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Med19 is targeted by miR-101–3p/miR-422a and promotes breast cancer progression by regulating the EGFR/MEK/ERK signaling pathway

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ABSTRACT

Our previous study found that mediator complex subunit 19 (Med19) is upregulated and involved in breast cancer tumorigenesis; however, the detailed effects and mechanism of Med19 in breast cancer require further study. In this study, we found that Med19 was obviously elevated in human breast cancer tissues, which was significantly associated with larger tumors, high-grade malignant features and poor prognosis. Furthermore, Med19 enhanced breast cancer cell proliferation, epithelial–mesenchymal transition, invasion and migration in vitro and in vivo. Med19 interacted with epidermal growth factor receptor (EGFR) and increased EGFR expression. Moreover, Med19 activated the EGFR/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway and exerted its oncogenic activity in an EGFR signaling-mediated manner. In addition, Med19 expression was regulated by miR-101–3p and miR-422a. Med19 expression positively correlated with EGFR expression and negatively correlated with miR-101–3p and miR-422a expression in human breast cancer tissues. Med19 mediated the crosstalk between miR-101–3p/miR-422a and the EGFR/MEK/ERK signaling pathway. This study revealed a new miR-101–3p/miR-422a-Med19-EGFR/MEK/ERK axis that plays a significant role in breast cancer progression. These results help elucidate the potential mechanisms of Med19 in human breast cancer progression.

1. Introduction

Breast cancer is the most commonly diagnosed cancer in women worldwide and the second cause of cancer-related death in women aged younger than 45 years in China [1,2]. Breast cancer is a complicated genetic disease caused by the accumulation of numerous genetic alterations and has a high rate of recurrence and metastasis. Surgery and multifarious adjuvant therapies, the traditional treatments for breast cancer, do not always block disease progression. Therefore, there is an urgent need to investigate the molecular mechanisms of breast cancer progression.

The Mediator, a large multisubunit complex, is involved in the process of mRNA transcription and plays a vital role in the regulation of genes transcribed by RNA Pol II [3,4]. In humans, the mutation or altered expression of Mediator subunits has extensive effects on cell physiological function and the induction of pathological diseases, including cancer [5]. Mediator complex subunit 19 (Med19) is a member of the Mediator complex. Our previous study demonstrated that Med19 is significantly upregulated in human breast cancer tissues. Furthermore, Med19 knockdown via RNA interference markedly suppresses the growth of breast cancer cells [6,7]. An increasing number of studies have indicated that the aberrant expression of Med19 correlates with tumor development and progression in various types of cancer. Yu et al. revealed that Med19 overexpression plays a role in prostate cancer progression and could regulate crucial genes involved in cell proliferation, cell cycle, and epithelial-mesenchymal transition (EMT) [8]. Med19 is also increased and acts as an oncogene in laryngocarcinoma, and Med19 knockdown induces apoptosis in HEp2 cells via activation of caspase-3, caspase-9 and Apaf-1 [9]. Yuan et al. showed that high Med19 protein levels are positively correlated with aggressive characteristics of bladder cancer and regulate the Wnt/β-catenin pathway [10]. However, the detailed mechanism by which Med19 promotes breast cancer progression and the molecular mechanisms of Med19 dysregulation in breast cancer are largely unknown.

Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein that belongs to the ERBB tyrosine kinase family. EGFR overexpression is often reported in breast carcinomas, and breast cancer with high EGFR expression is more aggressive and more likely to

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metastasize [11]. Furthermore, patients with high EGFR expression have a poorer clinical prognosis and higher recurrence and relapse rates after therapy [12]. EGFR dysregulation leads to abnormal activation of the downstream EGFR signaling pathway, which has been reported to be involved in the tamoxifen resistance and bone metastasis of breast carcinoma [13]. Exploring the regulatory mechanism of EGFR signaling is therefore of crucial importance in comprehending the molecular landscape of breast cancer.

MicroRNAs (miRNAs) are endogenously expressed small noncoding RNAs that bind to the 3′-untranslated region (3′-UTR) to negatively regulate gene expression [14]. An increasing number of studies have suggested that miRNAs are involved in almost every aspect of breast cancer development and progression, such as proliferation, invasion, metastasis and EMT [15–18]. Therefore, predicting and authenticating miRNAs that target Med19 could be a strategy to explain the mechanism of Med19 dysregulation in breast cancer.

In this study, we found that Med19 was upregulated in human breast cancer tissues, and this upregulation was significantly associated with larger tumors, high-grade malignant features and poor outcomes. We further comprehensively elucidated that Med19 not only promotes breast cancer proliferation but also enhances invasion and migration capacities in vitro and in vivo. For the first time, we identified the relationship between Med19 and EGFR, both of which have key roles in breast cancer progression. Our results demonstrate that Med19 dysregulation induces carcinogenesis and breast cancer progression by interacting with EGFR and activating the EGFR/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathway. In addition, miR-101–3p and miR-422a target Med19. Therefore, Med19 functions as a scaffolding molecule in the miR-101–3p/miR-422a/Med19/EGFR/MEK/ERK axis that plays vital roles in breast cancer progression. Above all, these results help elucidate the potential mechanisms of Med19 in human breast cancer progression and suggest Med19 as a potential therapeutic target in pharmacological strategies.

2. Materials and methods

2.1. Patient samples

The 247 human breast cancer tissues and adjacent normal tissues collected from 2003 to 2008 were obtained from the Affiliated Hospital of Jiangnan University. The 64 human breast cancer tissues for PCR analysis were collected from 2013 to 2017. Written informed consent was obtained from all patients. The study was approved by the Ethics Committee of the Affiliated Hospital of Jiangnan University.

2.2. Experimental procedures

The immunohistochemistry (IHC), cell culture, small interfering RNA (siRNA), RNA preparation and quantitative reverse transcription polymerase chain reaction (qRT-PCR), transwell invasion and migration, Western blotting, coimmunoprecipitation (Co-IP), liquid chromatography-tandem mass spectrometry (LC-MS/MS), immunofluorescence and luciferase reporter assays are described in the Supplementary Materials and Methods. The specific primers used are shown in Supplementary Table S1. The human Med19 shRNA and expression lentiviral vectors were constructed, and breast cancer cells were stably infected with these vectors according to our previous reports [6,7]. Cell proliferation was measured using a Cell Counting Kit-8 (CCK-8, Dojindo, Japan) at an absorbance of 450 nm. The CCK-8 and colony formation assays were performed according to our previous study [7].

2.3. Animal experiments

For the siRNA injection assay, MDA-MB-231 cells (5×10^6 cells in 250 μl) were injected subcutaneously into the right and left flanks of female severe combined immunodeficient (SCID) mice (Shanghai SLAC Laboratory Animal, China). One week after the tumor diameter reached 5 mm, the mice were randomly assigned into two groups (n = 6 each) to receive intratumor injections of 100 μl of Med19 siRNA or scramble sequences (modified with 2′—O-methyladenosine, injection dose shown in Supplementary Table S2). Intratumor injections were repeated twice a week. Tumors were measured (perpendicular diameters) on different days, and tumor volume was calculated. On day 27, the mice were killed, and the tumor weight and volume were measured.

MDA-MB-231 cells were infected with sh-Med19 or control lentivirus and resuspended in PBS at a concentration of 10^7 cells/ml. Female SCID mice were injected with 100 μl of each group via the tail vein. Animals were sacrificed and autopsied 5 weeks after cell injection. Lung metastasis was assessed by macroscopic observation and confirmed by HE staining. All animal experiments were carried out with approval from the Ethics Committee of the Affiliated Hospital of Jiangnan University.

2.4. Statistical analysis

SPSS17.0 (SPSS Inc, Chicago, IL, USA) and GraphPad Prism 6.0 (GraphPad software, Inc, La Jolla, CA, USA) were used for statistical analyses and figure processing. All experimental data are presented as the means ± SD of at least three independent experiments. Parametric or nonparametric analyses were performed before specific statistical tests were conducted. Differences between two groups were assessed using Student's t-test. One-way ANOVA with the Bonferroni *post hoc* test were employed to analyze the differences among three or more groups. IHC scores between normal and breast cancer tissues were compared using the Wilcoxon rank-sum test. The $\chi 2$ test was used to evaluate the relationship between Med19 expression and clinicopathological characteristics. The Kaplan–Meier method was used to evaluate the differences in survival rates, which were analyzed by the log-rank test. The correlations were analyzed using Pearson's correlation coefficients. All tests conducted were two-sided and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Upregulation of Med19 is associated with poor survival of breast cancer patients and promotes breast cancer cell proliferation

To investigate the role of Med19 in breast cancer oncogenesis, we detected Med19 expression in a cohort of 247 breast cancer samples and adjacent normal tissues through immunohistochemical staining (Table S3). Med19 was clearly upregulated in breast cancer tissues compared with adjacent normal tissues (Fig. 1A, Wilcoxon rank-sum test, $P < 0.001$). The relationship between Med19 expression and the clinicopathological characteristics of the breast cancer patients is summarized in Table 1. According to the statistical analyses, increased Med19 expression was significantly associated with the tumor size (Table 1, χ^2 test, $P = 0.019$) and tumor grade (Table 1, χ^2 test, $P = 0.036$). Moreover, Kaplan-Meier analysis revealed that breast cancer patients with high Med19 expression had a shorter overall survival time than those with low Med19 expression ($P = 0.0103$) (Fig. 1B).

We detected Med19 expression in 9 human breast cancer cell lines and the non-tumorigenic breast cell line MCF-10A using Western blotting, and found that Med19 expression was in higher in breast cancer cells compared with MCF-10A cells (Fig. 1C). Then, we selected the MDA-MB-231 and ZR-57-30 cell lines for Med19 knockdown and the BT-549 and Hs578T cell lines for Med19 overexpression. The efficiencies of overexpression and knockdown were confirmed by qRT-PCR and Western blotting (Fig. 1D). Then, we investigated the potential role of Med19 in breast cancer cells. The downregulation of Med19 inhibited the proliferation of MDA-MB-231 and ZR-57-30 cells, whereas

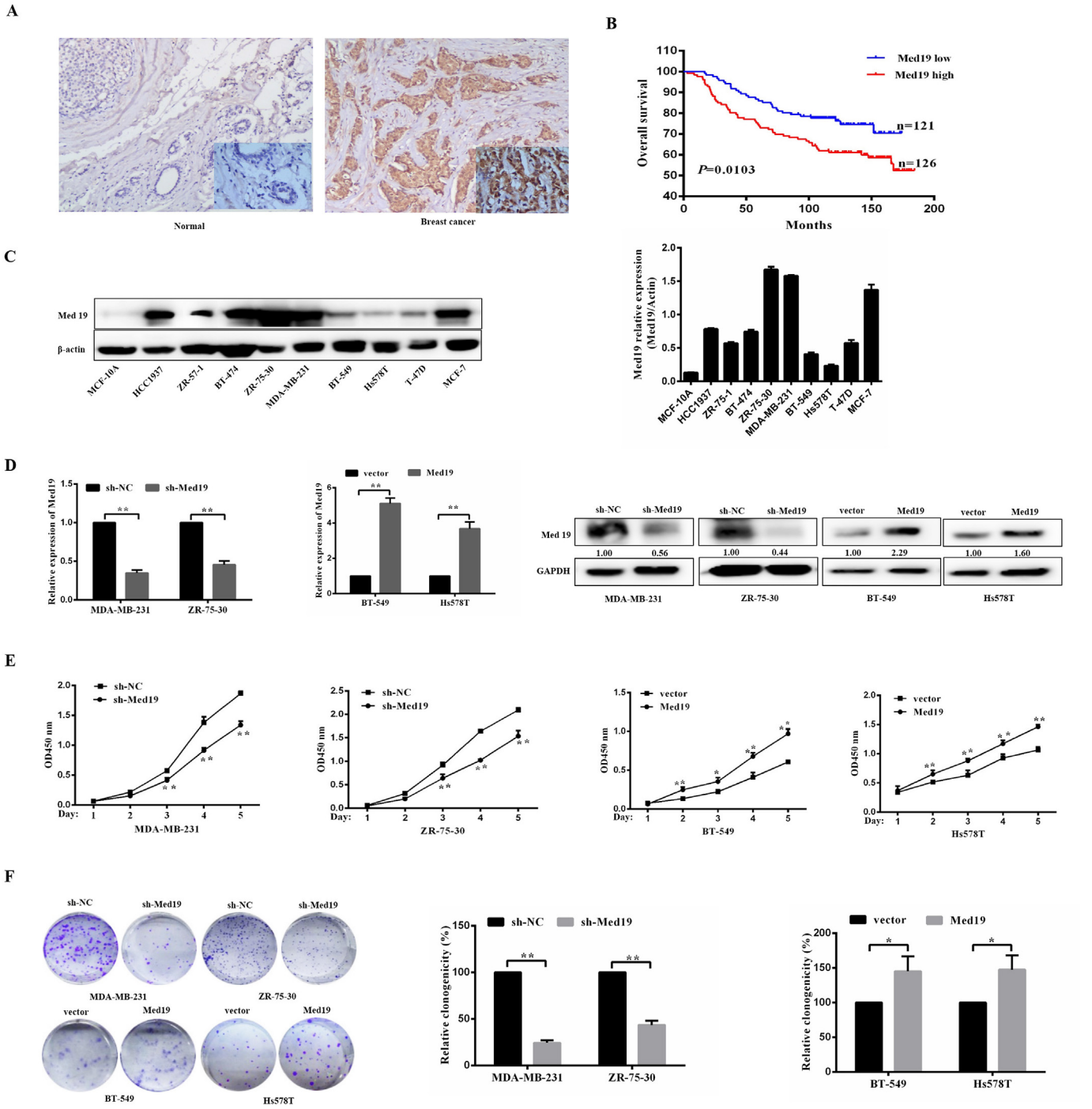

Fig. 1. Dysregulation of Med19 predicts a poor prognosis for patients with breast cancer and promotes breast cancer cell proliferation. (A) Med19 expression in breast cancer tissues and normal tissues was detected using immunohistochemical staining, magnification, × 100 and × 400 (bottom right panel). (B) Kaplan–Meier analysis of overall survival in two groups of patients with breast cancer stratified by low and high expression of Med19 ($P = 0.0103$). (C) Western blot analysis of Med19 expression in 9 breast cancer cell lines (n = 3). (D) QRT-PCR and Western blot analyses of Med19 expression after stable infection (means ± SD. n = 3. Student's *t*-test. $^{**}P < 0.01$). MDA-MB-231 and ZR-75-30 cells were infected with sh-Med19 or the negative control, and BT-549 and Hs578T cells were infected with Med19 overexpression vector or vector control. (E) CCK-8 assays and (F) colony formation assays were performed to detect the proliferation of MDA-MB-231 and ZR-75-30 cells following Med19 knockdown and of BT-549 and Hs578T cells overexpressing Med19 (means ± SD. n = 3. Student's *t*-test. $^{*}P < 0.05$ and $^{**}P < 0.01$).

the upregulation of Med19 facilitated the proliferation of BT-549 and Hs578T cells, as shown by CCK-8 assays (Fig. 1E) and colony formation assays (Fig. 1F). However, in MCF-10A cells, neither Med19 knockdown nor overexpression of Med19 significantly changed the cell proliferation abilities (Fig. S1). These results suggested that Med19 upregulation occurs during breast cancer development, promotes breast cancer cell proliferation and is associated with poor survival in breast cancer.

3.2. Med19 promotes breast cancer cell invasion, migration and EMT

To further detect the potential effects of Med19 on the malignant process of breast cancer, we performed a series of additional functional experiments. Transwell assays showed that Med19 knockdown decreased the migration and invasion capacities of MDA-MB-231 and ZR-57-30 cells, while Med19 overexpression in BT-549 and Hs578T cells

Table 1
Correlations between Med19 expression and clinical characteristics in breast cancer patients (n = 247).

Characteristics	Number of cases	Med19 expression		X^2	p
		Low	High		
		121	126		
Age (years)				0.025	0.874
< 50	89	43	46		
≥50	158	78	80		
Tumor size (cm)				5.489	0.019*
≤2	118	67	51		
> 2	129	54	75		
Tumor stage				0.624	0.43
I + II	197	99	98		
III + IV	50	22	28		
Grading					
I	41	27	14	6.673	0.036*
II	108	53	55		
III	98	41	57		
Lymph node metasis				0.222	0.637
Positive (absent)	114	54	60		
Negative (presence)	133	67	66		
ER				0.234	0.628
Positive	175	84	91		
Negative	72	37	35		
PR				0.042	0.837
Positive	207	102	105		
Negative	40	19	21		
Cerb-2				0.024	0.877
Positive	138	67	71		
Negative	109	54	55		

Med19 low expression: IHC score 0–1 +; Med19 high expression: IHC score 2 + -3 +. *Significant difference ($P < 0.05$); **significant difference ($P < 0.01$). ER, estrogen receptor; PR, progesterone receptor.

enhanced the migration and invasion abilities (Fig. 2A and B).

The EMT process can promote the invasion and metastasis of many cancers. Thus, we explored the role of Med19 in EMT. The expression of epithelial cell markers (E-cadherin and claudin-1) was increased in MDA-MB-231 and ZR-75-30 cells after Med19 knockdown, whereas the expression of mesenchymal markers (N-cadherin and vimentin) and EMT-related transcription factors (TCF8/ZEB1, Snail and Slug) was decreased in these cells (Fig. 2C). The converse results were obtained in BT-549 and Hs578T cells overexpressing Med19 (Fig. 2C). Taken together, the above results revealed that Med19 may play a crucial role in breast cancer invasion, migration and EMT.

3.3. Med19 interacts with EGFR and activates the EGFR/MEK/ERK signaling pathway

To explore the molecular mechanistic basis of the tumor-promoting effects of Med19 in breast cancer, we sought to identify its interacting partners. Co-IP and Coomassie brilliant blue staining revealed candidate bands (Fig. 3A) that were incised for further LC-MS analysis. Through data analysis of these 9 special bands, we authenticated approximate 900 proteins. Based on the effects of Med19 in breast cancer and literature-based knowledge, we chose RAF1, NF-κB-2, MAPK14, MAPK9, HMGB2 and EGFR for co-IP verification and demonstrated that RAF1, MAPK14 and EGFR could interact with Med19 (Fig. 3B). QRT-PCR analysis showed that knockdown of Med19 distinctly decreased EGFR mRNA levels but did not affect RAF1 and MAPK14 expression (Fig. 3C). To validate the endogenous interaction between Med19 and EGFR, co-IP was performed using a Med19 antibody in MDA-MB-231 and ZR-75-30 cells (Fig. 3D). Med19 could also be coprecipitated by an EGFR antibody in both cell lines, as shown by Western blotting analysis (Fig. 3D), confirming the physical interaction between Med19 and EGFR.

We next examined the expression and intracellular distribution of Med19 and EGFR by Western blotting of total, cytoplasmic and nuclear extracts, which showed that Med19 was localized in both the cytoplasm and the nucleus, whereas EGFR was mainly localized in the cytoplasm, with little expression in the nucleus (Fig. 3E). The localization of the two proteins was further confirmed by immunofluorescence in MDA-MB-231 and ZR-57-30 cells. Fluorescence microscopy images showed that Med19 and EGFR were both in the cytoplasm and nucleus of the two cell lines; EGFR was found mainly in the cytoplasm, where it co-localized with Med19 (Fig. 3F).

We detected Med19 and EGFR expression in the 4 breast cancer cell lines utilized in this study and MCF-10A cells, and the results showed that Med19 and EGFR expression were both in higher in breast cancer cells compared with MCF-10A cells (Fig. S2). More interestingly, we found that decreasing Med19 levels could downregulate the expression of EGFR at both the mRNA and protein levels in MDA-MB-231 and ZR-75-30 cells. The opposite results were observed in BT-549 and Hs578T cells, in which Med19 levels were increased (Fig. 3G). Then, we were interested in determining whether Med19 has an impact on the EGFR/MEK/ERK signaling pathway. Western blotting analysis showed that similar to the reduction in EGFR expression upon Med19 downregulation in MDA-MB-231 and ZR-75-30 cells, the activation of EGFR (p-EGFR), MEK and ERK1/2 (p-MEK and p-ERK1/2), as well as total MEK and ERK1/2 levels, were decreased (Fig. 3G). In contrast, upregulation of Med19 in BT-549 and Hs578T cells increased the expression of these proteins (Fig. 3G). Above all, these data provided evidence that Med19 interacts with EGFR and at least partially activates the EGFR/MEK/ERK signaling pathway.

3.4. Med19 facilitates breast cancer cell proliferation, invasion, migration and EMT in an EGFR/MEK/ERK signaling-mediated manner

To investigate whether Med19 functions in an EGFR/MEK/ERK signaling-mediated manner in breast cancer cells, we conducted rescue experiments. The CCK-8 and colony formation assay results showed that Med19 suppression inhibited the proliferation of MDA-MB-231 and ZR-57-30 cells, but this inhibition was impaired by simultaneous overexpression of EGFR (Fig. 4A and B). Additionally, EGFR overexpression partially attenuated the effects of Med19 knockdown on breast cancer cell migration and invasion, as shown by transwell assays (Fig. 4C). Western blotting results showed that EGFR overexpression could partially restore the inhibition of N-cadherin, vimentin, TCF8/ZEB1, Snail, and Slug expression mediated by Med19 knockdown (Fig. 4D, left panel). Nevertheless, the Med19 knockdown-induced upregulation of E-cadherin and claudin-1 was decreased by EGFR upregulation (Fig. 4D, left panel). Finally, we detected EGFR/MEK/ERK signaling molecules and found that EGFR overexpression could recover MEK and ERK1/2 activation and expression, which were inhibited by Med19 downregulation (Fig. 4D, right panel). Our results indicated that Med19 promotes breast cancer cell proliferation, invasion, migration and EMT in an EGFR/MEK/ERK signaling-mediated manner.

3.5. Inhibition of Med19 expression attenuates breast cancer malignant progression in vivo

To examine the in vivo effects of Med19 and explore the potential of Med19 as a therapeutic target, we used MDA-MB-231 cells to construct a xenograft mouse tumor model. Med19 siRNA and scramble sequences modified with 2′—O-methyladenosine were synthesized and injected into the mouse tumors. Mice were injected twice a week after the development of neoplasia and sacrificed on day 27 posttreatment. The mice injected with Med19 siRNA had significantly smaller tumors than did those in the control group (Fig. 5A). Our data indicated that Med19 may be a potential therapeutic target because knockdown of Med19 significantly inhibited tumor growth.

To further investigate the effects of Med19 on breast cancer progression in vivo, we performed a breast cancer lung metastasis assay in

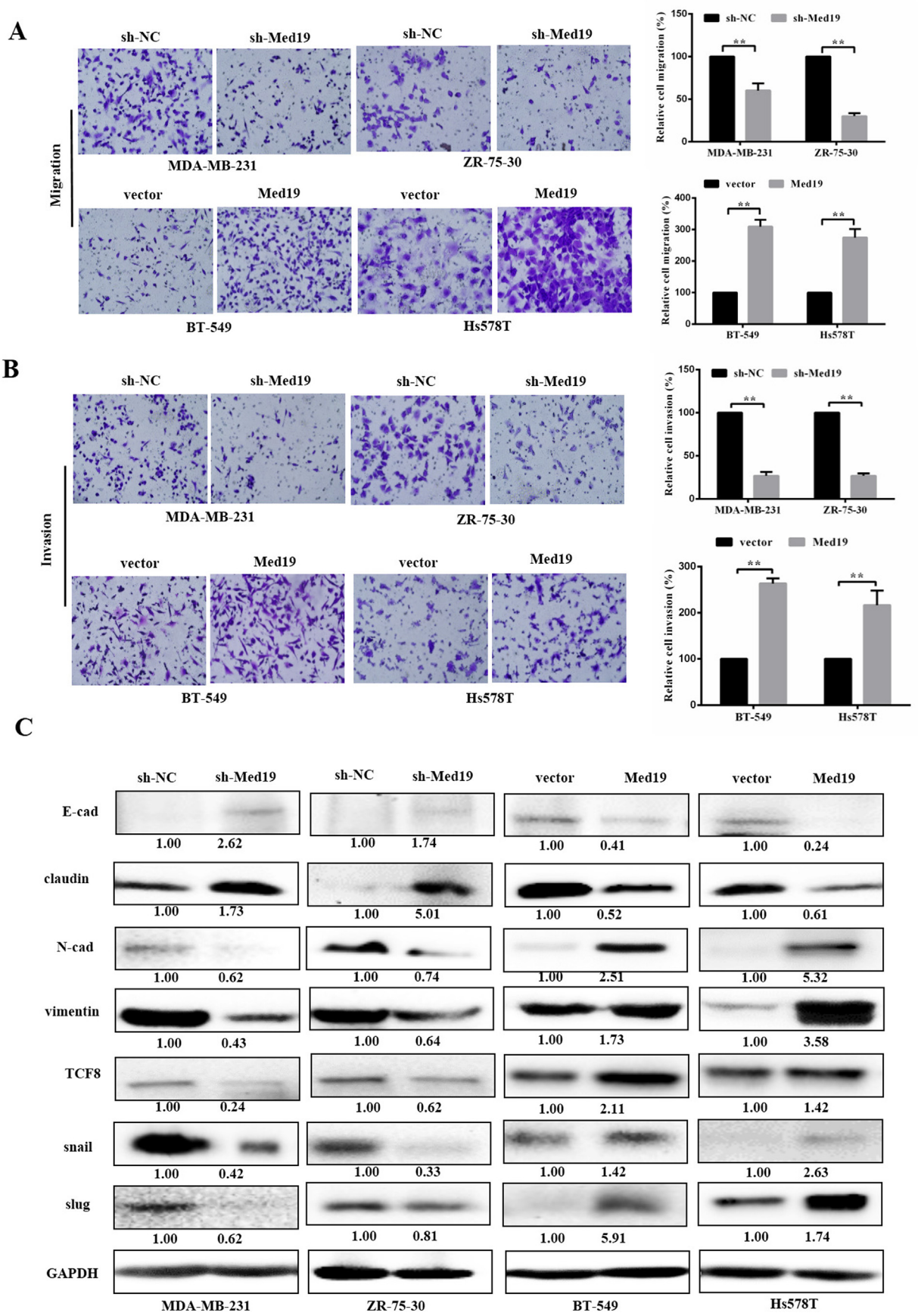

Fig. 2. Med19 promotes breast cancer cell invasion, migration and EMT. (A, B) Cell migration and invasion were evaluated using transwell migration and Matrigel invasion assays in MDA-MB-231 and ZR-75-30 cells with Med19 knockdown and in BT-549 and Hs578T cells overexpressing Med19 (200× magnification. mean ± SD. n = 3. Student's *t*-test. $^{**}P < 0.01$). (C) The levels of EMT-related proteins were analyzed in the four cell lines mentioned above (means ± SD. n = 3. Student's *t*-test).

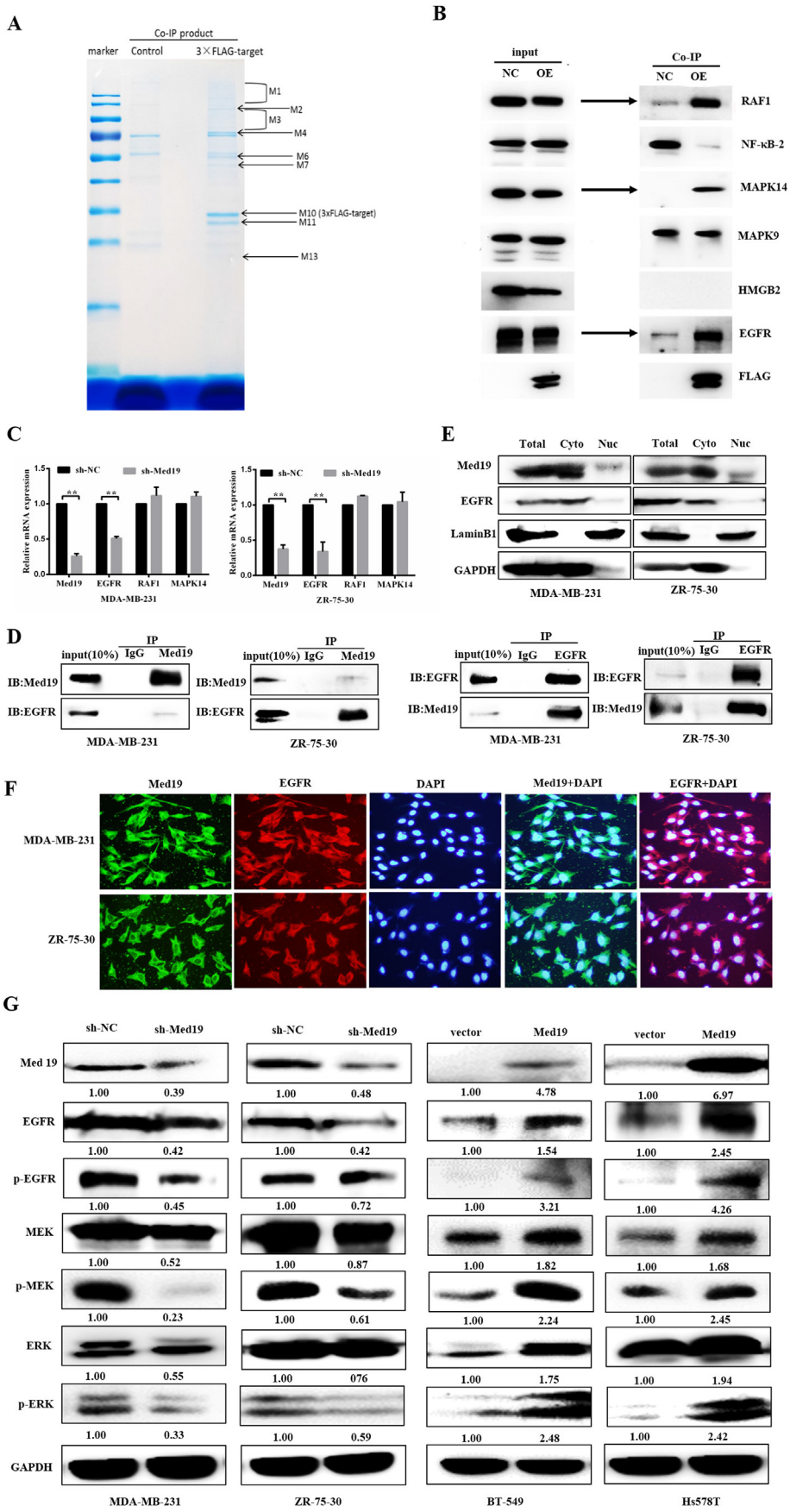

Fig. 3. Med19 interacts with EGFR and activates the EGFR signaling pathway. (A) The extracts isolated using 3 × FLAG-target were separated by SDS-PAGE and visualized using Coomassie brilliant blue staining. (B) A co-IP assay was performed to verify the proteins that interacted with Med19. The blots are shown (n = 3). (C)QRT-PCR analysis of Med19, EGFR, RAF1 and MAPK14 expression in Med19 knockdown MDA-MB-231 and ZR-75-30 cells (means ± SD. n = 3. Student's *t*-test. $^{**}P < 0.01$). (D) Co-IP of endogenous Med19 and EGFR in extracts of MDA-MB-231 and ZR-75-30 cells, which both have high Med19 expression. Western blotting was used to confirm the presence of Med19 and EGFR in the co-IPs (n = 3). (E) Western blots showing Med19 and EGFR levels in the total, cytoplasmic and nuclear fractions of MDA-MB-231 and ZR-75-30 cells (n = 3). (F) Immunofluorescence staining for Med19 and EGFR (200× magnification. n = 3). (G) Western blot analysis of EGFR, p-EGFR, p-MEK/MEK and p-ERK/ERK levels after the down-regulation of Med19 in MDA-MB-231 and ZR-75-30 cells (means ± SD. n = 3. Student's *t*-test). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

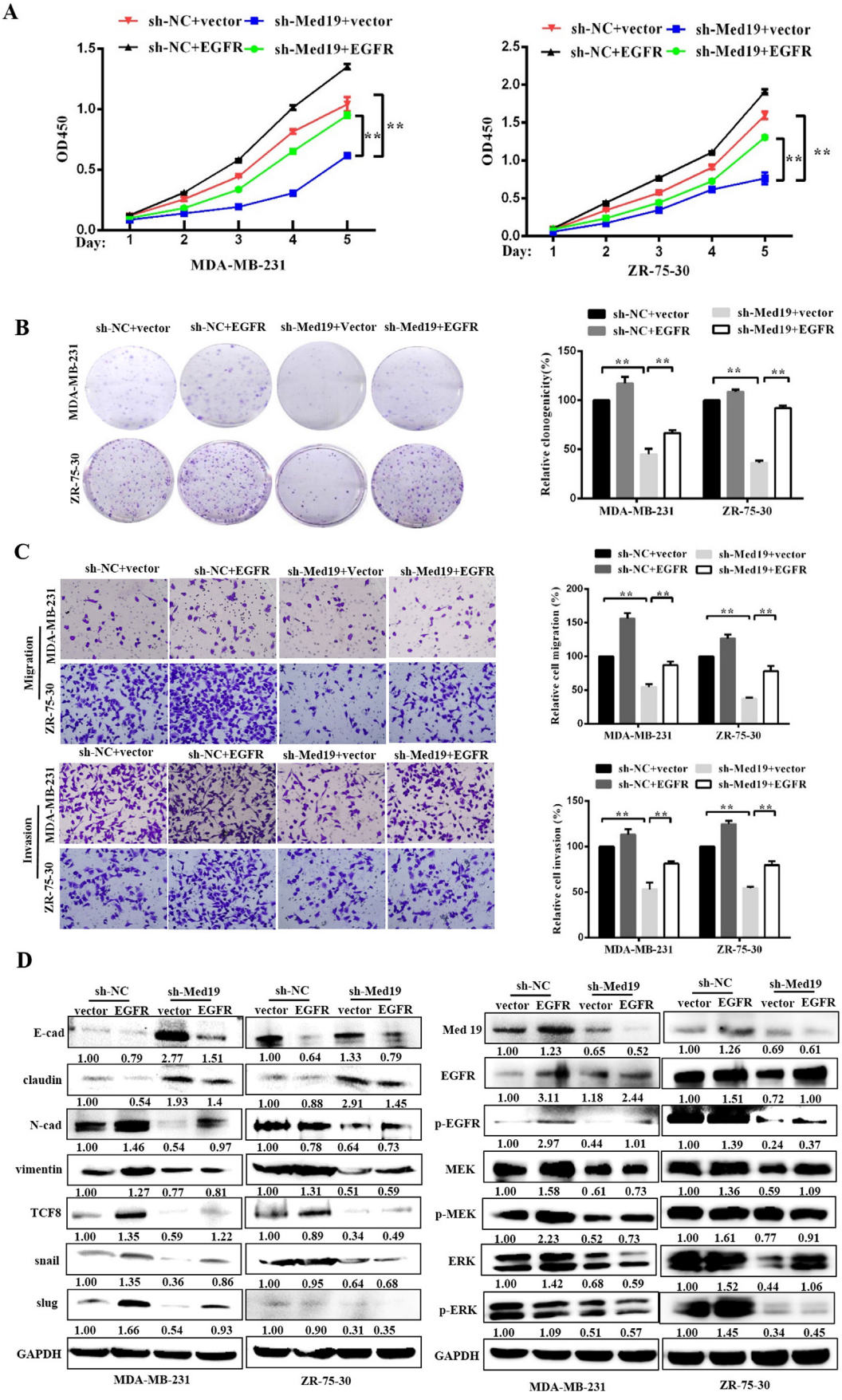

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Fig. 4. Overexpression of EGFR effectively reverses Med19-induced breast cancer cell progression. MDA-MB-231 and ZR-75-30 cells were transfected with sh-NC + vector, sh-NC + EGFR, sh-Med19 + vector, or sh-Med19 + EGFR. Cell proliferation, migration and invasion were detected using CCK-8 (A), colony formation (B), transwell migration (C, upper panel), and Matrigel invasion assays (C, lower panel), and the results of the quantitative analysis are shown (means ± SD. n = 3. One-way ANOVA with Bonferroni's test. $^{**}P < 0.01$). (D) Western blots were performed to evaluate protein levels (means ± SD. n = 3. One-way ANOVA with Bonferroni's test).

nude mice using MDA-MB-231 cells infected with Med19 shRNA lentiviral vector or control. The same number of control and Med19-knockdown cells was injected into the lateral tail vein of 4- to 6-week-old female nude mice. After 5 weeks, the mice were sacrificed, and lung metastatic nodules were observed (Fig. 5B and C). Med19-knockdown cells formed markedly fewer metastatic lung colonies that did control cells strongly (Fig. 5C). The HE staining results also revealed the same tendency (Fig. 5C). Thus, our data indicated that downregulation of Med19 inhibits breast cancer tumor metastasis in vivo.

3.6. *Med19 is targeted by miR-101-3p/miR-422a, which directly bind its 3′-UTR*

We used the TargetScan, miRBase, microRNA.org and DIANA LAB databases to predict the miR-34a-5p, miR-101–3p, miR-214–3p, miR-339–5p and miR-422a binding sites in the 3′-UTR of Med19 and identify the regulators of Med19 expression that were responsible for the dysregulation of Med19 in breast cancer; these miRNAs have been reported to suppress breast cancer development. Notably, miR-339–5p, miR-101–3p and miR-422a, particularly miR-101–3p and miR-422a, decreased the fluorescence intensity in the luciferase reporter assay compared with the negative control group (Fig. 6A). Then, we confirmed that miR-101–3p and miR-422a directly targeted the 3′-UTR of Med19 using luciferase reporter assays (Fig. 6B and C). The overexpression of miR-101–3p and miR-422a suppressed the luciferase activity of Med19 3′-UTR reporter constructs, whereas the effects were abolished when the binding sites were mutated (Fig. 6C). In addition, miR-101–3p and miR-422a mimics reduced the expression of the Med19 mRNA and protein in MDA-MB-231 cells, as determined using qRT-PCR and Western blotting analyses, whereas the miRNA inhibitors had the opposite effects on BT-549 cells (Fig. 6D and E).

To better understand the correlation of Med19, EGFR, miR-101–3p and miR-422a in breast cancer patients, we measured their expression using qRT-PCR in a cohort of 64 breast cancer tissues. EGFR expression positively correlated with Med19 expression (Fig. 6F, Pearson's correlation analysis, $r = 0.5624$, $P < 0.001$), suggesting that Med19 positively regulates EGFR expression. In addition, the expression of both miR-101–3p and miR-422a showed inverse correlations with Med19 expression in patients with breast cancer (Fig. 6G, Pearson's correlation analysis, $r = -0.2724$, $P = 0.0295$; Fig. 6H, Pearson's correlation analysis, $r = -0.3459$, $P = 0.0051$), indicating that miR-101–3p and miR-422a negatively regulate Med19 expression. Based on these results, Med19 upregulation in breast cancer may be caused by miR-101–3p and miR-422a downregulation.

4. Discussion

Although we previously reported that the inhibition of Med19 expression could augment the population of cells in G0/G1 phase and significantly reduce the growth of breast cancer cells [6], the effects and mechanism of Med19 in breast cancer progression required further investigation. In this study, we found that Med19 was upregulated in human breast cancer tissues and cells compared with adjacent non-tumor tissues and non-tumorigenic breast cells. High Med19 expression in patients with breast cancer predicted a poor prognosis and was associated with the tumor size and tumor grade. Our results showed that

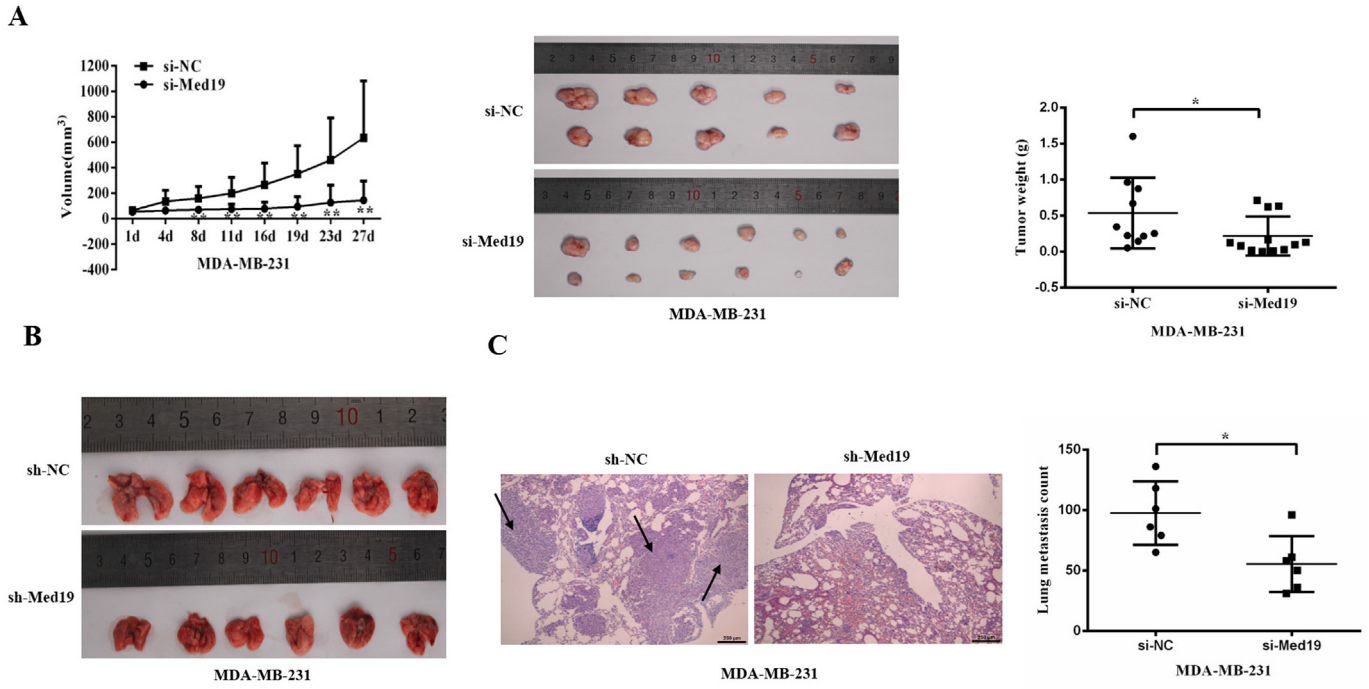

Fig. 5. Med19 knockdown attenuates the malignant progression of breast cancer in vivo. (A) The tumor volumes measured at different time points (means ± SD. n = 6. Student's *t*-test. $^{**}P < 0.01$). Photographs of neoplasms in xenograft mice after the injection of the Med19 siRNA or scrambled sequences and the final tumor weights are shown (means ± SD. n = 6. Student's *t*-test. $^{*}P < 0.05$). (B) Photographs of metastatic lung nodules in SCID mice injected with MDA-MB-231 cells infected with lentivirus-sh-Med19 or the control vector. (C) Arrowheads indicate metastatic nodules in the lung stained with HE. The number of metastatic nodules in the lung was calculated, and the results are shown in the right panel (means ± SD. n = 6. Student's *t*-test. $^{*}P < 0.05$).

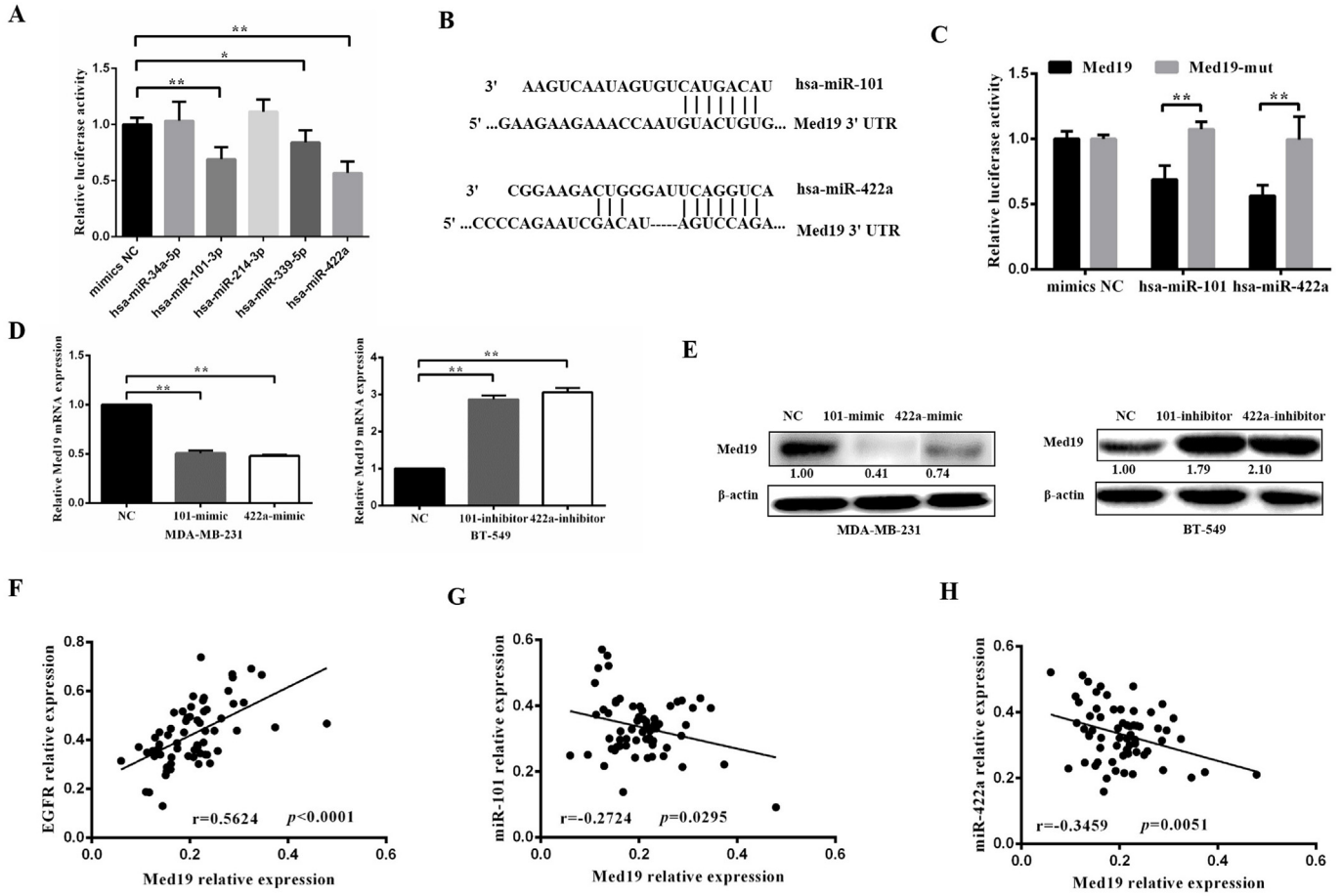

Fig. 6. MiR-101–3p and miR-422a regulate Med19 expression, and their expression levels negatively correlate with Med19 expression. (A) Luciferase activity in 293T cells cotransfected with a luciferase reporter containing the wild-type Med19 3′-UTR and miRNA mimics or the control (means ± SD. n = 3. One-way ANOVA with Bonferroni's test. $^{**}P < 0.01$, $^{*}P < 0.05$). (B) The potential binding sequences for miR-101–3p and miR-422a within the human Med19 3′-UTR (means ± SD. n = 3. One-way ANOVA with Bonferroni's test. $^{**}P < 0.01$). (C) Luciferase activity in 293T cells cotransfected with a luciferase reporter containing Med19 sequences with wild-type or mutated miR-101–3p/miR-422a binding sites and miR-101–3p/miR-422a mimics or control sequences (means ± SD. n = 3. One-way ANOVA with Bonferroni's test. $^{**}P < 0.01$). (D) Med19 mRNA levels in MDA-MB-231 cells transfected with miR-101–3p/miR-422a mimics and BT-549 cells transfected with miR-101–3p/miR-422a inhibitors were determined using qRT-PCR (means ± SD. n = 3. One-way ANOVA with Bonferroni's test. $^{**}P < 0.01$). (E) Western blot showing the levels of the Med19 protein in MDA-MB-231 cells transfected miR-101–3p/miR-422a mimics and in BT-549 cells transfected miR-101–3p/miR-422a inhibitors (means ± SD. n = 3. One-way ANOVA with Bonferroni's test). (F) Med19, EGFR, miR-101–3p and miR-422a levels were analyzed in 64 breast cancer tissue samples using qRT-PCR. Pearson's correlation coefficients were calculated to analyze the correlations. Analysis of the correlation between Med19 and EGFR expression, $r = 0.5624$, $P < 0.001$. (G) Analysis of the correlation between Med19 and miR-101–3p expression, $r = -0.2724$, $P = 0.0295$. (H) Analysis of the correlation between Med19 and miR-422a expression, $r = -0.3459$, $P = 0.0051$.

Med19 promotes cell proliferation and enhances the migration and invasion capacities of human breast cancer. In cancer, EMT plays a crucial role in the tumorigenic process and is involved in tumor metastasis [19,20]. Here, we first delineated the role of Med19 in facilitating EMT and enhancing the metastatic potential of breast cancer. In vivo assays revealed that knockdown of Med19 could suppress tumor growth and distant metastases, which indicated that Med19 could be a therapeutic target.

To elucidate how overexpressing Med19 promotes aggressive behavior in breast cancer and to identify its partners that cause carcinogenesis, we performed co-IP and LC-MS assays and found that EGFR interacted with Med19. We further examined the expression and intracellular distribution of Med19 and EGFR by Western blotting and immunofluorescence. The main EGFR signals were observed in the cytoplasm, with only a few foci in the nucleus, as reported in previous studies [21,22]. Med19 was localized in both the cytoplasm and nucleus and colocalized with EGFR in the cytoplasm. Dysregulation of EGFR has been observed in breast cancer and has been associated with aggressive phenotypes and a poor prognosis [23–25]. EGFR is a well-characterized receptor tyrosine kinase (RTK) that has kinase activity and consequently induces an intracellular signal transduction cascade that participates in biological processes such as cell proliferation, differentiation, migration, adhesion, and angiogenesis [26]. The MEK/ERK pathway is a major signaling pathway that can be activated by EGFR signaling [27]. The EGFR/MEK/ERK pathway has been reported to be involved in tumor progression in a variety of cancers [28–30]. Here, our results showed that knockdown of Med19 levels obviously decreased EGFR expression and activation, and EGFR downregulation could affect the downstream pathway that it transduces. Western blotting analysis indicated that EGFR downregulation caused by Med19 inhibition resulted in decreased expression and activation of molecules in the MEK/ERK pathway. Rescue experiments showed that Med19 plays a role in the malignant progression of breast cancer in an EGFR/MEK/ERK signaling-dependent manner. Furthermore, the results showed that p-MEK expression was changed dramatically by Med19 knockdown or EGFR overexpression in MDA-MB-231 cells; however, p-ERK appeared to be less sensitive to Med19 knockdown than p-MEK in MDA-MB-231 cells, although the results were significant ($P < 0.05$) compared with the control group, which indicated that Med19 plays its role, at least in part, via an EGFR/MEK/ERK signaling-dependent

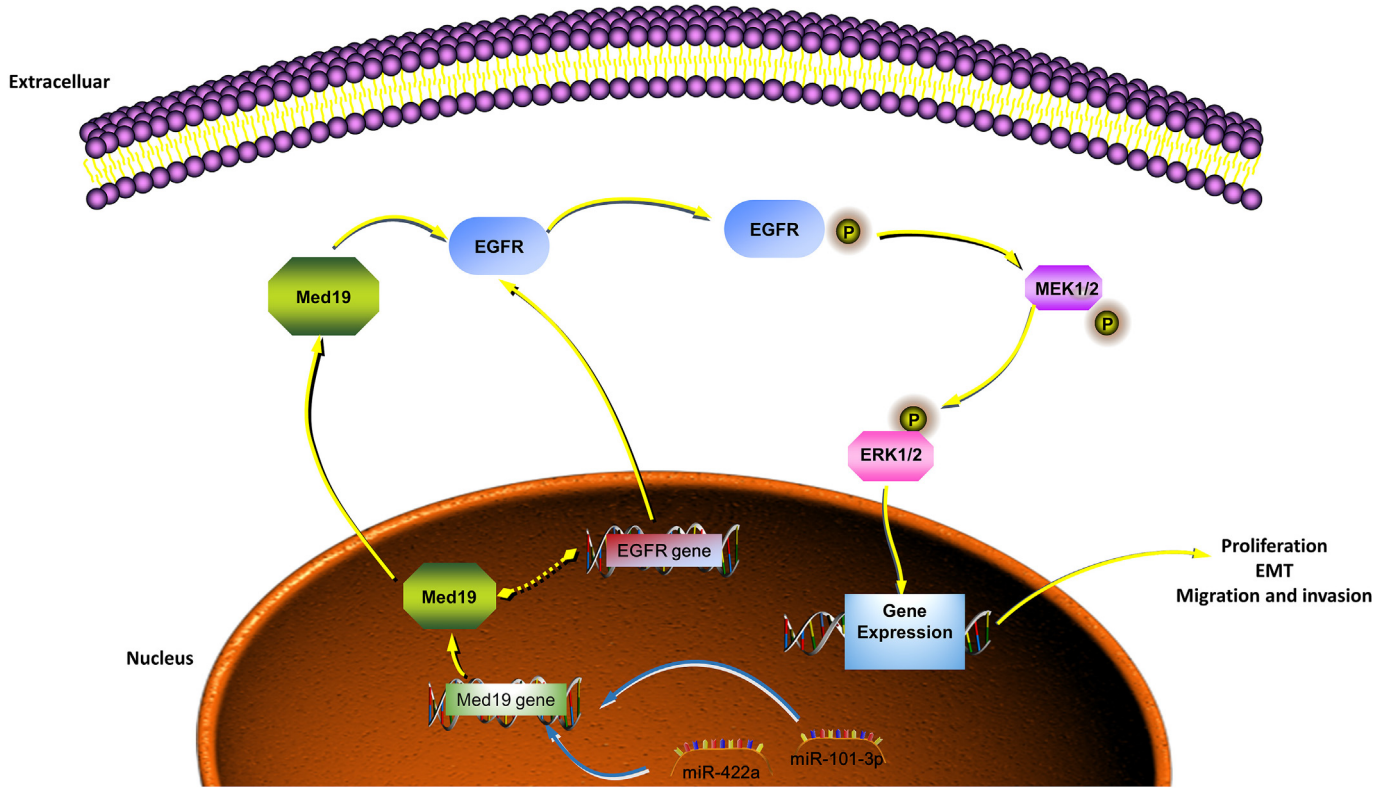

Fig. 7. Model of the mechanism by which Med19 promotes breast cancer progression.

manner, but another pathway may exist that mediates the function of Med19. The present study is the first to identify the partner of Med19. We speculate that, on the one hand, Med19 could physically interact with EGFR and activate EGFR/MEK/ERK signaling. On the other hand, Med19 could also regulate EGFR gene expression to further effect the EGFR pathway. In human prostate cancer, it has been reported that knockdown of Med19 suppresses androgen receptor target gene expression and inhibits the proliferation of androgen-dependent and castration-resistant prostate cancer cells [31]. Agaesse G. et al. reported that Med19 transcriptionally regulates Tspan8 gene expression and is implicated in cutaneous melanoma invasion [32], which suggests that Med19 may transcriptionally regulate EGFR gene expression or regulate other pathways that could affect EGFR gene expression. However, future investigations are required to further determine how Med19 regulates EGFR expression and activates EGFR/MEK/ERK signaling in breast cancer. Additionally, alterations in Med19 expression did not exert significantly effects on the proliferation capacity of non-tumorigenic breast cells, indicating that breast cancer cells are much more sensitive to Med19 regulation than non-tumorigenic human breast MCF-10A cells, possibly because EGFR is expressed at lower levels in non-tumorigenic MCF-10A human breast cells than in breast cancer cells.

We speculated that decreased expression of the negative regulator of Med19 might result in the upregulation of Med19 in breast cancer. MiRNAs negatively regulate gene expression via binding to complementary sequences in the 3′-UTR of target mRNAs, resulting in translational inhibition or degradation [14]. Accumulating evidence supports that miRNAs are frequently dysregulated in breast cancer; such miRNAs act as either tumor suppressors or oncogenes and are involved in tumorigenesis and cancer progression [33–35]. We predicted that several miRNAs would bind the 3′-UTR of Med19 and then confirmed that miR-101–3p and miR-422a targeted Med19 using a luciferase reporter assay. Levels of the Med19 mRNA and protein were decreased in MDA-MB-231 cells transfected with miR-101–3p and miR-422a mimics and increased in BT-549 cells transfected with miR-101–3p and miR-422a inhibitors. Recently, correlations between downregulated miR-101–3p and various cancers have increasingly been reported [36–38]. Notably, miR-101–3p is downregulated in breast cancer, and decreased miR-101–3p expression is associated with a poor prognosis [39]. MiR-422a has been frequently reported as a tumor suppressor in glioblastoma [40], osteosarcoma [41], colorectal cancer [42], gastric cancer [43] and breast cancer [44]. In our study, we detected the expression of both miR-101–3p and miR-422a in human breast cancer tissues and identified negative correlations with Med19 expression, indicating that Med19 upregulation in breast cancer might be mainly caused by miR-101–3p and miR-422a downregulation. Furthermore, there may be other signaling pathways, transcription factors and cancer suppressors that can negatively regulate Med19 expression. He et al. reported that miR-214 could target Med19 and suppress tumor growth and metastasis in colorectal cancer [45]. However, in our study, we could not verify that miR-214 binds the 3′-UTR of Med19 through a luciferase reporter assay in breast cancer, perhaps because the modulatory mechanism of miRNAs often involves many-to-many relationships and has tissue-specific characteristics.

In conclusion, Med19 expression was dramatically upregulated in human breast cancer tissue, at least partially due to the low expression of the tumor suppressors miR-101–3p and miR-422a. Med19 functions as a mediator of the crosstalk with and regulation of the EGFR/MEK/ERK signaling pathway to promote breast cancer progression (Fig. 7). Therefore, the miR-101–3p/miR-422a-Med19-EGFR/MEK/ERK axis exists in breast cancer, and Med19 represents a potential therapeutic target for breast cancer treatment.

Conflicts of interest

No potential conflicts of interest were disclosed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.12.008.

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