; CSC, cancer stem cell; EMT, epithelial–mesenchymal transition; EPI, epirubicin; HER2, Erb-B2 receptor tyrosine kinase 2; KLF4, KLF transcription factor 4; MED1, mediator complex subunit 1; MED27, mediator complex subunit 27; MMP-9, matrix metallopeptidase 9; RNA-seq, RNA-sequencing; SOX-2, SRY-box transcription factor 2; SP1, Sp1 transcription factor; TNBC, triple-negative breast cancer.

Ruozhu Wang, Wendan Yu, Tianhua Zhu, and Fei Lin contributed equally to this article.

1 | INTRODUCTION

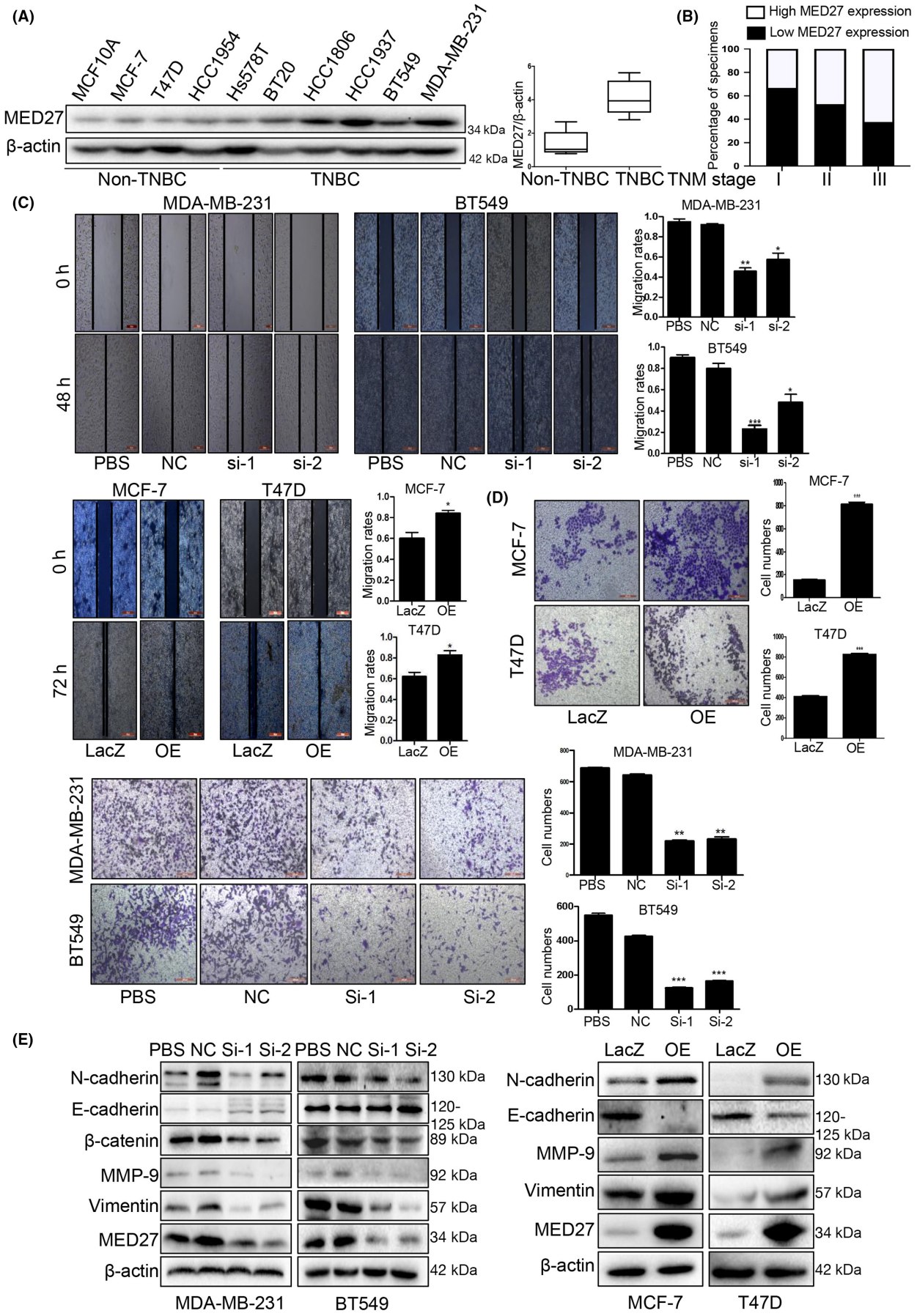
Breast cancer, and not lung cancer, has now become the leading cause of cancer incidence worldwide for women, based on the latest global burden of cancer statistical data released by the International Agency for Research on Cancer in 2020. New cases of breast cancer even reached 2.28 billion globally in 2020, which means that 11% of new patients with cancer had breast cancer. Moreover, ~20%–30% of patients when diagnosed with breast cancer[1] had developed remote metastasis and the survival rate of these patients was only 26%, despite various strategies including surgical resection, radiotherapy, chemotherapy, and hormone and molecular targeted therapies.[2–4] Based on gene-expression profiling, five main intrinsic or molecular subtypes of breast cancer (luminal A, luminal B, triple-negative/basal-like, HER2-enriched, and normal-like[5]) have been identified and studied intensely. Luminal A, Luminal B, Triple-negative/basal-like, HER2-enriched, and Normal-like,[5] among which triple-negative breast cancer is considered to be more aggressive, tends to be a higher grade, and has a poorer prognosis compared with other types. As this type of cancer tests negative for both hormone receptors and excess HER2, it is unlikely to respond to the commonly used target therapies such as hormonal therapy or agents targeting HER2. Hence, finding new key genes involved in breast cancer progression and elucidating the underlying molecular mechanisms of these genes seem necessary for the creation of new advanced therapeutic strategies and the improvement of therapeutic efficiency in breast cancer, especially in triple-negative breast cancer treatment.

The mediator complex is widely distributed in eukaryotes and plays an essential role in basal transcriptional machinery by interacting directly with RNA polymerase II (Pol II) and enhancer-bound transcriptional factors to promote the activation of many Pol II-transcribed genes.[6] The complex is composed of 30 subunits that constitute three main parts named the head, middle, and tail modules, respectively. Head and middle modules usually bind to Pol II, whereas the tail module binds to various transcription factors or co-factors.[7,8] By interacting with transcription factors, the mediator complex participates in transcription elongation, mRNA export, selective splicing, and the regulation of downstream gene expression.[9–11] As one subunit of this complex, MED27 has been previously found as a co-factor of transcription factor SP1. It has also been reported to coordinate with other transcriptional factors to regulate gene expression.[9,12] Reports regarding the function and the corresponding molecular mechanism of MED27 in tumorigenesis and development are still few. Our previous studies demonstrated the tumor-promoting role of MED27 in melanoma survival and its dedifferentiation function in thyroid cancer progression.[13] It has also been reported to promote the proliferation and metastasis of adrenal cortical carcinoma cells by affecting the EMT process.[14] In addition, microRNA-18a has been found to inhibit the growth of osteosarcoma cells by targeting MED27.[15] In breast cancer, although limited studies have focused on the function of MED27 in regulating tumor cell proliferation and apoptosis,[16] its precise functions and the corresponding mechanisms in mediating breast cancer cell metastasis, stemness, and chemoresistance are still poorly understood and deserve further exploration.

Based on RNA-seq and combined with previous reports, we looked at KLF4 as the possible downstream target of MED27 to promote breast cancer progression. KLF4 belongs to the SP/KLF factor family, which is characterized by three zinc finger motifs in its carboxyl-terminal sequence, a *trans*-activated domain (TAD), and an inhibitory domain at the N-terminus.[17] As an evolutionarily conserved zinc finger protein transcription factor, KLF4 has been reported to be involved in multiple cellular functions, including cell differentiation, proliferation, embryogenesis, and pluripotency.[18–21] It has also been reported to play a key, although a sometimes contradictory, role in cancer development. On the one hand, KLF4 functions as a tumor suppressor in the progression of liver, lung, and gastric carcinoma.[22–24] On the other hand, KLF4 promotes tumorigenesis in melanoma, breast, and prostate cancer under certain circumstances.[25–27] KLF4 is highly expressed in primary breast ductal carcinoma[28] and has a carcinogenic effect in breast cancer depending on the state of p21CIP1/WAF1.[29] Moreover, recent reports have found that ATXN3 promotes breast cancer metastasis by deubiquitinating KLF4.[30] Given the essential role of KLF4 in sustaining pluripotency of colorectal and breast stem cells,[31,32] we speculated that, most likely, MED27 promoted the stemness maintenance and subsequent metastasis of breast cancer cells via targeting KLF4.

Here, we explore the precise function and the underlying molecular mechanisms of MED27 in breast cancer development. We not only found high expression of MED27 in breast cancer samples, especially in triple-negative breast cancer, but also confirmed the key role of MED27 in sustaining breast cancer survival by promoting cancer cell metastasis, stemness maintenance, and desensitizing cancer cells to chemotherapy. Based on RNA-seq, we identified KLF4 as a possible downstream target of MED27. The effect of KLF4 itself on breast cancer cell metastasis and stemness-maintaining capacity was evaluated, and its key role in mediating MED27's oncogenic role was further verified *in vitro* and *in vivo*. Furthermore, we explored

FIGURE 1 MED27 is highly expressed in TNBC and promotes the migration and invasion of breast cancer cells. (A) Expression of MED27 in immortalized breast cells, triple-negative and non-triple-negative breast cancer cells was detected by western blot. (B) Expression of MED27 in human breast cancer tissues from patients with different TNM stages based on tissue microarray. (C) Wound-healing assays were performed after MED27 silencing in MDA-MB-231 and BT549 cells or MED27 overexpression in MCF7 and T47D cells, respectively, and the migration rates were calculated. (D) Cell invasion assays were performed after MED27 was overexpressed in MCF7 and T47D or knocked down in MDA-MB-231 and BT549 cells, and the numbers of invasion cells were counted. (E) Expression levels of MMP-9, N-cadherin, E-cadherin, β-catenin, and vimentin were measured by western blot after MED27 silencing or overexpression in breast cancer cells. For (A) and (B), the data are shown as means ± SD of three independent experiments, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.


(A)
MCF10A
MCF-7
T47D
HCC1954
Hs578T
BT20
HCC1806
HCC1937
BT549
MDA-MB-231
MED27
β-actin
34 kDa
42 kDa
Non-TNBC
TNBC
MED27/β-actin
Non-TNBC TNBC
(B)
High MED27 expression
Low MED27 expression
Percentage of specimens
TNM stage I II III
(C)
MDA-MB-231
BT549
0 h
48 h
PBS NC si-1 si-2
Migration rates
MCF-7
T47D
72 h
LacZ OE
(D)
Cell numbers
Si-1 Si-2
(E)
PBS NC Si-1 Si-2 PBS NC Si-1 Si-2
N-cadherin
E-cadherin
β-catenin
MMP-9
Vimentin
MED27
β-actin
130 kDa
120-125 kDa
89 kDa
92 kDa
57 kDa
34 kDa
42 kDa
LacZ OE LacZ OE

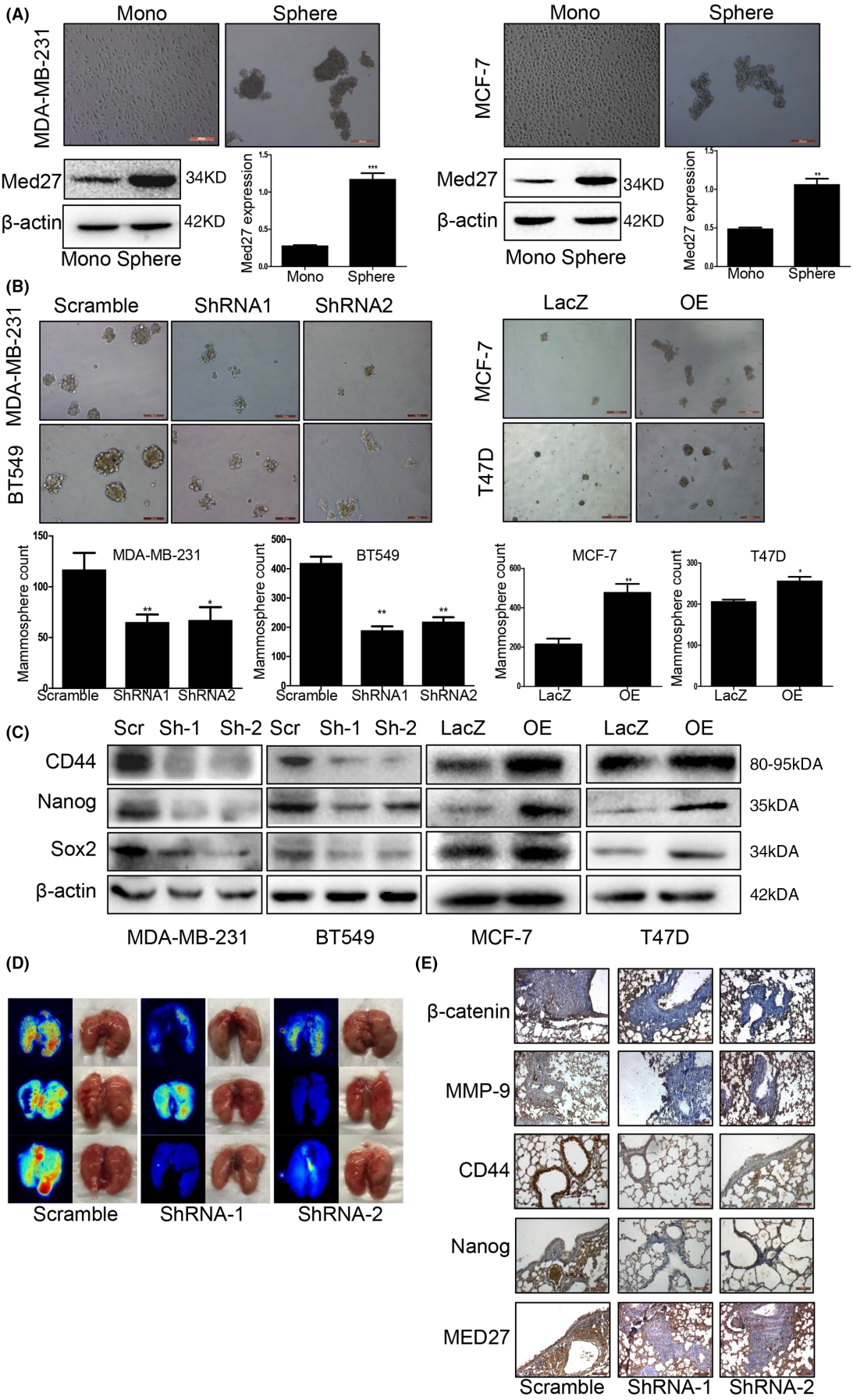

(A)
Mono
Sphere
MDA-MB-231
MCF-7
Med27
β-actin
34KD
42KD
Mono Sphere
Med27 expression
(B)
Scramble
ShRNA1
ShRNA2
LacZ
OE
BT549
T47D
Mammosphere count
(C)
Scr Sh-1 Sh-2 Scr Sh-1 Sh-2 LacZ OE LacZ OE
CD44
Nanog
Sox2
80-95kDA
35kDA
34kDA
42kDA
(D)
Scramble
ShRNA-1
ShRNA-2
(E)
β-catenin
MMP-9
CD44
Nanog
MED27

FIGURE 2 MED27 promoted stemness maintenance of breast cancer cells. (A) Representative images of MDA-MB-231 and MCF7 cells with adherence status or mammosphere status are shown; the expression of MED27 was detected by western blot and quantified. Scale bars, 200 μm. (B) Representative images of mammosphere formation are shown after MED27 was stably knocked down in MDA-MB-231 and BT549 cells or stably overexpressed in MCF7 and T47D cells, and the numbers of sphere were quantified. Scale bars, 100 μm. (C) Expression levels of stemness-related markers CD44, Nanog, and Sox2 were measured by western blot after MED27 knockdown or overexpression. (D) Bioluminescence images of tumor tissues in lungs from BALB/c nude mice 45 days after intravenous injection of MDA-MB-231 cells with stable MED27 silencing. (E) Immunohistochemistry staining of β-catenin, MMP9, CD44, Nanog, and MED27 in metastatic lung tissues from BALB/c nude mice. Scale bars, 100 μm.

and provided the responsive molecular mechanisms of MED27 in regulating KLF4 expression and clarified the precise binding site of MED27 as a specific transcriptional factor at the KLF4 promoter region. More notably, the correlation between MED27 and KLF4 expression in breast cancer tissue samples was analyzed and their clinical significance was evaluated and uncovered. The whole study aimed at providing new targets and strategies for the diagnosis and treatment of breast cancer and suggests that the MED27/KLF4 signaling axis is promising to become such a candidate.

2 | MATERIALS AND METHODS

2.1 | Antibodies

The antibodies against KLF4 (#4038), vimentin (#5741), Snail (#3879), c-Kit (#3074), Epcam (#2929), β-catenin (#8480), MMP-9 (#13667), cleaved-caspase-3 (#9664), caspase-3 (#14220), caspase-9 (#9508), cleaved-caspase-9 (#9505), and MED1 (#51613) were purchased from Cell Signaling Technology (Beverly, MA, USA). The antibodies against N-cadherin (22018-1-AP), E-cadherin (20874-1-AP), Nanog (#14295-1-AP), CD44 (#15675-1-AP), CD133 (#18470-1-AP), Sox2 (#11064-1-AP), and β-actin (20536-1-AP) were purchased from Proteintech Group (Wuhan, Hubei, China). MED27 (sc-390295) was purchased from Santa Cruz (Santa Cruz, Shanghai, China).

2.2 | Other Materials and Methods

Other materials and methods are found in the Supporting Information.

3 | RESULTS

3.1 | MED27 was highly expressed in breast cancer, especially in triple-negative breast cancer and promoted metastasis of cancer cells

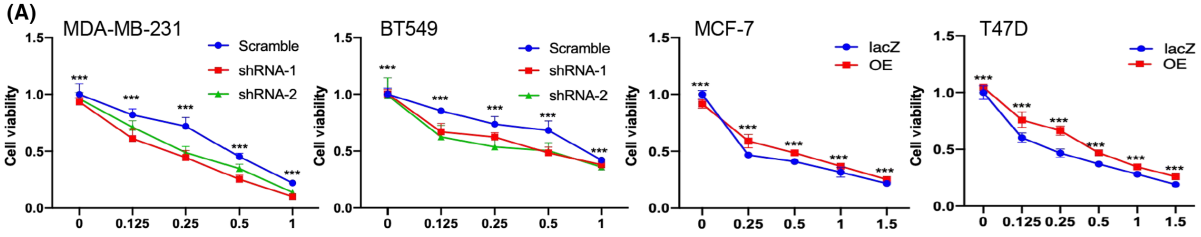
In order to evaluate the role of MED27 in breast cancer, we first detected the expression of MED27. Compared with normal breast epithelial cells (MCF10A), the expression of MED27 in breast cancer cells was much higher. Moreover, MED27 expression in triple-negative breast cancer cells was significantly increased compared with that in non-triple-negative breast cancer cells both in mRNA levels (Figure S1A) and in protein levels (Figure 1A). We also analyzed the expression of MED27 in breast cancer tissues based on TCGA database and immunohistochemistry (IHC) staining of tissue microarrays. Consistently, MED27 expression in breast cancer tissues was higher than that in normal tissues, especially in triple-negative breast cancer samples (Figure S1B). With the development of the clinical cancer stage, its expression increased accordingly (Figure 1B and Figure S1B). In addition, beyond our expectation, MED27 expression was positively correlated with Her2 expression in breast cancer tissues (Figure S1C). By analyzing the expression of other mediator subunits in breast cancer gene-expression databases, we found that MED27, MED30, and MED10 expression levels were higher in TNBC than in non-TNBC (Figure S1D). These results together preliminarily suggested the possible oncogenic role of MED27 in breast cancer.

Next, we further explored the function of MED27 in breast cancer. We first knocked down MED27 in triple-negative breast cancer cell lines, MDA-MB-231 and BT549, and overexpressed MED27 in non-triple-negative breast cancer cell lines, T47D and MCF-7, and analyzed its effect on metastasis of tumor cells. After 48 h transfection, MED27 silencing significantly inhibited the migration and invasion of breast cancer cells. In contrast, its overexpression markedly enhanced the migration and invasion of breast cancer cells (Figure 1C,D). In addition, as shown in Figure 1E, MED27 silenced the attenuated expression of MMP-9, N-cadherin, β-catenin, and vimentin, whereas MED27 overexpression caused the reverse trend.

3.2 | MED27 promoted stemness maintenance of breast cancer cells

Considering the close relationship between cancer metastasis and stemness maintenance,[33–35] we next evaluated the effects of MED27 on stemness phenotypes in breast cancer cells. First, we established breast cancer spheroids via *in vitro* culture and analyzed the expression of MED27 in attached cells and tumorspheres. The results showed that the expression level of MED27 in tumorspheres was significantly elevated compared with that in attached cells (Figure 2A), implying the possible involvement of MED27 in stemness maintenance. Therefore, we next observed the effects of MED27 expression change on tumorsphere formation. MED27 silencing inhibited the tumorsphere formation in breast cancer cells, whereas

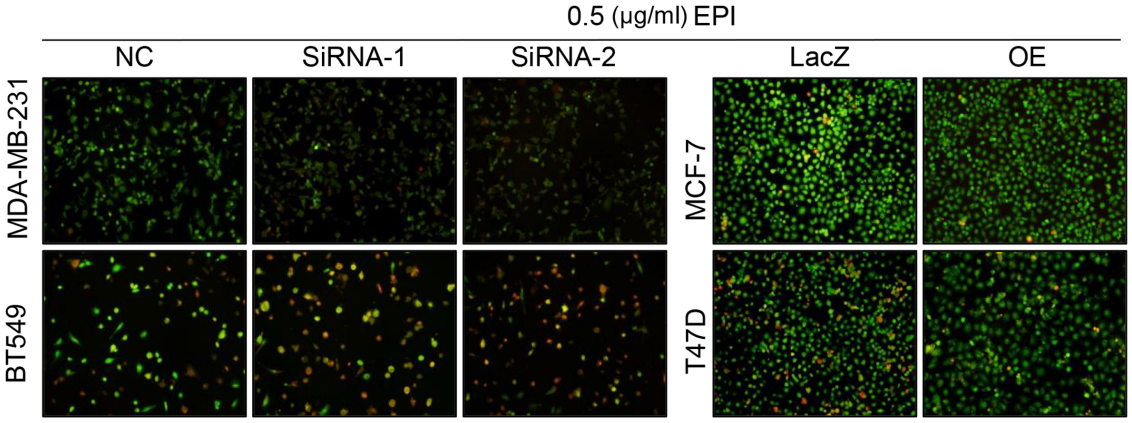
(A)



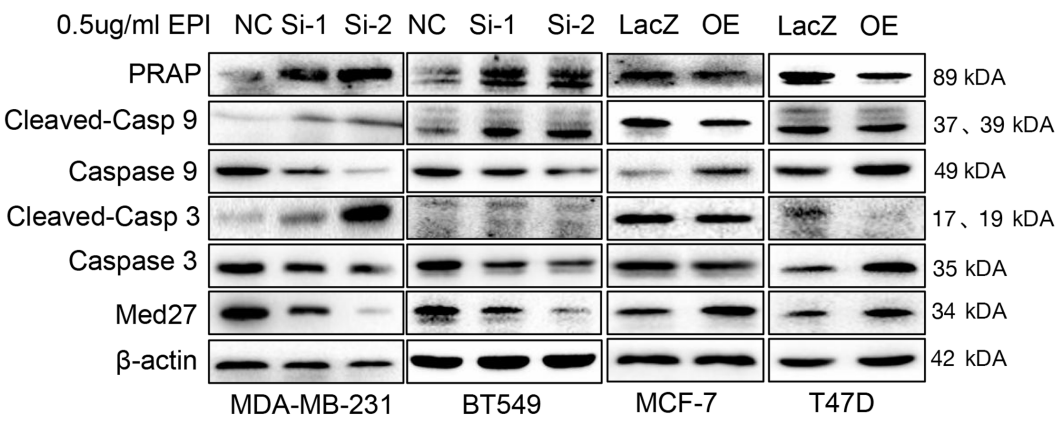
IC_{50}(μg/ml)					
MDA-MB-231			BT549		
Scramble	ShRNA-1	ShRNA-2	Scramble	ShRNA-1	ShRNA-2
0.814	0.415	0.544	0.997	0.639	0.553

IC_{50}(μg/ml)			
MCF-7		T47D	
LacZ	OE	LacZ	OE
0.541	0.854	0.403	0.744

(B)



(C)



(D)

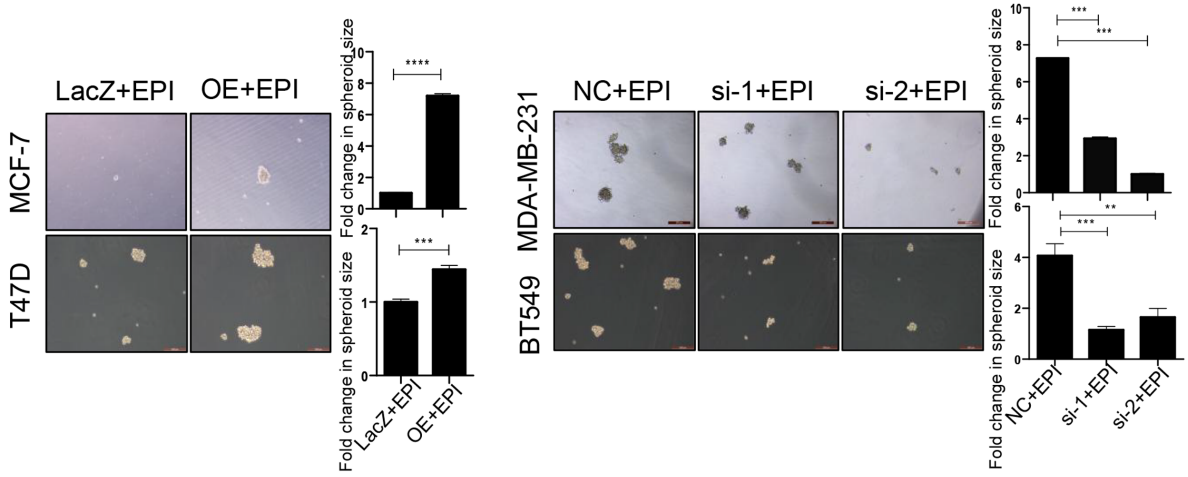


FIGURE 3 Knockdown of MED27 promotes the sensitivity of breast cancer cell to chemotherapeutic drugs. (A) Breast cancer cells with stable knockdown or overexpression of MED27 were treated with different concentrations of epirubicin and the cell viability was measured by MTT assay. The IC_{50} value of EPI was calculated. (B) Representative images of AO/EB assay are shown to evaluate cellular apoptosis upon MED27 silencing or overexpression followed by 0.5 μg/mL EPI treatment. (C) Expression levels of PARP, cleaved-caspase-9, and cleaved-caspase-3 were detected by western blot after MED27 silencing or overexpression followed by EPI treatment. (D) Representative images of mammosphere formation in breast cancer cells upon MED27 overexpression or knockdown followed by EPI treatment, and the numbers of spheres were counted. Scale bars, 200 μm. For (A) and (D), the data are shown as means ± SD of three independent experiments, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. EPI, epirubicin.

its overexpression displayed the opposite effect (Figure 2B). In addition, the expression levels of stemness-related proteins, CD44, Nanog, and Sox2, were decreased in MDA-MB-231 and BT549 cells with MED27 silencing, but increased in T47D and MCF-7 cells upon MED27 overexpression (Figure 2C). Combined with the high expression of MED27 in tumorspheres, these results confirmed that MED27 sustained stemness phenotypes in breast cancer cells.

Considering the association between cancer stemness and EMT again, we next assessed the metastatic potential of MDA-MB-231 cells with stable knockdown of MED27 in mice. Compared with the scrambled group, the MED27 knockdown group displayed fewer lung tumor nodes accompanied by a lower expression of MMP-9, β-catenin, CD44, and Nanog in the metastases tissues (Figure 2D,E). Combined with the *in vitro* study performed above, these results collectively indicated that MED27 promoted breast cancer cell survival by contributing to enhanced self-renewal and metastatic capacity.

3.3 | Knockdown of MED27 sensitized breast cancer cells to epirubicin therapy

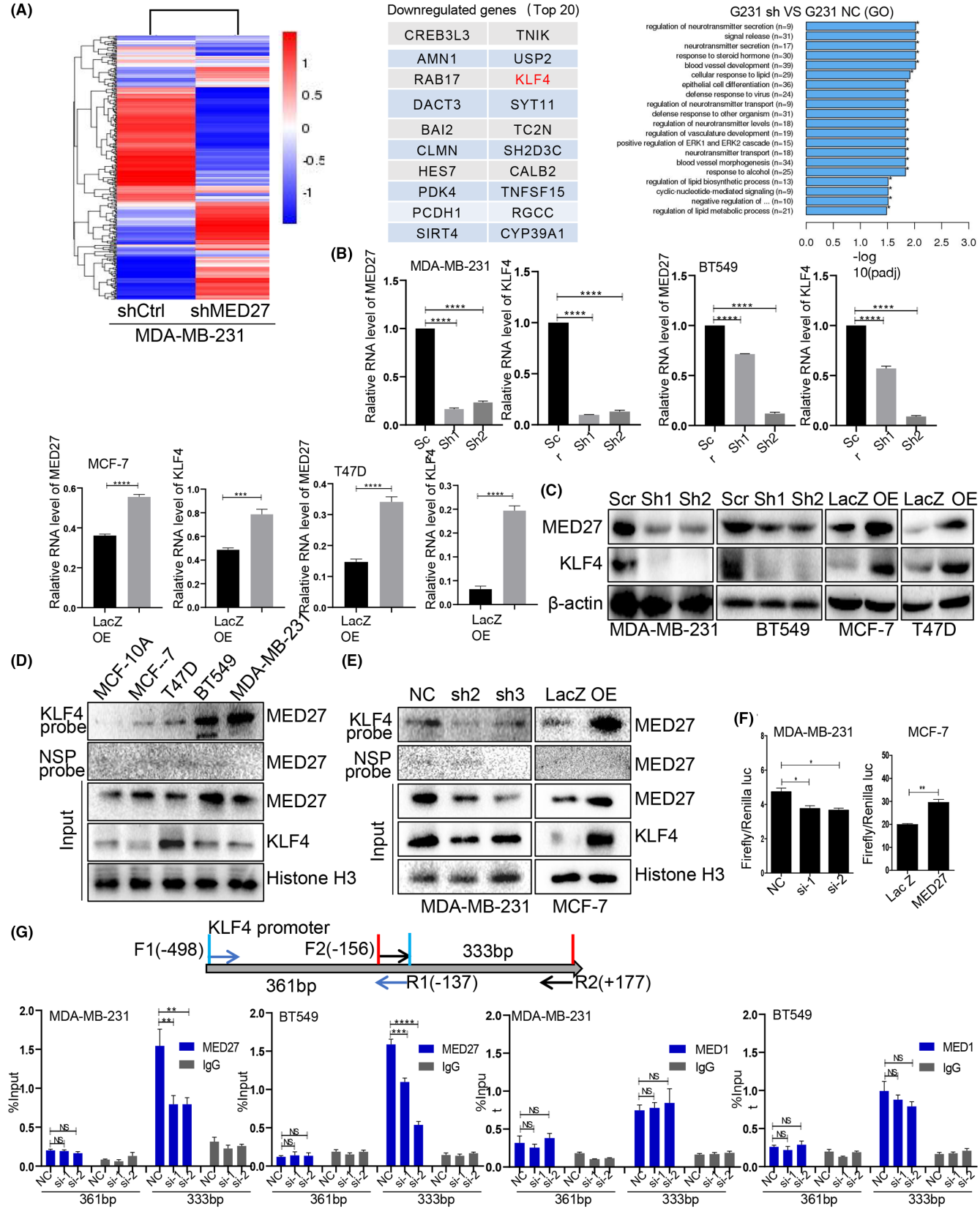
Given the stemness-promoting role of MED27 in breast cancer described above, and the responsibility of the CSC subpopulation within the tumoral mass for tumor metastasis and resistance to conventional therapy, we next analyzed the effect of MED27 on the chemotherapeutic sensitivity of breast cancer cells. Knockdown of MED27 increased the sensitivity of breast cancer cells to EPI, whereas its overexpression reduced such sensitivity (Figure 3A), suggesting the contribution of MED27 to the chemotherapeutic response of breast cancer cells.

To provide insights into the possible mechanisms of MED27 in affecting EPI sensitivity, cell apoptosis status was initially analyzed. The results showed that MED27 knockdown in MDA-MB-231 and BT549 cells induced more apoptosis under EPI treatment, whereas MED27 overexpression in T47D and MCF-7 cells reduced cell apoptosis upon EPI treatment (Figure 3B). In line with this, the expression levels of apoptosis-related proteins in breast cancer cells with EPI treatment, including poly(ADP-ribose)polymerase (PARP), cleaved-caspase-9, and cleaved-caspase-3, were increased or decreased accordingly upon MED27 knockdown or overexpression (Figure 3C). Subsequently, a tumorsphere formation assay was performed to detect the effect of MED27 on stemness in breast cancer cells with EPI treatment. MED27 overexpression increased the tumorsphere formation ability in T47D and MCF-7 cells treated with EPI, and its silencing caused the opposite effect in MDA-MB-231 and BT549 cells (Figure 3D). Therefore, we found conclusively that MED27 promoted chemotherapeutic resistance in breast cancer cells, at least partially, by inhibiting cell apoptosis and increasing stemness phenotypes.

3.4 | MED27 transcriptionally regulates KLF4 by binding to its promoter region in breast cancer cells

To further elucidate the underlying molecular mechanism of MED27 in regulating breast cancer progression, we performed an RNA-seq assay in MDA-MB-231 cells with stable knockdown of MED27 or not. The top 20 most downregulated genes and Gene Ontology (GO) enrichment analysis of the differentially expressed genes after MED27 silencing from RNA-seq data are shown in Figure 4A. KLF4 was selected as one of the downstream genes of MED27 among the significantly downregulated genes based on its known function in breast cancer survival.[28–30] Further analysis demonstrated that knocking down MED27 significantly downregulated the expression of KLF4, while its overexpression upregulated KLF4 levels (Figure 4B,C), confirming the positive regulation of KLF4 by MED27 in breast cancer cells.

Given the essential role of the mediator complex in regulating gene transcription, we deduced the possible transcriptional regulation of MED27 on KLF4 in breast cancer cells. To confirm this, we first used a DNA-pulldown assay to investigate the possible binding of MED27 on the KLF4 promoter region in different breast cancer cells. The results showed that MED27 could be pulled down by the promoter probe of KLF4, and its binding in triple-negative breast cancer cell lines was higher than that in non-triple-negative breast cancer cell lines (Figure 4D). Moreover, MED27 knockdown reduced its binding to the KLF4 promoter, whereas its overexpression increased such binding (Figure 4E), again proving binding of MED27 to the KLF4 promoter. We then constructed a KLF4 promoter (−498/+177)-driven luciferase plasmid and transfected MDA-MB-231 or MCF-7 cells with this plasmid and MED27-specific siRNAs or its overexpression plasmids for 48 h. A luciferase expression assay showed that MED27 knockdown reduced luciferase activity, whereas MED27 overexpression enhanced such activity (Figure 4F), confirming the hypothesis that MED27 transcriptionally regulated KLF4. Subsequently, a ChIP assay was performed. As shown in Figure 4G, the KLF4 promoter region is divided into two fragments. When MED27 was silenced, its binding at the KLF4 promoter (−156



to +177 position) was also decreased, but nearly no binding was found at the KLF4 promoter region (−498 to −137 position), suggesting that, most likely, MED27 anchors at the KLF4 promoter fragment (−156 to +177 position) as a specific transcriptional factor to regulate KLF4 expression in breast cancer cells. To investigate if another mediator complex subunits could also be recruited at the KLF4 promoter, we performed another ChIP assay with MED1 antibody. Although binding of MED1 at the −156/+177 position of the KLF4

FIGURE 4 MED27 transcriptionally regulates KLF4 by binding to its promoter region in breast cancer cells. (A) Heat map displays the differentially expressed genes identified by RNA-seq after MED27 was knocked down in MDA-MB-231 cells. The top 20 genes that were most downregulated and GO enrichment analysis of the differential genes are shown after MED27 silencing. (B, C) Expression of MED27 and KLF4 was measured by qPCR and western blot after MED27 silencing or overexpression. (D, E) DNA-pulldown assay was performed in different breast cancer cell lines or in cells with MED27 knockdown or overexpression. (F) MED27 siRNAs or MED27 overexpression plasmids and pGL3-KLF4 promoter plasmid were co-transfected into MDA-MB-231 or MCF-7 cells, and the KLF4 promoter activity was measured using the dual-luciferase assay. (G) ChIP assay in MDA-MB-231 and BT549 cells using MED27 or MED1 antibody after MED27 or MED1 knockdown, respectively, with their specific siRNAs was performed to demonstrate the enrichment of MED27 or MED1 at the KLF4 promoter (−156 to +177 region). IgG was used as a negative control. The results are shown as the percentage input. For (B, F, G), the data are shown as means ± SD of three independent experiments, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$.

promoter region was also seen, but weaker than MED27, such binding was not affected by MED1 knockdown with its specific siRNAs (Figure 4G). In line with MED27, MED1 showed almost no binding at the −498/−137 position of the KLF4 promoter region. These results preliminarily indicated that MED27, but not MED1, mainly contributed to the transcriptional regulation of KLF4 in breast cancer cells.

3.5 | MED27-mediated promotion of cell invasion and stemness in breast cancer cells are partially dependent on KLF4

As KLF4 was identified to be transcriptionally regulated by MED27 and has been reported to be required for maintenance of breast cancer cell stemness, migration and invasion,[32,36] very possibly, MED27 performs its oncogenic role in breast cancer through targeting KLF4. We first evaluated the function of KLF4 itself in breast cancer. In agreement with known reports, KLF4 displayed a tumor-promoting role by enhancing the invasive and stemness-maintaining capacity of breast cancer cells (Figure S2A–D). Next, rescue experiments were conducted by knocking down MED27 and overexpressing KLF4 simultaneously in MDA-MB-231 cells, or knocking down KLF4 and overexpressing MED27 simultaneously in MCF-7 cells. The results showed that KLF4 knockdown or overexpression significantly reversed the MED27 overexpression- or knockdown-mediated promotion or inhibition of cellular invasion and tumorsphere formation (Figure 5A,C). Moreover, the western blot assay showed again that KLF4 knockdown or overexpression rescued MED27 overexpression- or knockdown-mediated expression changes of EMT and stemness-associated proteins (Figure 5B,D). Altogether, these data supported the idea that MED27 promoted breast cancer cell metastasis and stemness was at least partially dependent on its regulation of KLF4.

3.6 | MED27 maintained cell stemness traits by upregulating KLF4 in the mouse xenograft model of human breast cancer cells

In order to further verify the role of MED27/KLF4 signaling in the expansion of breast cancer, we constructed an orthotopic mouse xenograft model of human breast cancer cells with stable knockdown of MED27 and/or stable overexpression of KLF4 by injecting such cells into the mammary fat pad of BALB/c nude mouse for continuous observation. As expected, knocking down MED27 alone inhibited tumorigenesis and growth, while overexpression of KLF4 significantly promoted tumor incidence and growth. More notably, compared with the control group, KLF4 overexpression reversed the delayed tumor growth mediated by MED27 silencing (Figure 6A–C). Western blot analysis of tissue lysates from the formed tumors showed that the overexpression of KLF4 reversed the MED27 knockdown-mediated expression decrease of stemness-related markers CD44 and Nanog (Figure 6D). The same trend was observed based on immunohistochemistry (Figure 6E). Collectively, these data further confirmed that MED27 promoted breast cancer progression by targeting KLF4.

3.7 | MED27 expression is positively correlated with KLF4 expression in human breast cancer tissues and predicts poor prognosis of patients with breast cancer

Considering the essential role of MED27 in breast cancer cells by regulating KLF4 demonstrated above, we finally evaluated their clinical significance in breast cancer patients. Immunohistochemistry based on the tissue microarray of 128 breast cancer patients showed that 47 patients displayed high expression levels in both MED27 and KLF4, and 42 patients displayed low expressions in both proteins. Representative staining results for high and low expression are shown in Figure 7A and a positive correlation between their expression was found ($p<0.0001$, $r=0.454$) (Figure 7B). Further analysis of the clinicopathological data showed that the expression of both MED27 and KLF4 was associated with the differentiation status of tumor tissues in breast cancer patients (Table 1). Moreover, consistent with the *in vitro* and *in vivo* studies above, the overall survival rate of patients with high expression of MED27 or KLF4 was lower than the patients who had low expression ($p<0.0001$ and $p=0.027$, respectively) (Figure 7C,D). The overall survival of patients with high expression of both MED27 and KLF4 was significantly lower than for the other groups ($p=4.5e{-}5$) (Figure 7E), Furthermore, log-rank test analysis showed that the survival rate was lower in MED27 high expression patients in both the TNBC and non-TNBC groups ($p=0.002$ and $p=2.47e{-}9$, respectively) (Figure 7F). These results suggest the potential of MED27/KLF4 signaling axis as breast cancer prognostic biomarkers.

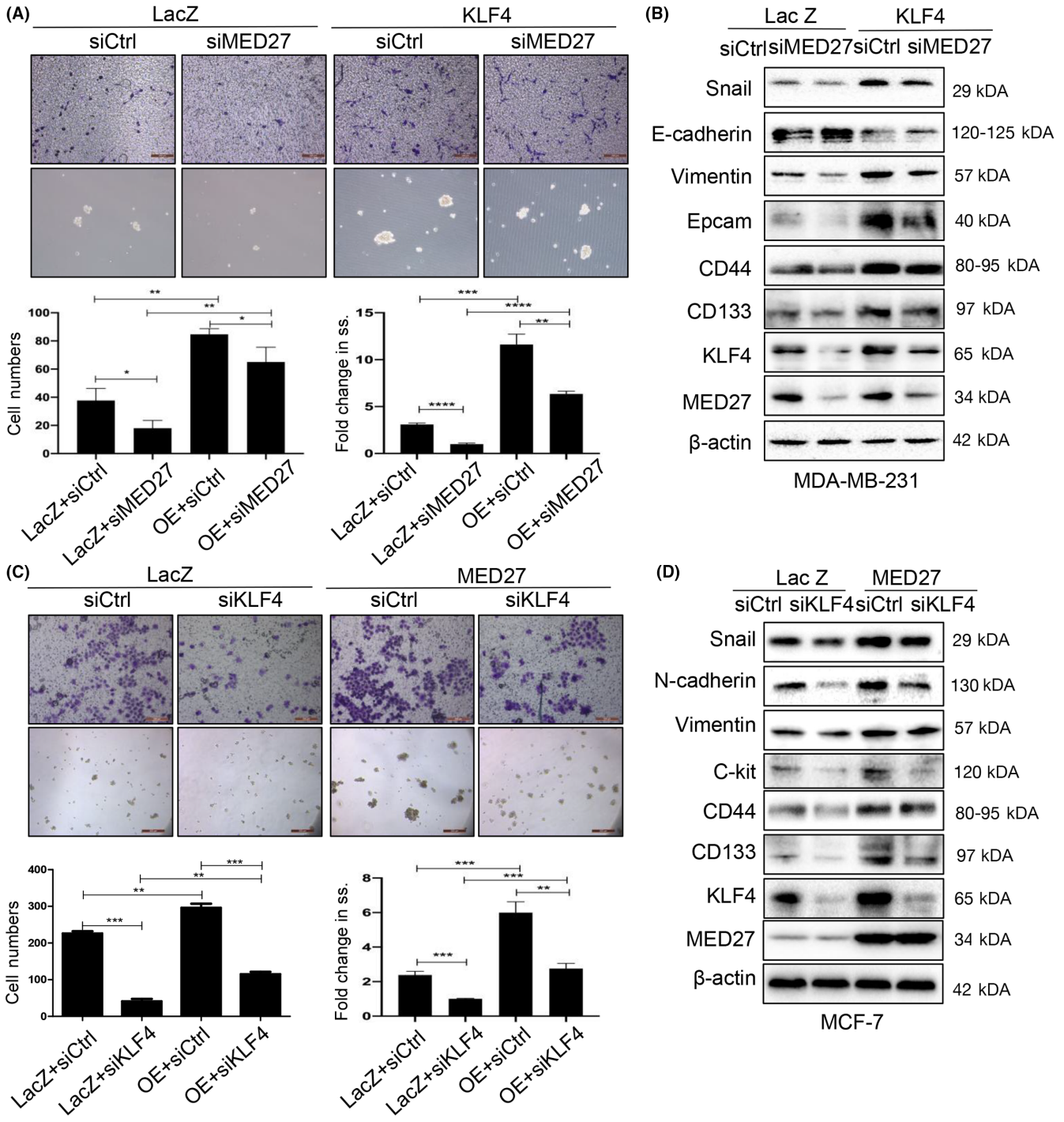
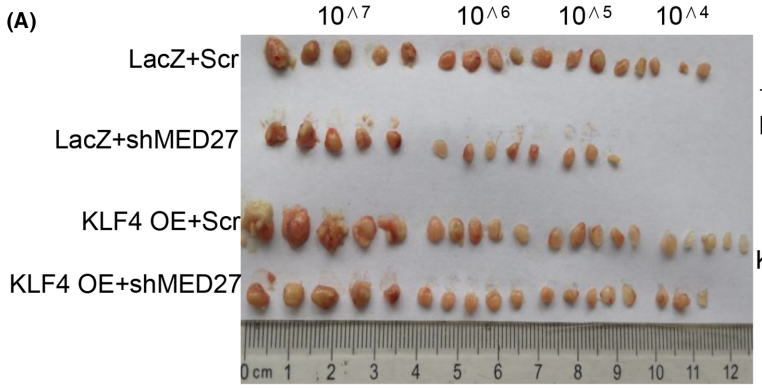
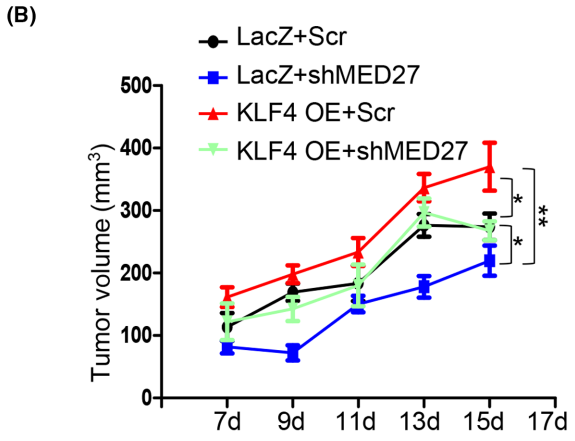
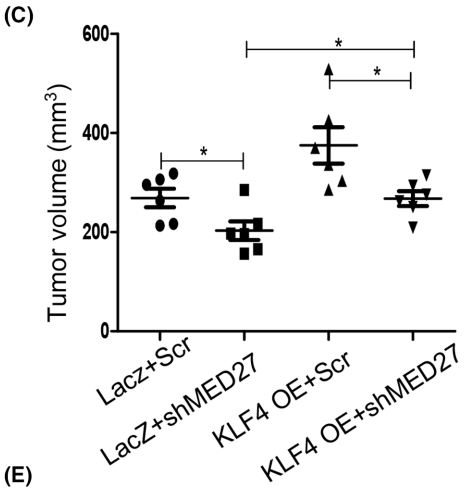


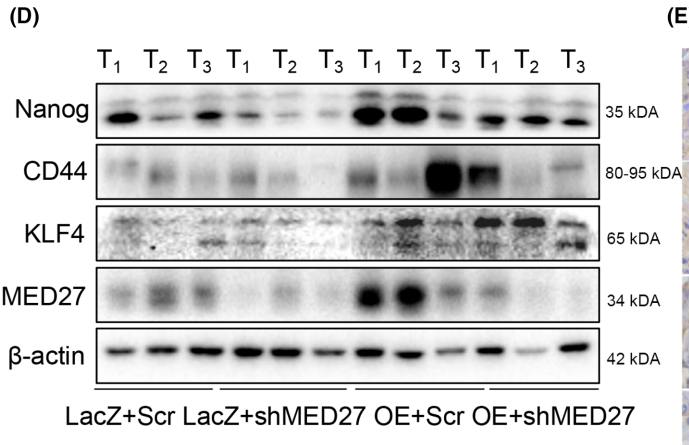
FIGURE 5 MED27-mediated promotion of cell invasion and stemness in breast cancer cells are partially dependent on KLF4. (A) Transwell and mammosphere formation assays were performed in MDA-MB-231 cells with MED27 silencing and/or KLF4 overexpression, and the invasive cell number and fold change of tumorsphere number was quantified, respectively. Scale bars, 200 μm. (B) Expression levels of Snail, E-cadherin, vimentin, Epcam, CD44, and CD133 in MDA-MB-231 cells with MED27 silencing and/or KLF4 overexpression were measured by western blot. (C) Transwell and mammosphere formation assays were performed in MCF-7 cells with KLF4 silencing and/or MED27 overexpression, and the invasive cell number and fold change of tumorsphere number, respectively, were counted. Scale bars, 200 μm. (D) Expression levels of Snail, N-cadherin, vimentin, c-kit, CD44, and CD133 were measured using western blot in MCF-7 cells with KLF4 silencing and/or MED27 overexpression.



		10^7	10^6	10^5	10^4
LacZ	Scr	5	5	5	2
	shMED27	5	5	3	0
KLF4 OE	Scr	5	5	5	5
	shMED27	5	5	5	3







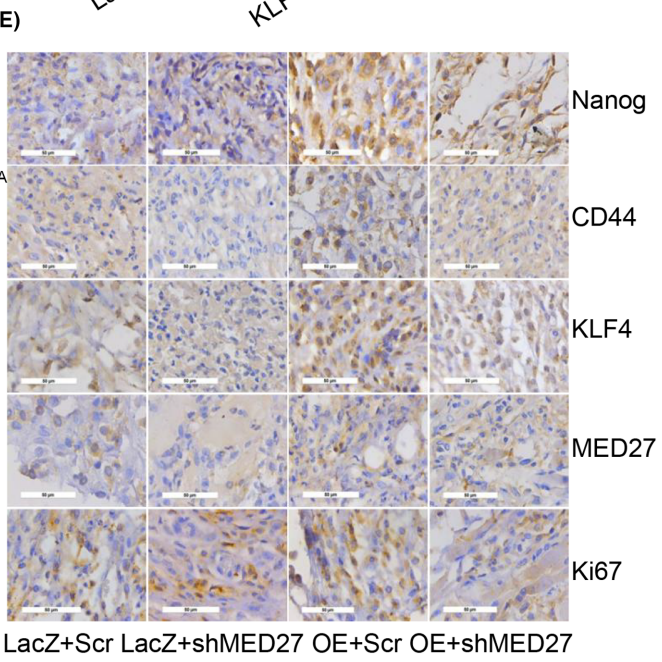
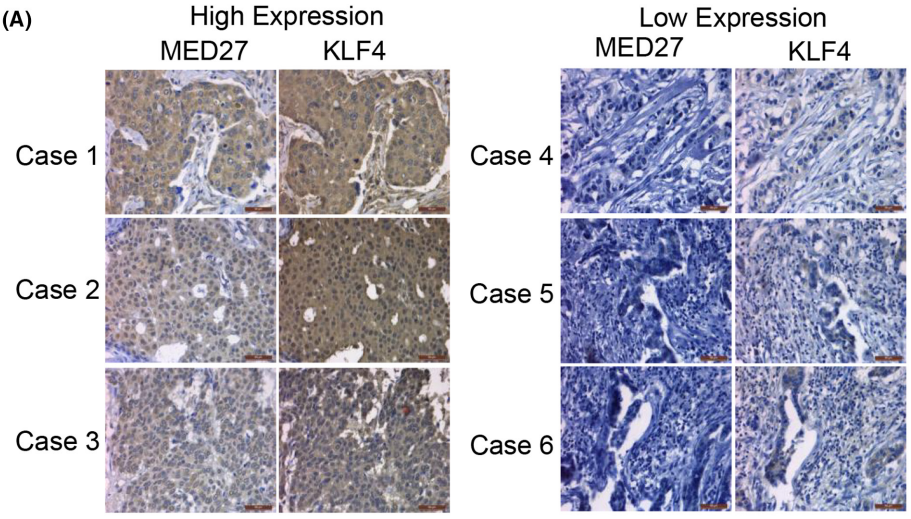


FIGURE 6 MED27 maintained cell stemness traits by upregulating the expression of KLF4 in a mouse xenograft model of human breast cancer cells. (A) Morphology of tumor xenografts from each mouse in different treatment groups was photographed, and the tumor incidence with different cell dilution ratios was recorded. (B) Diameters of the formed tumors in each nude mouse with 10^7 cells injected from different groups were measured at an interval of 2 days after 7 days of injection and the corresponding tumor volumes were calculated. (C) Tumor volume was obtained in 10^7 cells by injection in four groups after the mice were sacrificed. Tumor volume = (width^2 × length)/2. (D) Expression levels of Nanog, CD44, KLF4, and MED27 in three representative tumor tissues of each group were measured by western blot. (E) Expression levels of Nanog, CD44, KLF4, MED27, and Ki67 in mouse tumor tissues were measured by immunohistochemistry staining. $N = 5$ mice/group, Scale bars, 50 μm, original magnification: ×40. The level of significance was indicated by $^*p < 0.05$, $^{**}p < 0.01$.

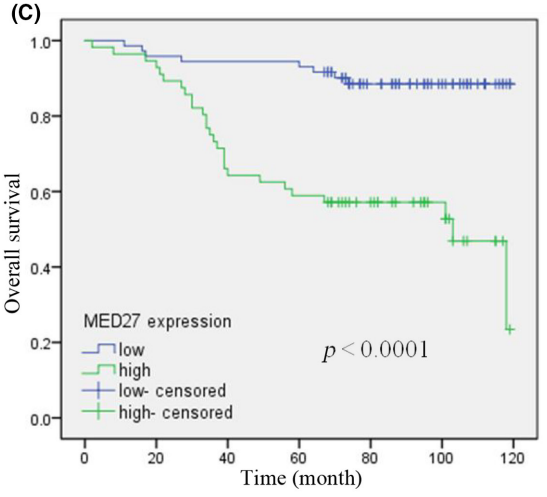
(A)



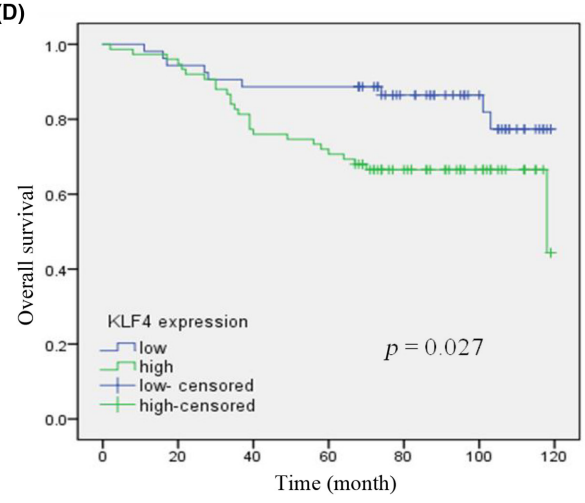
(B)

$r = 0.454$	Med27 expression		
KLF4 expression	High(%)	Low(%)	*p*-value
High	47 (61)	30 (39)	$p < 0.0001$
Low	9 (17.6)	42 (82.4)	

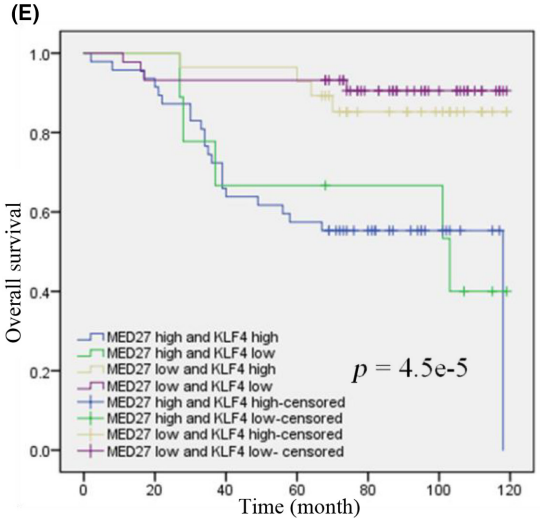
(C)



(D)



(E)



(F)

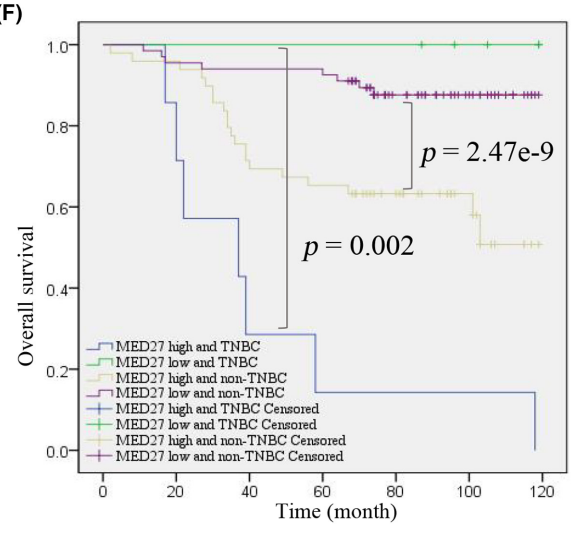


FIGURE 7 Expression of MED27 was positively correlated with KLF4 and predicted the poor prognosis of breast cancer patients. (A) Representative IHC staining images of breast cancer patient tissues showed consistent MED27 and KLF4 expression based on tissue microarray. (B) Correlation between the expression of MED27 and KLF4 in human breast cancer tissues from 128 patients. (C–E) Relevance between overall survival of patients with breast cancer and the expression of MED27 and/or KLF4 in tumor tissues were analyzed using Kaplan–Meier analysis. (F) Correlation between overall survival of TNBC ($n = 12$) or non-TNBC ($n = 116$) patients and MED27 expression based on breast cancer tissue microarray was analyzed.

TABLE 1 Relationship between the level of MED27 or KLF4 expression and clinicopathologic characteristics in breast cancer.

	MED27			KLF4		
Characteristics	**High**	**Low**	***p***	**High**	**Low**	***p***
Age (median)						
≤57	31	36	0.546	40	27	0.79
>57	25	36		35	26	
Differentiation						
High	7	2	0.027	8	1	0.004
Middle	32	35		45	22	
Low	17	35		22	30	
T			0.47			0.149
T_1	20	25		26	18	
T_2	30	44		41	33	
T_3	5	2		7	0	
T_4	1	1		1	1	
N						
N_0	33	32	0.088	38	27	0.868
N_1	14	24		21	17	
N_2	3	12		9	6	
N_3	6	4		7	3	
TNM stage						
I	11	13	0.808	16	8	0.557
II	33	40		40	33	
III	12	19		19	12	

4 | DISCUSSION

MED27 function as an oncogene involved in tumorigenesis and development has been reported recently in various cancers including adrenocortical, osteosarcoma, oropharyngeal, and breast cancer.[14–16,37] In our previous studies, for the first time, we successively reported the tumor-promoting role of MED27 in melanoma and thyroid cancer by activating the NF-κB/iNOS signaling pathways and antagonizing IKKα-induced cell differentiation, respectively.[13,38] Of note, in terms of the function of MED27 in breast cancer, although a recent report found that MED27 promoted the malignant behavior of cells by affecting Sp1 in breast cancer,[16] its effects were mainly explored for the proliferation and apoptosis of breast cancer cells. As for its role in maintaining breast cancer cell metastasis and stemness and the responsible molecular mechanisms, these points have been relatively unexplored. Here, to our knowledge, for the first time, we identify MED27 as a tumor-promoter in breast cancer driving cancer cell metastasis and stemness. Furthermore, our results also suggest that KLF4, transcriptionally regulated by MED27, is necessary to maintain the tumor-promoting role of MED27 in breast cancer (Figure 8).

Based on RNA-seq, KLF4 was selected as the downstream target gene regulated by MED27. Rescue experiments further confirmed the key role of KLF4 in mediating the tumor-promoting function of MED27 in breast cancer cells, because KLF4 overexpression or knockdown significantly reversed the impairment or enhancement of cancer cell metastasis and stemness caused by MED27 knockdown or overexpression. According to previous studies, KLF4 has been shown to promote the EMT process by enhancing cell stemness. For example, in pancreatic cancer, KLF4 promotes the EMT processes of cancer cells by functioning as a type of stemness maintenance factor. Related findings have also been reported in endometrial cancer, human nasopharyngeal carcinoma, and non–small-cell lung cancer, in which KLF4 acts similarly as a stemness maintenance factor to promote the EMT process by interacting with TWIST1 and JAGGED1.[39–41] Combined with a recent study showing that KLF4

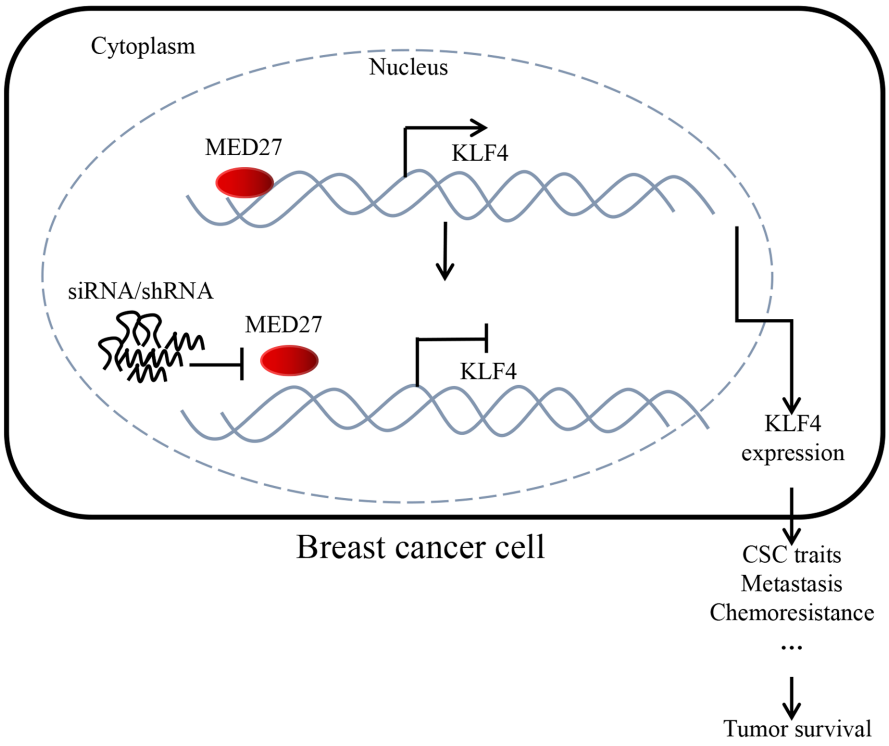


FIGURE 8 Schematic diagram illustrates the function of MED27 in promoting breast cancer progression and the underlying molecular mechanism. The symbol (⊣) indicates negative regulation. The arrow (→) indicates direct or indirect positive regulation.

promoted the stemness and invasiveness of breast cancer cells,[42] our results not only proved once again the capacity of KLF4 to maintain stemness and metastasis in breast cancer, but also revealed its expression regulatory mechanism upstream, further enriching our understanding of KLF4's involvement in tumorigenesis and development and increasing the potential to develop the MED27/KLF4 signaling axis as a candidate therapeutic target in cancer, especially in breast cancer.

By further investigating the underlying mechanisms of MED27 in regulating KLF4 based on pulldown and dual-luciferase reporter assays, we found that MED27 bound at the promoter region of KLF4 as a specific transcriptional factor to affect KLF4 transcription. More accurate analysis from ChIP identified the actual binding site of MED27 in the KLF4 promoter, at the −156/+177 position. Of course, segmented testing within this fragment (−156/+177) to clarify the more precise binding site for MED27 deserves further exploration. In addition, the binding pattern of MED27 at the KLF4 promoter, for instance directly binding to the KLF4 promoter or indirectly binding to the KLF4 promoter by interacting with other co-factors, needs to be investigated further. Most likely, based on the common role of the mediator complex in the basal transcriptional machinery by interacting with RNA polymerase II and enhancer-bound transcription factors, MED27 does not regulate KLF4 expression as an independent transcription factor, but acts synergistically with other transcription factors. What are these factors? Are they synergistic or antagonistic in the transcriptional regulation of MED27 on KLF4 in breast cancer cells? Are they oncogenic or antineoplastic? To answer these questions, further studies seem to be necessary. As to the contribution of the whole mediator complex in regulating KLF4 expression mediated by MED27 in breast cancer cells, based on our current data that only weak binding of MED1at KLF4 specific promoter region was found and that such binding was unaffected by the level of MED1 itself, we do not exclude the possibility that, although the entire complex is recruited to the KLF4 promoter region, some of its components, such as MED27, could play a more important role in transcription regulation of KLF4.

In summary, we validated the critical role of MED27 in sustaining breast cancer cell survival and refined the possible responsive molecular mechanism for this role. We found that MED27 promoted breast cancer cell invasion and stemness maintenance mainly via transcriptionally regulating its downstream target gene KLF4. More significantly, MED27/KLF4 signaling showed potential diagnostic and prognostic values for the clinic. Both MED27 and KLF4 were highly expressed in breast cancer tissues and the expression of these two genes was positively correlated with each other. Moreover, high expression levels of MED27 or KLF4 were highly correlated with tumor differentiation status and predicted a poor prognosis for these patients with breast cancer. Taken together, our study suggests that the MED27/KLF4 signaling axis could very possibly serve as a key candidate target for breast cancer diagnosis and treatment, and further emphasizes the critical role of targeting of MED27 and KLF4, sequentially or simultaneously, in breast cancer treatment.

AUTHOR CONTRIBUTIONS

Ruozhu Wang, Wei Guo, and Wuguo Deng conceived the study and designed the experiment. Ruozhu Wang, Wendan Yu, Tianhua Zhu, Fei Lin, Chunyu Hua, Liyuan Ru, Ping Guo, and Xinyu Wan performed the experiments. Guoqing Xue, Shilong Han, Ziyue Guo, Kuan Lv, Hanxiao Ge, and Guohui Zhang analyzed the data. Wei Guo, Lingzhi Xu and Wuguo Deng contributed to drafting the manuscript, interpreting data, and coordinating the study. All authors have read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ETHICS STATEMENT

Approval of the research protocol by an Institutional Reviewer Board: N/A.

Informed Consent: N/A.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: This animal study was approved by the Medical Ethics Committee of the Dalian Medical University with procedure number AEE17033.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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