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ORIGINAL ARTICLE

Knockdown of FAM64A suppresses proliferation and migration of breast cancer cells

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Abstract

Background FAM64A is a mitotic regulator promoting cell metaphase–anaphase transition, and it is frequently reported to be highly expressed in cancer cells. However, the role of FAM64A in human breast cancer (BrC) is poorly studied.

Methods The expression of *FAM64A* mRNA in BrC samples was determined by RT-qPCR assay and TCGA database mining. Kaplan–Meier plotter was used to analyze whether FAM64A expression impacted prognosis. Then, the expression of FAM64A was silenced using RNA interference. Cell-counting assay, colony formation assay and flow cytometry assay were conducted to detect proliferation; transwell migration assay, EMT-related proteins expression (E-cadherin, N-cadherin and vimentin), and EMT-related transcription factors mRNA expression (*Snail*, *Twist*, *Slug*) were conducted to evaluate the migration ability.

Results FAM64A was highly expressed in human BrC samples, which was negatively associated with poor survival time. Analysis of FAM64A expression in BrC cell lines demonstrated that the expression of FAM64A was significantly correlated with the proliferation rate and migration ability of BrC cells. Indeed, knockdown of FAM64A suppressed the proliferation of MDA-MB-231 and MCF-7 cells. Importantly, we also found that silencing of FAM64A inhibited the migration of BrC cells via impeding epithelial–mesenchymal transition.

Conclusions Our findings suggest that FAM64A plays an important role in the proliferation and migration of BrC cells, which might serve as a potential target for BrC treatment.

Keywords Breast cancer · FAM64A · Proliferation · Migration · EMT

Introduction

The incidence of human breast cancer (BrC) has been rising since the 1970s, accounting for 24.2% of cancer global incidence and 15% of cancer-related mortality in females [1, 2]. It was estimated that there would be about 2.1 million newly diagnosed female BrC cases in 2018 [2]. Despite the earlier diagnosis and improvement in surgical techniques and adjuvant medical therapies, the prognosis of advanced BrC and metastasis issues are still a problem [3]. Therefore, exploring new targets for the development of BrC has become a compelling issue.

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FAM64A, also known as CATS [4] and RCS1 [5], is located on chromosome 17 band p13. Initially, FAM64A was first identified as a novel clathrin assembly lymphoid myeloid (CALM) interactor that influences the subcellular localization of the leukemogenic fusion protein CALM/AF10, suggesting that FAM64A plays an important role in CALM/AF10-mediated transformation [6]. Later, a study from Zhao et al. demonstrated that FAM64A is a substrate of the anaphase-promoting complex/cyclosome (APC/C) and a mitotic regulator that controls the transition of cells from metaphase to anaphase [5]. Besides, FAM64A was found to be strongly expressed in multiple cancers, including breast cancer [4, 7]. Previous reports mainly suggested that FAM64A promotes tumor growth and cell cycle progression [8–10]. Despite the study from Cheng et al. which indicated that FAM64A is significantly up-regulated in triple-negative BrC (TNBC) and predicts poor clinical outcome [7], whether FAM64A regulates the proliferation and migration of breast cancer cells remain elusive.

Thus, this present study aimed to determine the roles of FAM64A in mediating BrC cells proliferation and migration. To address these issues, the FAM64A expression was then knocked down in BrC MDA-MB-231 and MCF-7 cell lines using FAM64A siRNA, and the effect of FAM64A knockdown on BrC cells proliferation and migration was determined. This study identified a novel role of FAM64A in regulating BrC cell proliferation and migration and might provide a potential novel target for the treatment of BrC patients.

Bioinformatics analysis

The data of *FAM64A* mRNA expression in BrC tissues and normal tissues were obtained from the TCGA visual database (GEPIA [11] and cBioPortal [12]). The intrinsic subtype of BrC was classified based on estrogen receptor (ER), progesterone receptor (PR), and human epithelial growth factor receptor 2 (HER2) expression. BrC survival was analyzed using a publicly available database: Kaplan–Meier Plotter software (www.kmplot.com). FAM64A Affy id: 221591_s_at. The expression levels of *FAM64A* mRNA were standardized by TCGA and Kaplan–Meier Plotter database, respectively.

Cell lines and cell culture

The human BrC cell lines MDA-MB-231, BT-549, ZR-75-30, MCF-7, T-47D, and BT-474 were purchased from the American Type Culture Collection (Manassas, VA, USA) and the intrinsic subtypes of BrC cell lines are shown in Table 1. Each cell line was cultured in Dulbecco’s modified eagle’s medium (DMEM; ThermoFisher Scientific, USA) containing 10% fetal bovine serum (FBS; PAN-Biotech, Germany) and 1% penicillin/streptomycin in a 5% CO_2 humidified incubator at 37 °C. Different concentrations of insulin were added to BT-549, MCF-7 and T-47D cell medium to maintain cells’ healthy growth according to the instructions of American type culture collection (ATCC, https://www.atcc.org/).

Table 1 Intrinsic subtypes of BrC cell lines

Cell lines	ER[a]	PR[b]	HER2[c]	Intrinsic subtype
MDA-MB-231	–[d]	–	–	Basal-like
BT-549	–	–	–	Basal-like
MCF-7	+[e]	+	–	Luminal A
T-47D	+	+	–	Luminal A
ZR-75-30	+	–	+	Luminal B
BT-474	+	+	+	Luminal B

[a] Estrogen receptor

[b] Progesterone receptor

[c] Human epidermal growth factor receptor 2

[d] Negative

[e] Positive

Table 2 Primers of real-time PCR

Primers	Sequences
FAM64A-F	5′-TTCTCGGTGGCAGAACATGG-3′
FAM64A-R	5′-GTCTCCTGATGGCTGACCAC-3′
Snail-F	5′-CACTATGCCGCGCTCTTTC-3′
Snail-R	5′-GCTGGAAGGTAAACTCTGGATTAGA-3′
Slug-F	5′-CGAACTGGACACACATACAGTG-3′
Slug-R	5′-CTGAGGATCTCTGGTTGTGGT-3′
Twist-F	5′-GTCCGCAGTCTTACGAGGAG-3′
Twist-R	5′-GCTTGAGGGTCTGAATCTTGCT-3′
β-actin-F	5′-CACCACACCTTCTACAATGAGCTGC-3′
β-actin-R	5′-ACAGCCTGGATAGCAACGTACATGG-3′

RNA extraction, reverse transcription-polymerase chain reaction (PCR), and real-time quantitative PCR (RT-qPCR)

Total mRNA was extracted using Trizol reagent (Takara, Japan). cDNA was generated using HiScript II Q RT SuperMix for qPCR + gDNA wiper(Vazyme Biotech, China), according to the manufacturer’s instructions. RT-qPCR was performed using 2 × SYBR Green qPCR Master Mix (Bimake, USA) in a LightCycler96 (Roche, Switzerland). RT-qPCR was conducted using the primers shown in Table 2. Changes in the mRNA levels were determined by the $2^{-\Delta\Delta Cq}$ method using *β*-actin as the loading control.

Western blot analysis

Samples were lysed on ice in RIPA lysis buffer (KeyGEN, China) supplemented with protease inhibitors (1 mM PMSF). In all, equal amounts of protein per lane were run in 10% sodium dodecyl sulfate-polyacrylamide electrophoresis gels and subsequently transferred to a polyvinylidene difluoride membranes (Merck Millipore, Germany) via wet transfer. The membranes were blocked using 5% bovine serum albumin (dissolved in Tris buffered saline containing 0.1% Tween 20). The membranes were incubated overnight at 4 °C with various primary antibodies after blocking. Finally, the membrane was incubated with fluorescence secondary antibodies (LI-COR Biosciences, USA) for 1 h at room temperature (RT), according to the manufacturer's instructions. Specific immunoblotting signals were detected using the LI-COR Odyssey Near Infrared Imaging System (LI-COR Biosciences). The antibodies used were the following: anti-β-Actin (AC004; ABclonal), anti-FAM64A (R59860; Sigma), N-cadherin (Protein Tech, 22018-1-AP), vimentin (Protein Tech, 10366-1-AP), E-cadherin (Protein Tech, 60335-1-Ig), IRDye 680RD Goat anti-mouse (926-68070; LI-COR), and IRDye 800RD Goat anti-Rabbit (926-68071; LI-COR).

SiRNA transfection

MDA-MB-231 and MCF-7 cells were grown in six-well plates to almost 70–80% confluence. Cells were transfected with 30 pmol of the negative control siRNA or FAM64A siRNA using the Lipofectamine 3000 Transfection Reagent (Invitrogen, USA), according to the manufacturer's instructions. Three different target siRNA sequences of FAM64A were obtained from Synbio Co. Ltd. The siRNA transfection was conducted using the primer sequences shown in Table 3. After 48 h, the cells were harvested, washed two times with cold phosphate-buffered saline (PBS), and lysed in lysis buffer.

Cell-counting assay

MDA-MB-231 and MCF-7 cells were seeded at 4000 cells per well in 96-well plates. Si-FAM64A and Si control was transfected according to the number of cells after 12 h of seeding. The cell-counting kit-8 (CCK-8; Bimake) was used to measure the optical densities (ODs) at 0, 24, 48, 72, and 96 h. CCK-8 (10 μl) reagent was added to each well followed by a 2-h incubation at 37 °C, and the absorbance was measured at 450 nm wavelength. Highest and lowest OD values in each set of data were eliminated to reduce the standard deviation.

Table 3 Primers of siRNA transfection

Primers	Sequences
FAM64A Si-1	5′-UCCUGGAAACGCCUGGAAATT-3′
FAM64A Si-2	5′-GCUCAGCUAAGAGUGCUUUTT-3′
FAM64A Si-3	5′-UCUCCAGAAGCUGUCCCAATT-3′
Negative control	5′-UUCUCCGAACGUGUCACGUT-3′

Plate colony formation assay

Cells were transfected with siRNA for 48 h according to the method described above, digested with trypsin and cell counting was performed after transfection. Cells were seeded at 200 cells per well in 6-well plates. MDA-MB-231 cells were cultured for 10 days, whereas MCF-7 cells were cultured for 20 days. The cells were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet solution, and washed with deionized water to obtain an image. Colonies visible to the eye were counted.

Flow cytometry analysis

Cells were trypsinized, centrifuged at 1000 rpm for 5 min at RT, and washed twice with cold PBS. The cells were fixed in ice-cold 70% ethanol at 4 °C overnight. Each sample was washed two times with cold PBS and incubated in propidium iodide/RNase staining buffer solution in the dark for 1 h at RT. The cell cycles were detected by flow cytometry (BD LSRFortessa X-20; USA) using a 488-nm excitation wavelength and analyzed using FlowJo version 7.6 software.

Transwell migration assay

5×10^4 cells were seeded in the upper chamber (24-well insert, 8 μm, Corning Costar). A medium with 10% FBS was used as an attractant in the lower chamber. After being incubated for 12 h, cells invaded through the membrane were fixed with 4% polyformaldehyde and stained with crystal violet, then the stained cell images were captured by a microscope (NiKon, Japan) and nine random fields at 20× magnification were counted. Results shown were the average from at least three independent experiments. Error bars represented the standard deviation.

Statistical analysis

Statistical analysis was performed using SPSS 20.0 software. The error bars in the graphical data represent the mean ± standard deviation (SD), which was obtained from at least three independent experiments. Two-tailed student's *t* test was used to analyze the significance of two individual groups. One-way ANOVA was used to compare multiple groups. Correlations were determined by Pearson's correlation. $P < 0.05$ was considered statistically significant.

Results

FAM64A was overexpressed in breast tumor samples and associated with poor prognosis of BrC patients

To explore the relationship between FAM64A expression and BrC malignancy, the TCGA visual database GEPIA and cBioPortal were adopted. As shown in Fig. 1a, the *FAM64A* mRNA expression level in patients with BrC ($n = 1085$) was significantly higher than that in patients without BrC ($n = 291$) ($P < 0.05$). Consistent with the findings in BrC tissues, the mRNA level of *FAM64A* in BrC cell lines was markedly higher than that in non-cancerous breast epithelial cell line (MCF-10A) ($P < 0.0001$, Fig. 1b). Also, a Kaplan–Meier plotter survival analysis of breast carcinoma tissues revealed that higher *FAM64A* expression was associated with reduced overall survival (OS) (HR 1.25, 95% CI 1.01–1.55, $P = 0.038$), distance metastasis-free survival

Fig. 2 The expression of FAM64A was correlated with the proliferation and migration of BrC cells. **a** *FAM64A* mRNA expression from TCGA RNA-Seq in four BrC intrinsic subtypes was shown, $^{****}P < 0.0001$ vs. basal-like, one-way ANOVA was used. **b** The expression of FAM64A was detected by Western blot assay in six BrC cell lines. $^{**}P < 0.01$ vs. basal-like, one-way ANOVA was used. **c** OD value of six BrC cell lines was detected by CCK-8 assay. OD value represented the cell proliferation. **d** Migration cells of six BrC cell lines were detected by transwell migration assay. Migration cells represented cell migration ability. **e** Linear correlation analysis was shown between both FAM64A expression and CCK-8 OD value. $r = 0.6467$, $P = 0.0037$, Pearson's correlation was used. **f** Linear correlation analysis was shown between both FAM64A expression and migration cells. $r = 0.7641$, $P = 0.0002$, Pearson's correlation was used. Data represent the mean ± SD of triplicate samples ▶

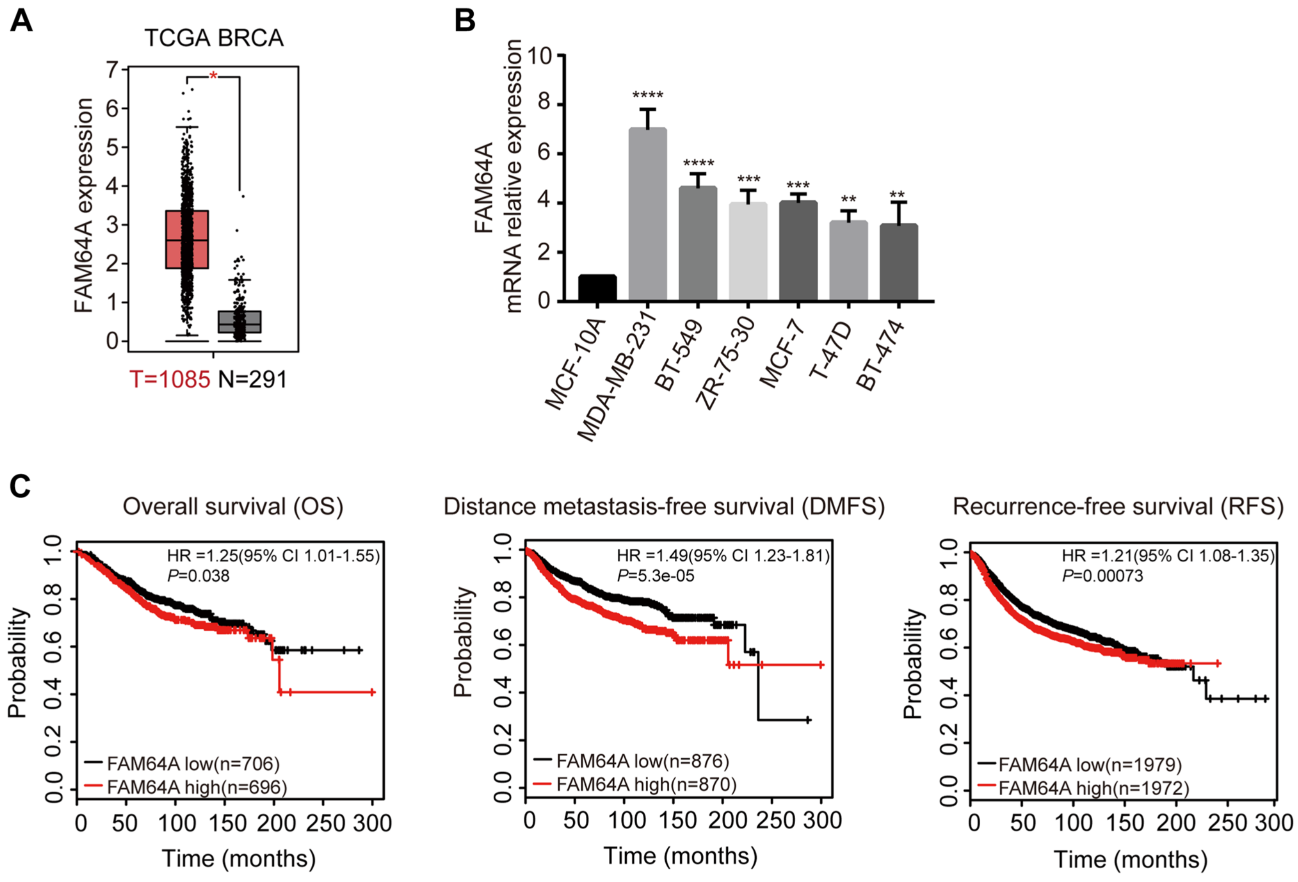
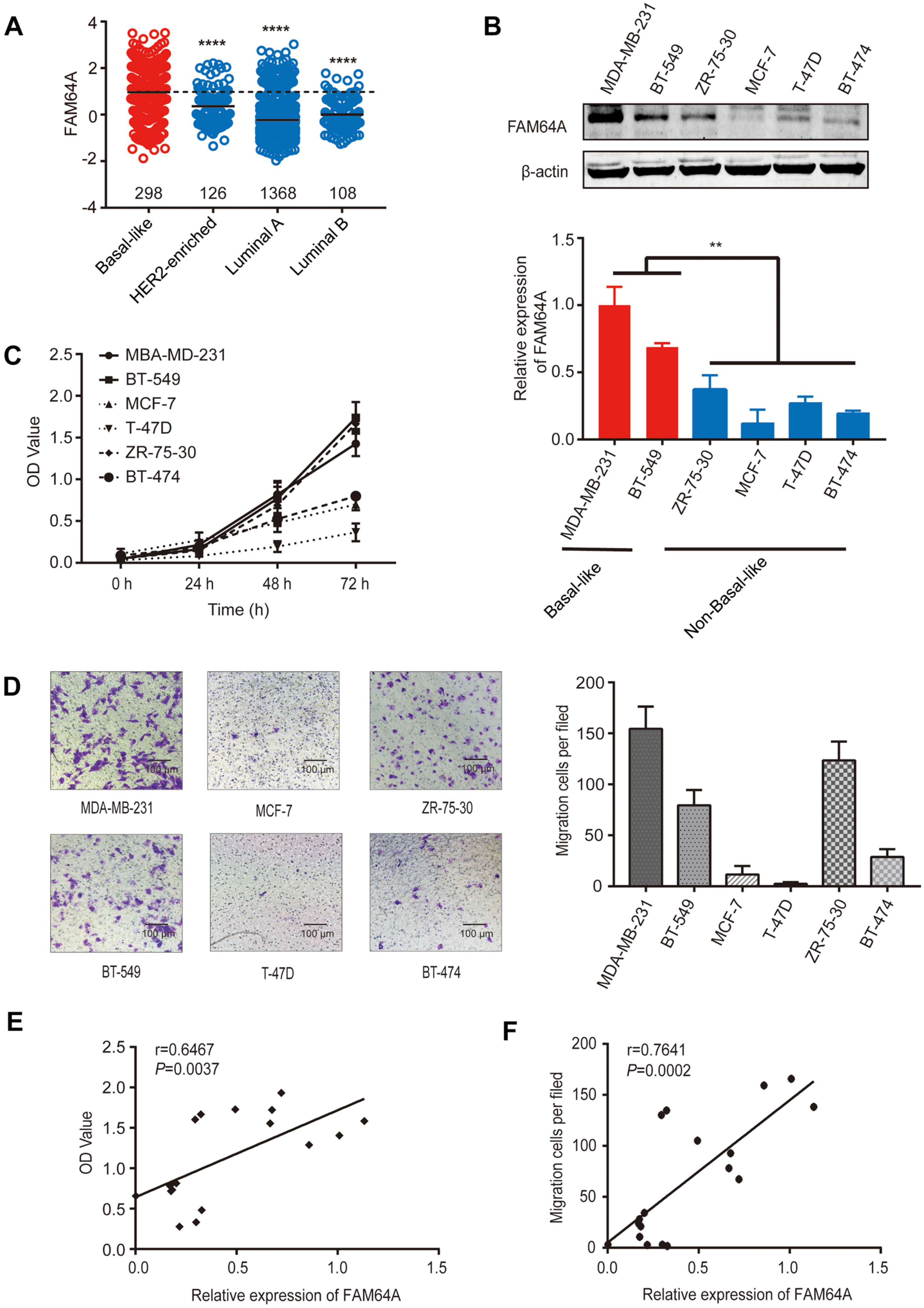


Fig. 1 FAM64A expression was elevated in human BrC and correlated with poor prognosis. **a** *FAM64A* mRNA expression from patients with BrC ($n = 1085$) in contrast to patients without BrC ($n = 291$) in TCGA data set, $^{*}P < 0.05$ vs. N (normal), *t* test was used. **b** The relative expression of *FAM64A* mRNA was detected by RT-qPCR in both non-cancerous breast cell and BrC cell lines. Data represent the mean ± SD of triplicate samples, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ vs. MCF-10A, one-way ANOVA was used. **c** Survival analysis (OS, DRFS, RFS) of breast carcinoma tissues from Kaplan–Meier plotter database are shown, respectively. The mRNA expression of *FAM64A* above or below the median divides the cases into high and low expression, respectively. *HR* hazard ratio, *CI* confidence interval, log-rank test was used in survival analysis


A
FAM64A
4
2
0
-2
-4
****
****
****
298
126
1368
108
Basal-like
HER2-enriched
Luminal A
Luminal B
B
MDA-MB-231
BT-549
ZR-75-30
MCF-7
T-47D
BT-474
FAM64A
β-actin
Relative expression of FAM64A
1.5
1.0
0.5
0.0
**
MDA-MB-231
BT-549
ZR-75-30
MCF-7
T-47D
BT-474
Basal-like
Non-Basal-like
C
OD Value
2.5
2.0
1.5
1.0
0.5
0.0
MBA-MD-231
BT-549
MCF-7
T-47D
ZR-75-30
BT-474
0 h
24 h
48 h
72 h
Time (h)
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100 μm
MDA-MB-231
MCF-7
ZR-75-30
BT-549
T-47D
BT-474
Migration cells per filed
200
150
100
50
0
MDA-MB-231
BT-549
MCF-7
T-47D
ZR-75-30
BT-474
E
OD Value
2.5
2.0
1.5
1.0
0.5
0.0
r=0.6467
P=0.0037
0.0
0.5
1.0
1.5
Relative expression of FAM64A
F
Migration cells per filed
200
150
100
50
0
r=0.7641
P=0.0002
0.0
0.5
1.0
1.5
Relative expression of FAM64A

(DMFS) (HR 1.49, 95% CI 1.23–1.81, $P = 5.3e{-}05$), and recurrence-free survival (RFS) (HR 1.21, 95% CI 1.08–1.35, $P = 0.00073$) in patients with BrC, respectively (Fig. 1c). In brief, these results suggested that *FAM64A* mRNA is overexpressed in BrC and could serve as a cancer biomarker in predicting a worse outcome for patients with BrC.

The expression of FAM64A was correlated with the proliferation and migration of BrC cells

As our data shown, FAM64A was overexpressed in breast tumor samples and associated with poor prognosis. More importantly, the *FAM64A* mRNA expression in basal-like intrinsic subtype BrC, which led to a worst outcome [13–15], was significantly higher than that in other subtypes BrC ($P < 0.0001$, Fig. 2a). Consistent with the finding in TCGA database, the FAM64A protein expression in basal-like subtype MDA-MB-231 and BT-549 cells was significantly higher than that in non-basal-like subtypes BrC ZR-75-30, MCF-7, T-47D, and BT-474 cells too ($P < 0.001$, Fig. 2b). Since the basal-like intrinsic subtype BrC is usually associated with rapid growth and tumor metastasis [13], we next investigated whether the expression of FAM64A correlated with the proliferation and migration of BrC cells. Cell-counting assay and transwell migration assay were conducted to determine the proliferation rate (Fig. 2c) and migration ability (Fig. 2d) of six BrC cell lines, respectively. Interestingly, Pearson's correlation analysis indicated that the expression of FAM64A significantly correlated with the proliferation rate ($r = 0.6467$, $P = 0.0037$, Fig. 2e) and migration ability ($r = 0.7641$, $P = 0.0002$, Fig. 2f) of BrC cells. These results suggested that FAM64A could be involved in regulating the proliferation and migration of BrC cells. Notably, MDA-MB-231 cells exhibited relatively highest FAM64A expression, whereas MCF-7 cells exhibited relatively lowest FAM64A expression among the six BrC cell lines and thus were chosen as the experimental model for further study.

Fig. 4 Knockdown of FAM64A inhibited the proliferation of BrC ▶ cells by prolonging the G1 cell phase. **a**, **b** Cell proliferation OD values were detected by CCK-8 assay in both MDA-MB-231 and MCF-7 cells. **c**, **d** Plate colony formation assays were detected in both MDA-MB-231 and MCF-7 cells. **e**, **f** The percentage of cells on different cell cycle phases was confirmed by flow cytometry assay in both the MDA-MD-231 and MCF-7 cells. Data represent the mean ± SD of triplicate samples, *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$, ns = no significant vs. Si control, *t* test was used to compare two individual groups and one-way ANOVA was used to compare multiple groups

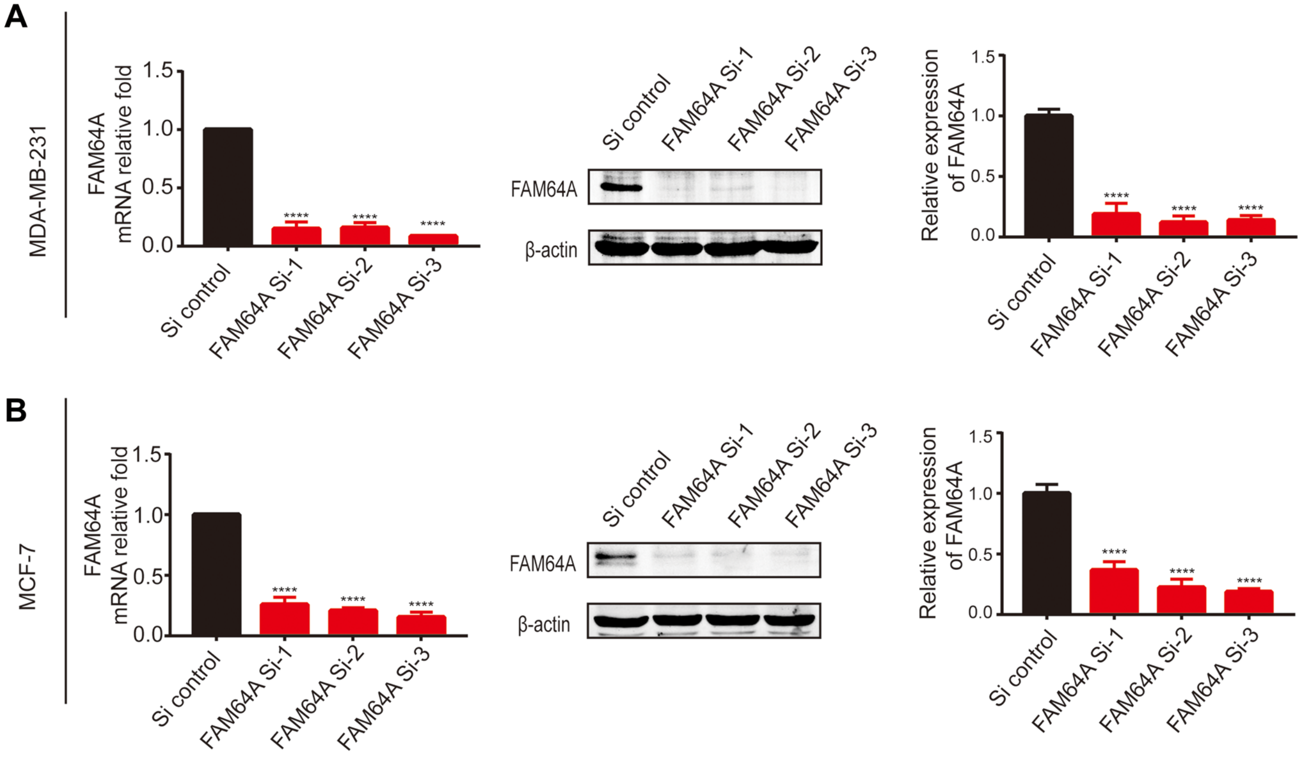
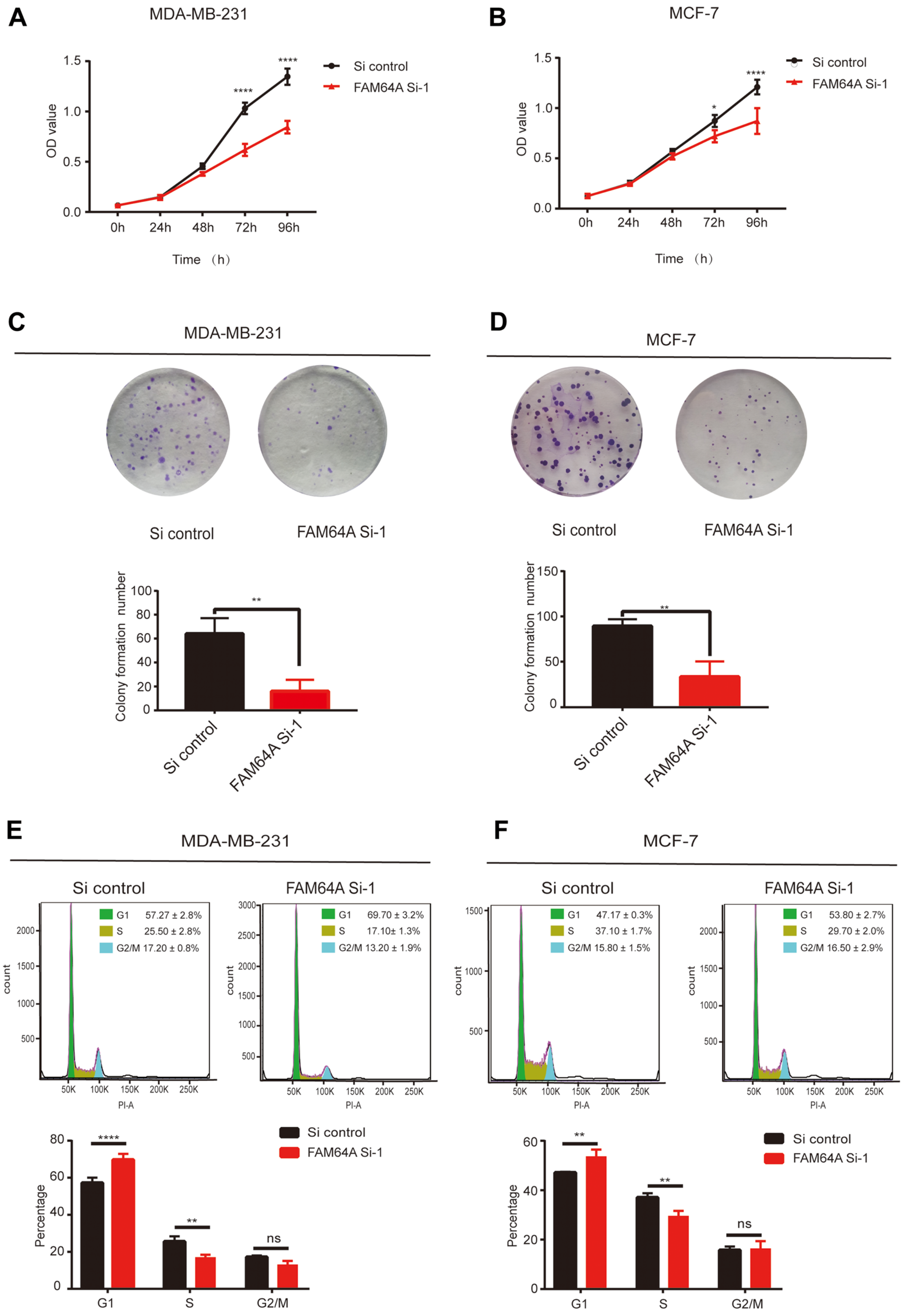


Fig. 3 Expression of FAM64A was inhibited successfully in both MDA-MB-231 and MCF-7 cells. **a** Knockdown efficiency of FAM64A in MDA-MB-231 cells, as assessed by RT-qPCR and Western blot assays. **b** Knockdown efficiency of FAM64A in MCF-7 cells, as assessed by RT-qPCR and Western blot assays. Data represent the mean ± SD of triplicate samples, ****$P < 0.0001$ vs. Si control, Si control: siRNA Negative control, one-way ANOVA was used


A
MDA-MB-231
OD value
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1.0
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0.0
0h
24h
48h
72h
96h
Time （h）
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Si control
FAM64A Si-1
B
MCF-7
OD value
1.5
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96h
Time （h）
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Si control
FAM64A Si-1
C
MDA-MB-231
Si control
FAM64A Si-1
Colony formation number
100
80
60
40
20
0
**
Si control
FAM64A Si-1
D
MCF-7
Si control
FAM64A Si-1
Colony formation number
150
100
50
0
**
Si control
FAM64A Si-1
E
MDA-MB-231
Si control
G1 57.27 ± 2.8%
S 25.50 ± 2.8%
G2/M 17.20 ± 0.8%
count
PI-A
FAM64A Si-1
G1 69.70 ± 3.2%
S 17.10± 1.3%
G2/M 13.20 ± 1.9%
count
PI-A
50K
100K
150K
200K
250K
Percentage
80
60
40
20
0
****
**
ns
G1
S
G2/M
Si control
FAM64A Si-1
F
MCF-7
Si control
G1 47.17 ± 0.3%
S 37.10 ± 1.7%
G2/M 15.80 ± 1.5%
count
PI-A
FAM64A Si-1
G1 53.80 ± 2.7%
S 29.70 ± 2.0%
G2/M 16.50 ± 2.9%
count
PI-A
Percentage
60
40
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ns
G1
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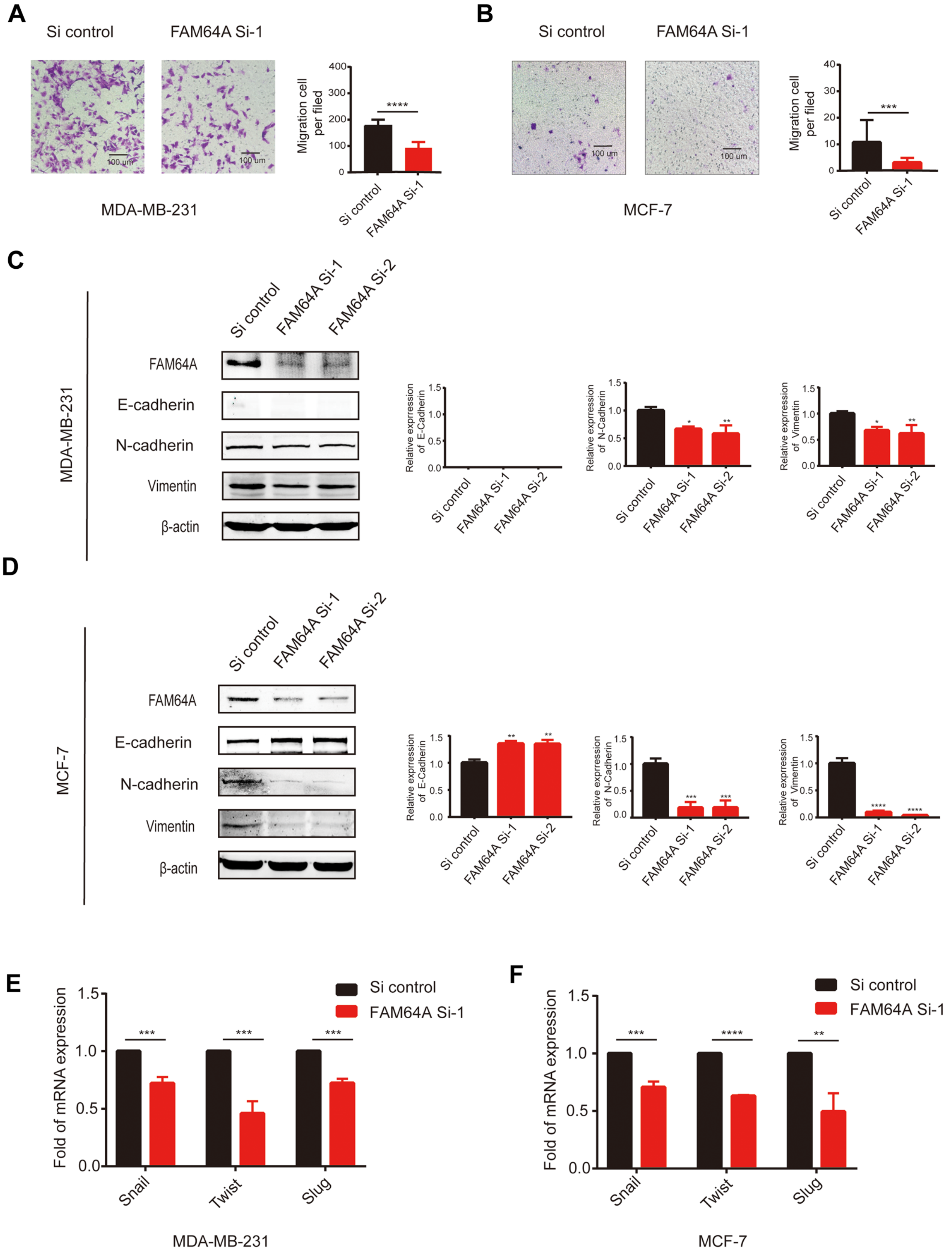
G2/M
Si control
FAM64A Si-1


A
Si control
FAM64A Si-1
100 um
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Migration cell per filed
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300
200
100
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Si control
FAM64A Si-1
MDA-MB-231
B
Si control
FAM64A Si-1
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Migration cell per filed
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Si control
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MDA-MB-231
Si control
FAM64A Si-1
FAM64A Si-2
FAM64A
E-cadherin
N-cadherin
Vimentin
β-actin
Relative expression of E-Cadherin
Relative expression of N-Cadherin
Relative expression of Vimentin
1.5
1.0
0.5
0.0
*
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D
MCF-7
Si control
FAM64A Si-1
FAM64A Si-2
FAM64A
E-cadherin
N-cadherin
Vimentin
β-actin
Relative expression of E-Cadherin
Relative expression of N-Cadherin
Relative expression of Vimentin
**
***
****
E
Fold of mRNA expression
1.5
1.0
0.5
0.0
Si control
FAM64A Si-1
***
Snail
Twist
Slug
MDA-MB-231
F
Fold of mRNA expression
Si control
FAM64A Si-1
***
****
**
Snail
Twist
Slug
MCF-7

◂**Fig. 5** Knockdown of FAM64A inhibited the migration of BrC cells. **a**, **b** Migration ability was detected by transwell assay in MDA-MB-231 and MCF-7 FAM64A-Si cells. **c**, **d** Western blot analysis of total cell extracts in MDA-MB-231 and MCF-7 FAM64A-Si cells was shown. Membrane was blotted against anti-FAM64A, anti-E-cadherin, anti-N-cadherin, anti-vimentin. **e**, **f** Relative expression of *Snail, Twist, Slug* mRNA in MDA-MB-231 and MCF-7 FAM64A-Si cells was detected by RT-qPCR assay. Data represent the mean ± SD of triplicate samples, $*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$ vs. Si control, *t* test was used to compare two individual groups and one-way ANOVA was used to compare multiple groups

Knockdown of FAM64 suppressed the proliferation of BrC cells by arrested cell cycle

To further identify the biological significance of FAM64A in BrC cells, we used siRNA to knockdown the expression of FAM64A in BrC cells. As shown in the results, FAM64A expression at both the mRNA and protein levels was markedly lower in the MDA-MB-231 ($P<0.0001$, Fig. 3a) and MCF-7 cells ($P<0.0001$, Fig. 3b) after transfection with FAM64A siRNA, indicating that we successfully suppressed the expression of FAM64A using small interfering RNAs. Results from cell-counting assay and plate colony formation assay demonstrated that knockdown of FAM64A inhibited the proliferation (MDA-MB-231: $P<0.0001$, Fig. 4a; MCF-7: $P<0.0001$, Fig. 4b) and colony formation ability (MDA-MB-231: $P<0.01$, Fig. 4c; MCF-7: $P<0.01$, Fig. 4d) of MDA-MB-231 and MCF-7 cells. Consistently, flow cytometry assay showed that knockdown of FAM64A arrested the cell cycle progression at G1 phase in both MDA-MB-231 (57.27 ± 2.8% vs. 69.70 ± 3.2%, $P<0.0001$, Fig. 4e) and MCF-7 cells (47.17 ± 0.3% vs. 53.80 ± 2.7%, $P<0.01$, Fig. 4f). These results indicated that knockdown of FAM64 suppressed the proliferation and colony formation ability of BrC cells by arresting the cell cycle progression in G1 phase.

Knockdown of FAM64A inhibited the migration of BrC cells

To further investigate whether FAM64A involved cell migration, transwell migration assay was used to assess the alteration of BrC cells migration ability after knockdown of FAM64A. Results from transwell migration assay demonstrated that knockdown of FAM64A significantly inhibited migration of BrC cells of both MDA-MB-231 ($P<0.0001$, Fig. 5a) and MCF-7 cells ($P<0.001$, Fig. 5b). As widely accepted, epithelial–mesenchymal transition (EMT) has a great effect on cell migration, thus we detected the expression of EMT marker, E-cadherin, N-cadherin and vimentin, by Western blot analysis. The results revealed that knockdown of FAM64A significantly down-regulated mesenchymal marker N-cadherin (MDA-MB-231: $P<0.01$, Fig. 5c; MCF-7: $P<0.001$, Fig. 5d) and vimentin (MDA-MB-231: $P<0.01$, Fig. 5c; MCF-7: $P<0.0001$, Fig. 5d) expression and up-regulated epithelial marker E-cadherin (MCF-7: $P<0.01$, Fig. 5d) expression in MDA-MB-231 and MCF-7 cells. Consistently, RT-qPCR showed that mRNA expression of EMT transcriptional factors (TFs), *Snail* (MDA-MB-231: $P<0.001$, Fig. 5e; MCF-7: $P<0.001$, Fig. 5f), *Twist* (MDA-MB-231: $P<0.001$, Fig. 5e; MCF-7: $P<0.0001$, Fig. 5f), *Slug* (MDA-MB-231: $P<0.001$, Fig. 5e; MCF-7: $P<0.01$, Fig. 5f) were down-regulated when FAM64A was knocked down in both MDA-MB-231 and MCF-7 cells. These results suggested that knockdown of FAM64A suppressed migration of BrC cells via impeding EMT transition.

Discussion

Previous studies have shown that FAM64A is involved in the development and progression of leukemia and other tumors [4, 6, 9, 16], but there was no direct evidence of the carcinogenic mechanism of the FAM64A gene in BrC, especially in migration. Therefore, the function of FAM64A in BrC is still far from clear.

FAM64A was reported to be highly expressed in cancer cell lines from a previous report [16]. Consistently, our results also revealed that FAM64A was highly expressed in BrC samples, suggesting that FAM64A may be closely associated with BrC development and progression. Additionally, data from Kaplan–Meier plotter demonstrated that increased expression of FAM64A was a risk factor associated with poor prognosis in patients with BrC. Therefore, these results suggested that *FAM64A* gene can serve as a specific oncogene involved in BrC progression.

BrC consists of four different intrinsic subtypes, each with different biological characteristics [17]. It is worth noting that HER2 gene amplification type BrC ZR-75-30 cells also proliferates and migrates aggressively, but the expression of FAM64A protein is less induced than that of triple-negative breast cancer (TNBC) MDA-MB-231 and BT-549 cells (TNBC cell lines). TNBC is characterized by poor overall survival, and most importantly, frequent distant metastasis [18]. Data in this study demonstrated FAM64A protein was highly expressed in TNBC cells, suggesting the effect of FAM64A is more inclined to mediate cells migration than proliferation.

The study of Arcangelo et al. revealed that FAM64A is highly expressed in proliferating cells, but not in quiescent cells, suggesting a role of FAM64A in the control of cell proliferation and can be viewed as a marker of proliferation [16]. In our study, a high expression of FAM64A was also found in rapid growth cell lines, which suggested that

FAM64A plays a role in excessive cancer cell proliferation. Thus, we knockdown the expression of FAM64A and the results demonstrated that FAM64A depletion inhibited the proliferation and colony formation of MDA-MB-231 and MCF-7 cells as the result of G_1 phase arrested, and these results suggested that FAM64A might serve as an oncogene in BrC cells and may greatly facilitate the proliferation and cell cycle progression. Cell cycle is orchestrated by a complex network of interactions between proteins, including cyclins, CDK activating kinase, CDK inhibitors, E3 ubiquitin ligase complexes and CDC25 phosphatases [19]. Any disturbance of the network could potentially lead to excess cell proliferation and tumor formation [20]. A study from Isabella Barbutti et al. demonstrated that FAM64A depletion reduced proliferation of U937 cells as the result of decreased expression of Cyclin A, E and B1 proteins, which is responsible for cell entry and progression in the S-phase and mitosis, respectively [9]. Besides, as the substrate of the APC/C, FAM64A controlled the metaphase-to-anaphase transition of HeLa cells [5]. Although the study of Zhao et al. demonstrated that FAM64A depletion resulted in a faster mitosis metaphase-to-anaphase transition [5], our data showed no additional difference in the G_2/M-phase but G_1-phase was prolonged. We reasoned that the pathway FAM64A affected could have been changed and resulted in the cells being arrested in G_1-phase, while the G_2/M-phase is too short to detect the alteration.

BrC is also a disease with frequent recurrence and metastasis, and 6% of patients were found to have an increased risk of metastasis when diagnosed with BrC [21]. At the onset of a metastatic cascade, epithelial cells requires EMT [22, 23], which is an essential hallmark during the development of cancer [24]. The process of EMT has been associated with the loss of epithelial features such as the cell junction molecule E-cadherin, and acquiring mesenchymal features such as the migration molecule N-cadherin and vimentin [18, 25]. In our study, knockdown of FAM64A protein inhibited the migration of BrC cells as the result of down-regulation of vimentin and up-regulation of E-cadherin. Besides, the mRNA of EMT-related TFs including *Snail*, *Twist*, *Slug*, which promote EMT via suppressing expression of E-cadherin in BrC cells, were detected to make our result more convincing [26–28]. Since the mRNA expression of *Snail, Twist, Slug* can be down-regulated in FAM64A knockdown cells, we reasoned that knockdown of FAM64A impeded the transcriptional level of Snail, Twist, Slug and resulted in up-regulation of E-cadherin. Our study suggested that targeting of FAM64A may potentially inhibited BrC metastasis. Therefore, we hypothesize that FAM64A inhibitors may be proved to be a potential strategy for BrC treatment, especially in TNBC. It should be noted that this study was still limited to in vitro evidences. Additional in vivo experiments are warranted to thoroughly identify the action of FAM64A in BrC.

Conclusion

In summary, our results suggest that FAM64A is highly expressed and correlate with poor prognosis in BrC. FAM64A knockdown suppresses proliferation and colony formation is thought to arrest the G_1 cell cycle and it also inhibits the migration via impeding EMT in BrC cells. These findings might prove that it is an important potential therapeutic target for BrC treatment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest in association with this study.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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