

# KIN17 promotes tumor metastasis by activating EMT signaling in luminal-A breast cancer

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## Abstract

**Background:** Breast cancer (BC), the most common cause of cancer death in women, overtook lung cancer as the leading cause of cancer worldwide in 2020. Although many studies have proposed KIN17 as a biomarker of tumorigenesis in different cancer types, its role in tumor metastasis, particularly in BC metastasis, has been under-explored. This study aimed to explore the role of KIN17 in BC metastasis.

**Methods:** Survival analyses was performed to identify the association between KIN17 expression and BC patient survival *in silico*. Using lentivirus constructs, we developed bidirectional KIN17 expression (KD, knockdown; OE, overexpression) cellular models of luminal-A (Lum-A) breast cancer MCF-7 cells. We performed *in vitro* wound healing, transwell with and without Matrigel assays, and *in vivo* tail-vein metastasis assay to evaluate the migration and invasion abilities of MCF-7 with stable KIN17 knockdown or overexpression. Western blotting was performed to compare the changes in protein expression.

**Results:** We found that KIN17 expression was associated with poor overall survival (OS), relapse-free survival (RFS), distant metastasis-free survival (DMFS) and post-progression survival (PPS), particularly in Lum-A breast cancer patients. Later, we found that KIN17 knockdown inhibited migration and invasion of MCF-7 cells via regulating EMT-associated signaling pathways *in vitro* and decreases metastatic spread of the disease *in vivo*. In contrast, KIN17 overexpression promoted migration and invasion of MCF-7 cells *in vitro* and increased the metastatic spread of the disease *in vivo*.

**Conclusions:** Overall, our findings provide preliminary data which suggests KIN17 of importance to target in metastatic Lum-A patients.

## KEYWORDS

EMT, KIN17, Lum-A breast cancer, metastasis, migration

## INTRODUCTION

Although the last two decades have witnessed huge success in diagnosis, prevention, and therapeutic advancements, cancer is still among the leading cause of death worldwide. Breast cancer has become the most common malignancy and leading cause of cancer-associated death among women worldwide.<sup>1</sup> Metastatic spread of disease is one of the major hallmarks of cancer and is the main culprit behind cancer associated deaths.<sup>2</sup> The mortality rate of breast cancer patients has increased in China probably due to the increase in the number of patients with metastatic disease.<sup>3</sup> More than 70% of breast cancer patients belong to the estrogen receptor (ER) positive subtype or luminal (Lum) subtype and are treated with adjuvant endocrine/hormonal therapy. Despite tumors in most patients being less aggressive and of low grade, cancer cells still evade therapeutic treatments and metastasize to other organs.<sup>4,5</sup> Therefore, better understanding of the molecular mechanism behind breast cancer progression and metastasis is needed for devising effective breast cancer treatments.

*KIN17* gene was first identified by Angulo et al. in 1989 as being conserved from yeast to mammals.<sup>6</sup> In man, *KIN17* harbors an N-terminal zinc finger domain (27–50 nt) and a C-terminal KOW motif (335–373 nt).<sup>7–10</sup> It is primarily expressed in heart, testicular and skeletal muscle cells with lower expression in other organs. It has been found to be involved in a diverse range of important physiological and pathological processes including DNA replication and transcription, DNA damage response to ionizing radiations, immunoglobulin isotype switching and, interestingly, tumor cell proliferation in different cancer types.<sup>11–14</sup>

Previous studies have proposed elevated *KIN17* expression as a promising biomarker of tumorigenesis. For instance, *KIN17* has been reported to drive DNA replication and cellular proliferation during the course of breast cancer development.<sup>15</sup> In line with this, knockdown of *KIN17* expression has been found to promote apoptosis in triple negative breast cancer (TNBC) cells.<sup>16</sup> *KIN17* protein is highly expressed in hepatocellular carcinoma tissues where it has been reported to increase the expression of cyclinD1 and p27Kip1, thereby promoting the proliferation of cancer cells.<sup>17</sup> In cervical cancer cells, *KIN17* protein expression has been found to be markedly increased and its knockdown inhibited cell proliferation and invasion, and promoted apoptotic cell death.<sup>13</sup> Recently, an independent study has found a role for *KIN17* in the metastatic spread of cervical cancer where it regulated the NF- $\kappa$ B-Snail pathway to promote tumor invasion and metastasis.<sup>18</sup> Overexpression of *KIN17* in non-small cell lung cancer (NSCLC) has been linked to lymph node metastasis while silencing of *KIN17* inhibited the invasion and metastasis of NSCLC cells.<sup>14</sup> Furthermore, *KIN17* has been reported to promote proliferation, migration and invasion of thyroid cancer cells by activating the p38 MAPK signaling pathway.<sup>19</sup>

Although these studies strongly suggest *KIN17* as an oncogene, tumor promoting and prometastatic marker in

different cancer types, *KIN17* regulated molecular mechanisms involved in tumor metastasis, in general, and in breast cancer metastasis, in particular, are poorly understood. In this study, we initially identified that *KIN17* expression was associated with poor overall survival (OS), relapse-free survival (RFS), distant metastasis-free survival (DMFS) and post-progression survival (PPS), particularly in luminal-A (Lum-A) breast cancer patients. In this line, we explored the role of *KIN17* in Lum-A and found that *KIN17* knockdown inhibited migration and invasion of Lum-A MCF-7 cells via regulating EMT-associated signaling pathways *in vitro* and decreased metastatic spread of the disease *in vivo*. On the other hand, *KIN17* overexpression promoted migration and invasion of Lum-A MCF-7 cells *in vitro* and increased the metastatic spread of the disease *in vivo*. Overall, our findings provide preliminary data which suggest *KIN17* of importance to target in metastatic Lum-A breast cancer patients.

## METHODS

### Survival analyses

Data for survival analyses were retrieved from online freely available KM-plotter survival analysis database (<https://kmplot.com/analysis/>) and were replotted in GraphPad Prism 6.0.

### Cell culture

A human breast cancer cell line MCF-7 was obtained from National Collection of Authenticated Cell Cultures of Chinese Academy of Sciences, Shanghai, China (Serial: TCHu74). The cells were then cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc) supplemented with 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc.), 100  $\mu$ g/ml penicillin, and 100  $\mu$ g/ml streptomycin (Hyclone, USA). MCF-7 cells were maintained in a humidified incubator at 37°C with 5% CO<sub>2</sub>. Subculture was performed when cells reached 80%–90% confluence.

### Construction and infection of lentivirus particles containing shRNA-KIN17

Lentivirus particles containing *KIN17* targeting shRNA (5'-GGAATTCGAAATGACTTT-3') and respective nontargeting control (5'-TTCTCCGAACGTGTACAGT-3') were constructed as described previously<sup>13</sup> (Figure S1(A)). Exponentially growing MCF-7 cells were subcultured and seeded in six-well plates at a density of  $1.3 \times 10^5$  cells/well. The following day, cells were incubated in Opti-MEM (Gibco; Thermo Fisher Scientific, Inc.) for 30 min and then transfected with lentivirus vector containing shRNA-*KIN17* (hereon termed as MCF-7<sup>KD</sup>) or nontargeting

control vector (hereon termed as MCF-7<sup>NC1</sup>) (Shanghai GeneChem Co., Ltd.), according to the manufacturer's protocol. Untransfected MCF-7 cells (MCF-7<sup>Mock</sup>) were used as a blank control. All the cells were cultured in DMEM media supplemented with 1.5 µg/ml puromycin in 5% CO<sub>2</sub> and 95% humidity at 37°C. Enhanced green fluorescent protein (EGFP) and puromycin selection were used to measure the transfection efficiency and screen successfully transfected cells, respectively. Subsequent experiments were conducted when >90% of cells exhibited a fluorescent signal (Figure S1(B)) and the KIN17 knock-down levels were verified by Western blot (Figure S1(C)).

## Construction and infection of lentivirus particles containing human gene KIN17

Lentivirus particles for KIN17 overexpression were constructed by inserting the human gene *KIN17* (5'-CGCAAATGGG-CGGTAGGCGTG-3'[GenBank ID:NM\_012311]) to GV248 plasmid as GV248-OE (Shanghai GeneChem Co., Ltd.) and control particles were constructed as GV248-NC without inserting any sequence in GV248 plasmid (Shanghai GeneChem Co., Ltd. (Figure S1(D)). Cells were seeded and transfected as previously described. Untransfected MCF-7 cells (MCF-7<sup>Mock</sup>) were used as a blank control. All cells were cultured in DMEM media supplemented with 1.5 µg/ml puromycin in 5% CO<sub>2</sub> and 95% humidity at 37°C. Enhanced green fluorescent protein (EGFP) and puromycin selection were used to measure the transfection efficiency and to screen successfully transfected cells respectively. Subsequent experiments were conducted when the >90% of cells exhibited fluorescent signal (Figure S1(E)) and the KIN17 overexpression levels were verified by western blot (Figure S1(F)).

## Wound healing assay

Exponentially growing cells were seeded in culture medium containing 90% DMEM and 10% FBS at a density of  $10 \times 10^6$  cells/well in six-well plates overnight at 37°C. When the cells reached about 90% confluence, the cell layer was scratched with a 200 µl sterile pipette tip to make a wound and the wells were washed with PBS three times. Subsequently, 2 ml DMEM free of serum was added to each well. The migration ability is the gap between the two edges of the wound, which was measured under the microscope (magnification,  $\times 10$  objective lens) at 0 h, 24 h, and 48 h after scratching. All assays were done in duplicate and repeated at least three times. All the images were analyzed on Image J.

## Transwell migration assay

Cell migration capacity was measured by culturing cells in 8.0 µm transwell chambers (Costar, Corning Incorporated), which was put into the 24-well cell culture plate. Briefly, the upper chambers were seeded with equal numbers of cells ( $5 \times 10^5$  cells/200 µl) in DMEM free of serum culture

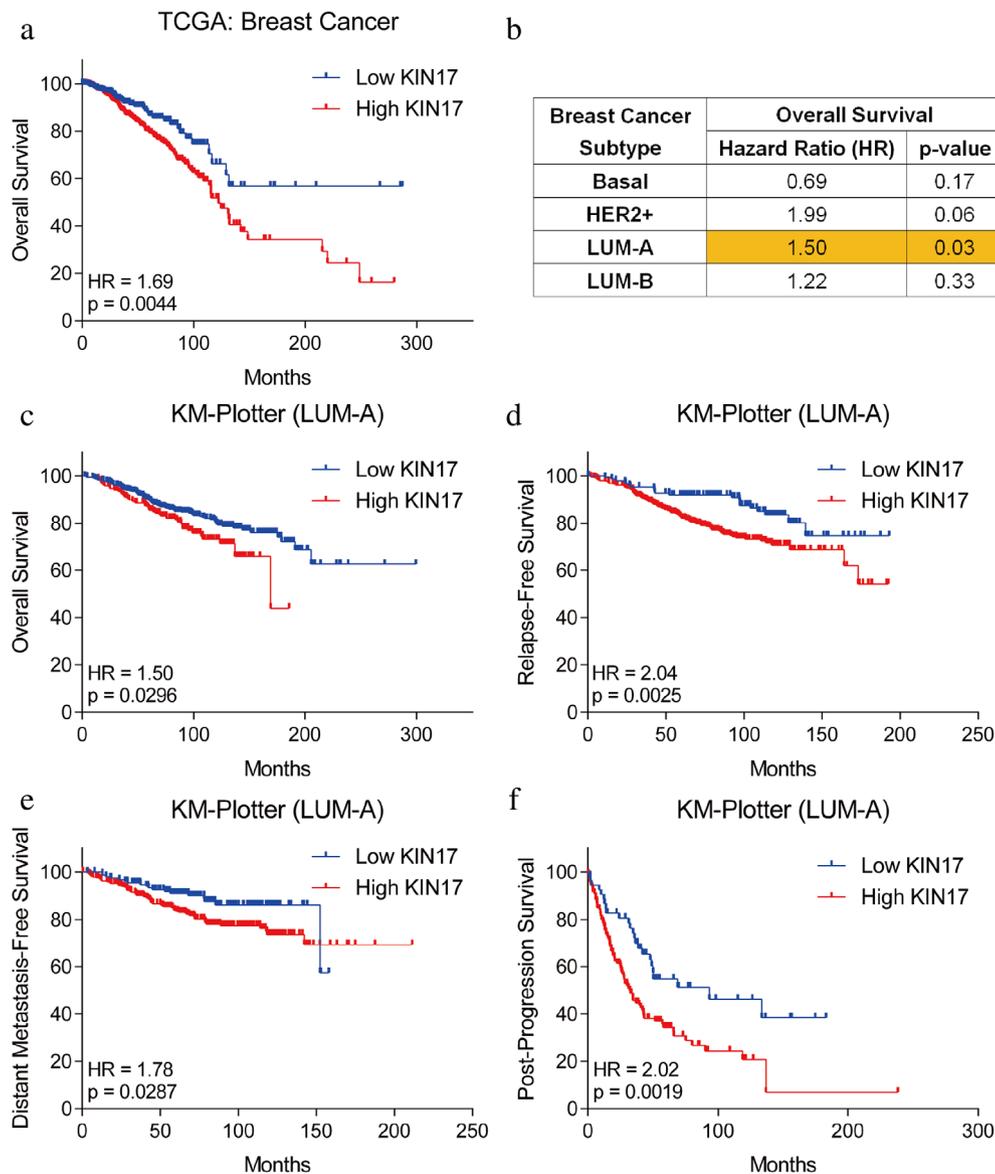
medium, and the lower chambers were filled with DMEM medium supplemented with 30% FBS as a chemoattractant. The plates were incubated in a humidified incubator at 37°C for 24 h. Cells that migrated through the membrane filter to the lower surface were fixed with methanol (Guangdong Guanghua Sci-Tech Co., Ltd.) at room temperature for 30 min and then stained with 0.1% crystal violet solution (Shanghai GeneChem Co., Ltd.) at room temperature for 30 min. After washing three times with PBS, the cells in the upper chambers were then wiped away and images of the migrated cells taken under inverted microscope (magnification,  $\times 20$  objective lens). Numbers of migrated cells were counted on Image J. All the assays were done in duplicate and repeated at least three times.

## Transwell invasion assay

The transwell invasion assay was performed in transwell chambers (Costar, Corning Incorporated) on 24-well cell culture plates. The upper and lower culture compartments were separated by polycarbonate filters with 8-µm pore diameter. Different from the transwell migration assay, the filters were precoated with Matrigel (BD Biosciences) for 1 h at 37°C. Equal numbers of cells ( $5 \times 10^5$  cells) in serum-free DMEM were added to the upper chamber. The lower chamber was filled with DMEM medium containing 30% FBS for inducing cell invasion, and plates were incubated at 37°C for 48 h. Invaded cells were fixed, stained, photographed and counted following the protocol described in the transwell migration assay. All the assays were performed in duplicate and repeated at least three times.

## Western blotting

Protein extraction from tissues and cells and western blot assay was performed as previously described.<sup>20</sup> Briefly, using RIPA lysis buffer (Keygen Biotech) to cleave cells or tissues and extract their total protein, the total protein concentration was then quantified on NanoDrop 2000. Then, 100 µg protein were loaded and separated by 10%–12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (EMD Millipore), and incubated with antibodies: KIN17 (K58) (1:1000, Santa Cruz Biotechnology, cat. no. sc-32 769); GAPDH (1:25000; proteintech, cat. no. 60004-1-Ig); AKT (1:1000, DB BIOTECH, cat. no.DB126-0.1); p-AKT(1:1000, DB BIOTECH, cat. no. DB127-0. 1); TCF8/ZEB1 (1:1000, Cell Signaling Technology, cat. no.3396); N-cadherin (1:1000, Cell Signaling Technology, cat. no.#13 116); E-cadherin (1:1000, Cell Signaling Technology, cat. no.3195);  $\beta$ -catenin (1:1000, Cell Signaling Technology, cat. no.8480); Vimentin (1:1000, Cell Signaling Technology, cat. no.5741); Snail (1:1000, Cell Signaling Technology, cat. no.3879); Claudin-1 (1:1000, Cell Signaling Technology, cat. no.13 255), HRP-linked antibody anti-rabbit IgG (1:5000, Cell Signaling Technology, cat. no.7074); HRP-linked antibody anti-mouse IgG (1:5000, Cell Signaling



**FIGURE 1** KIN17 is associated with poor survival in luminal-A (Lum-A) breast cancer patients. (a) Kaplan–Meier survival plot representing the percentage of overall survival (OS) in breast cancer patients from TCGA database based on low versus high KIN17 expression. (b) HR and *p*-value associated with OS in different subtypes of breast cancer based on low versus high KIN17 expression. (c–f) Kaplan–Meier survival plots representing the percentage OS (c), relapse-free survival (RFS) (d), distant metastasis-free survival (DMFS) (e) and post-progression survival (PPS) (f) in Lum-A breast cancer patients based on low versus high KIN17 expression. Data for (a–f) were retrieved from the KM-plotter online survival analysis tool

Technology, cat. no.7076 were used as secondary antibodies and signals were detected by ECL enhanced chemiluminescence substrate (Thermo Fisher Scientific, Inc.). Images were captured on the Image Quant RT ECL imager (GE Healthcare Life Sciences). For analysis, protein levels were normalized to GAPDH on the same gels. All the band intensities were quantified by Image Lab 6.0 imaging software (Bio-Rad Laboratories, Inc.)

## Xenograft tumor model

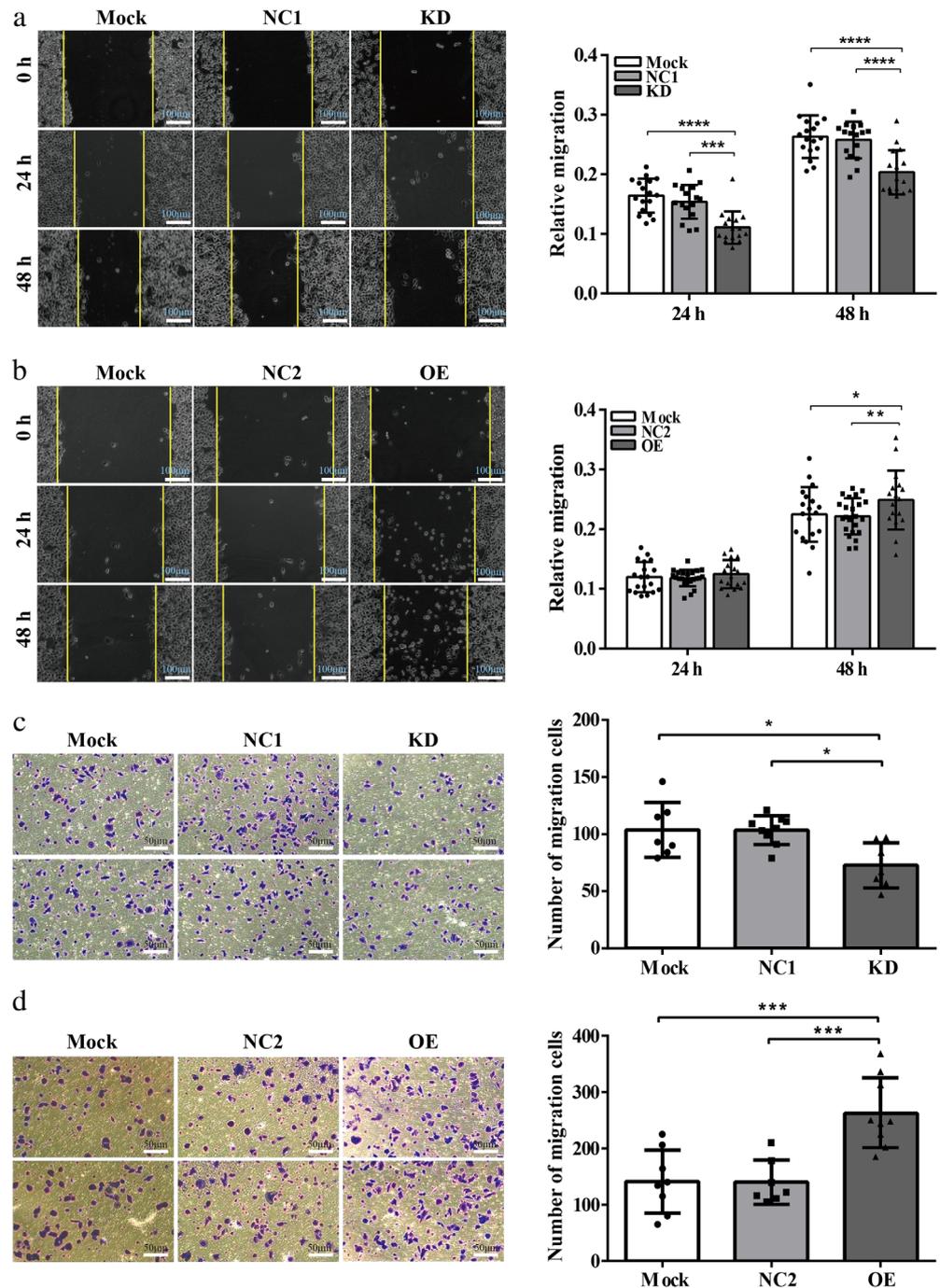
Five weeks old female BALB/c nude mice were purchased from the Southern Medical University Laboratory Animal Center and raised under standard laboratory conditions. After one week of adaptation, the metastasis experiment was performed. Breast cancer cells of MCF-7 in KD and OE groups ( $2.0 \times 10^6$  cells/mice in  $200 \mu\text{l}$  DMEM) were intravenously injected through the tail vein of the mice. The mice were weighed and received estradiol supplementation (0.4 mg/kg) every

seven days for 35 days after cell injection.<sup>21</sup> After six weeks, the mice were anesthetized and anatomized, and the metastatic liver nodules were calculated. Livers were excised for western blotting and hematoxylin and eosin staining. All animal experiments were conducted according to the instructions approved by the Nanfang Hospital Animal Ethics committee of Southern Medical University.

## Statistical analysis

All the experiments were duplicated and repeated at least three times. Statistical analyses were performed using Graph Pad prism 7.0. Two-tailed Student's *t*-tests were used for the comparison between two groups of independent samples. One-way ANOVA was used for the comparison of the mean values between multiple groups. Under the condition of homogeneity of variance, LSD was used for the multiple comparison analysis between groups. In the case of heterogeneity of variances,

**FIGURE 2** KIN17 promotes migration of luminal-A (Lum-A) breast cancer cells *in vitro*. (a and b) Representative images (10x) (left) and quantification (right) of scratch assays performed using MCF-7<sup>KD</sup> (a), MCF-7<sup>OE</sup> (b) cells compared with respective controls. Images were taken at 0, 24 and 48 h time points. (c and d) Representative images (20x) and quantification of transwell assays without Matrigel performed using MCF-7<sup>KD</sup> (c), MCF-7<sup>OE</sup> (d) cells compared with respective controls. Mock, untransfected MCF-7 cells, blank control; NC, negative control; KD, KIN17 knockdown; OE, KIN17 overexpression. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ,  $n = 3-4$



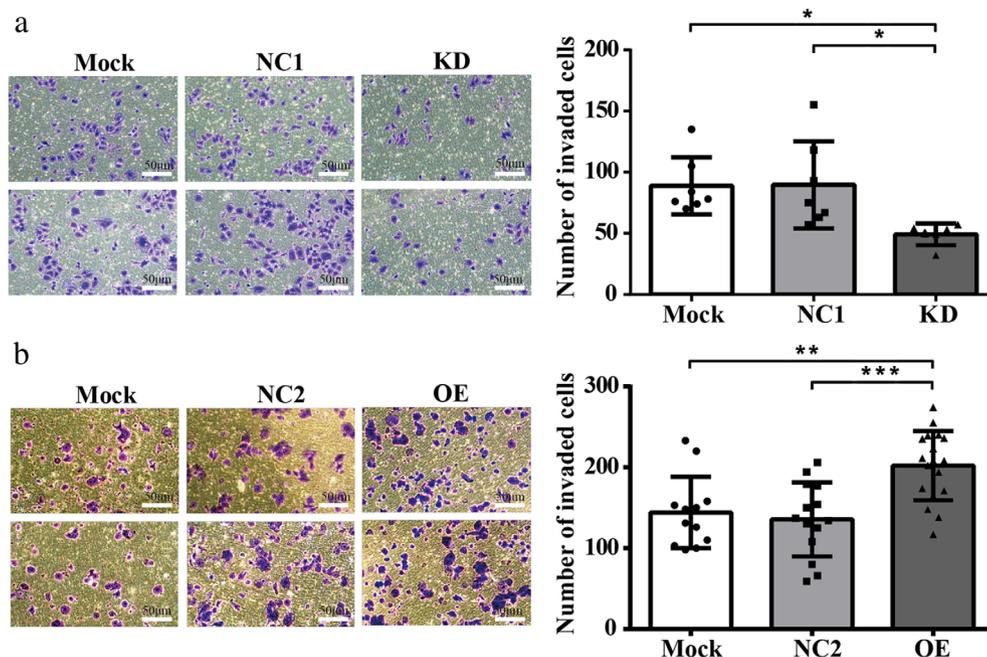
Dunnett's T3 method was used for multiple comparative analyses among groups. Data are presented as mean  $\pm$  SEM.  $p < 0.05$  was considered statistically significant. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , and \*\*\*\* $p < 0.0001$ .

## RESULTS

### KIN17 is associated with poor survival in Lum-A breast cancer patients

In order to study KIN17 in the context of breast cancer metastasis, we first confirmed the association between

KIN17 expression and breast cancer patient survival, and found that high KIN17 expression is associated with poor overall survival in breast cancer patients (Figure 1(a)). As breast cancer is divided into four major subtypes, we checked the association of KIN17 expression with the survival of breast cancer patients representing different subtypes and found that high KIN17 in particular is significantly associated with poor OS in Lum-A breast cancer patients (Figure 1(b), (c)). Surprisingly, high KIN17 expression was also found to be associated with poor relapse-free survival (RFS), distant metastasis-free survival (DMFS) and post-progression survival (PPS) (Figure 1(d)-(f)) in Lum-A breast cancer patients suggesting a close link



**FIGURE 3** KIN17 promotes invasion capacity of luminal-A (Lum-A) breast cancer cells *in vitro*. (a and b) Representative images (20x) and quantification of transwell assays with Matrigel performed using MCF-7<sup>KD</sup> (a), MCF-7<sup>OE</sup> (b) cells compared with respective controls. Mock, untransfected MCF-7 cells, blank control; NC, negative control; KD, KIN17 knockdown; OE, KIN17 overexpression. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ,  $n = 4$

between KIN17 expression and metastatic spread of the disease.

### KIN17 promotes migration of Lum-A breast cancer cells *in vitro*

In order to investigate the role of KIN17 in Lum-A breast cancer metastasis *in vitro* and *in vivo*, we first developed a stable KIN17 knockdown and overexpression cell line using well-established Lum-A breast cancer MCF-7 cell line models; herein referred to as MCF-7<sup>KD</sup> and MCF-7<sup>OE</sup>, respectively (Figure S1; For details, see Supplementary Information). We then investigated the effect of KIN17 knockdown and overexpression of Lum-A breast cancer cell migration using wound healing and transwell migration assays. MCF-7<sup>KD</sup> cells showed lower wound healing ability compared with MCF-7<sup>NC1</sup> cells and MCF-7<sup>Mock</sup> cells after 24 and 48 h of the scratch assay, while no significant change was observed between MCF-7<sup>NC1</sup> and MCF-7<sup>Mock</sup> cells (Figure 2(a)). In contrast, MCF-7<sup>OE</sup> cells exhibited notably higher wound healing ability compared with MCF-7<sup>NC2</sup> cells and MCF-7<sup>Mock</sup> cells after 48 h of the scratch assay, while no change was found between MCF-7<sup>NC2</sup> and MCF-7<sup>Mock</sup> cells (Figure 2(b)). We also observed similar results in the transwell migration assay where we found that KIN17 knockdown evidently inhibited migration (Figure 2(c)), whereas KIN17 overexpression significantly promoted migration of MCF-7 cells (Figure 2(d)). Overall, these results suggest that high KIN17 expression promotes migration in Lum-A breast cancer MCF-7 cells.

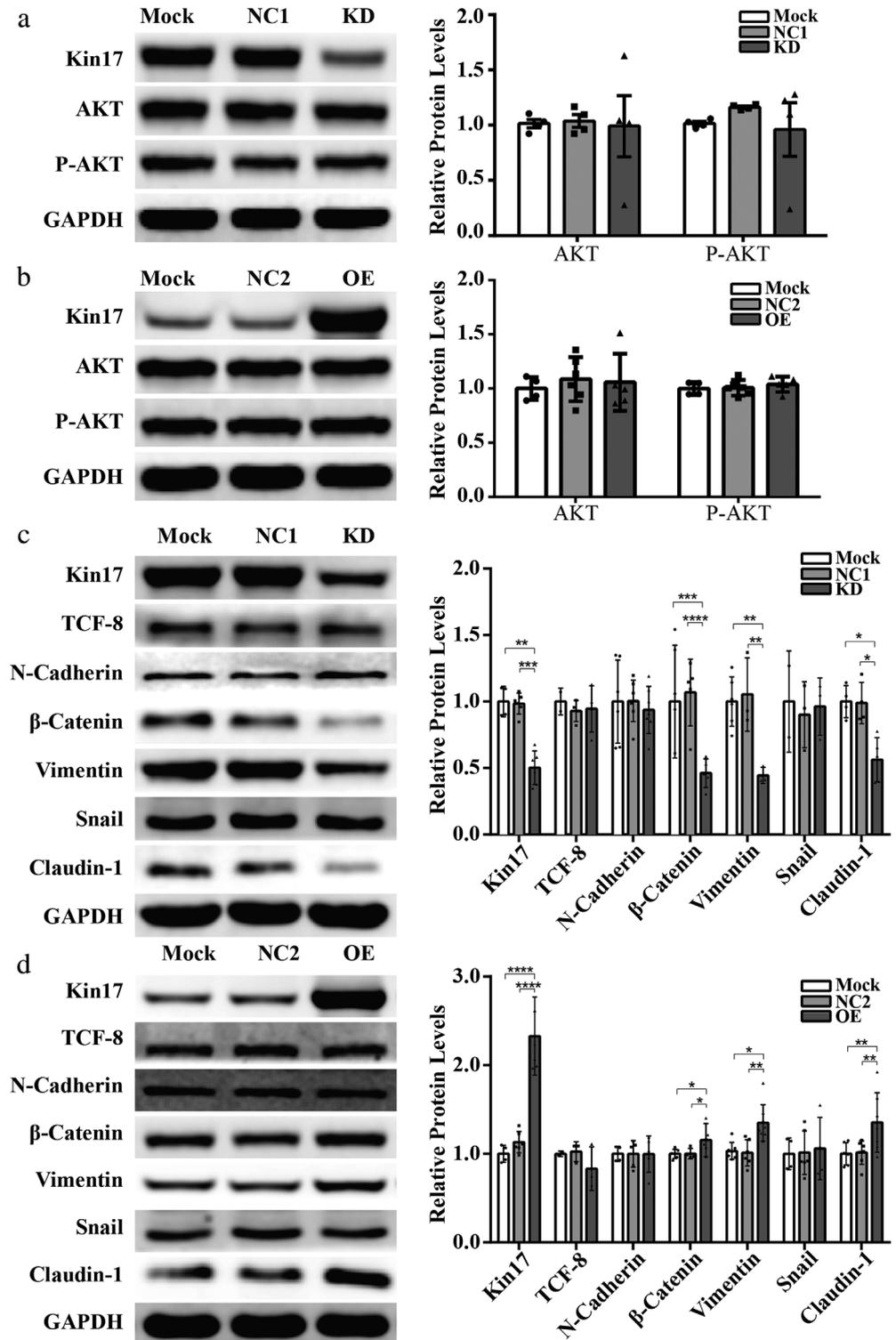
### KIN17 promotes invasion in Lum-A breast cancer cells *in vitro*

Next, to determine the role of KIN17 in regulating invasion of Lum-A breast cancer MCF-7 cells, we performed transwell invasion assay with Matrigel. The results showed that knockdown of KIN17 significantly suppressed the invasion of MCF-7 cells through Matrigel in contrast to MCF-7<sup>NC1</sup> and MCF-7<sup>Mock</sup> cells (Figure 3 (a)). In line with this, we also performed the transwell invasion assay in the OE group of MCF-7 cells. Results showed that increased expression of KIN17 significantly upregulated the invasion ability of MCF-7 cells. In contrast, there was no significant change between the MCF-7<sup>NC2</sup> and MCF-7<sup>Mock</sup> cells in terms of number of cells invaded through Matrigel (Figure 3(b)). Together, these data suggest that high KIN17 expression promotes invasion in Lum-A breast cancer MCF-7 cells.

### KIN17 hyperactivates EMT-associated signaling pathways in Lum-A breast cancer cells *in vitro*

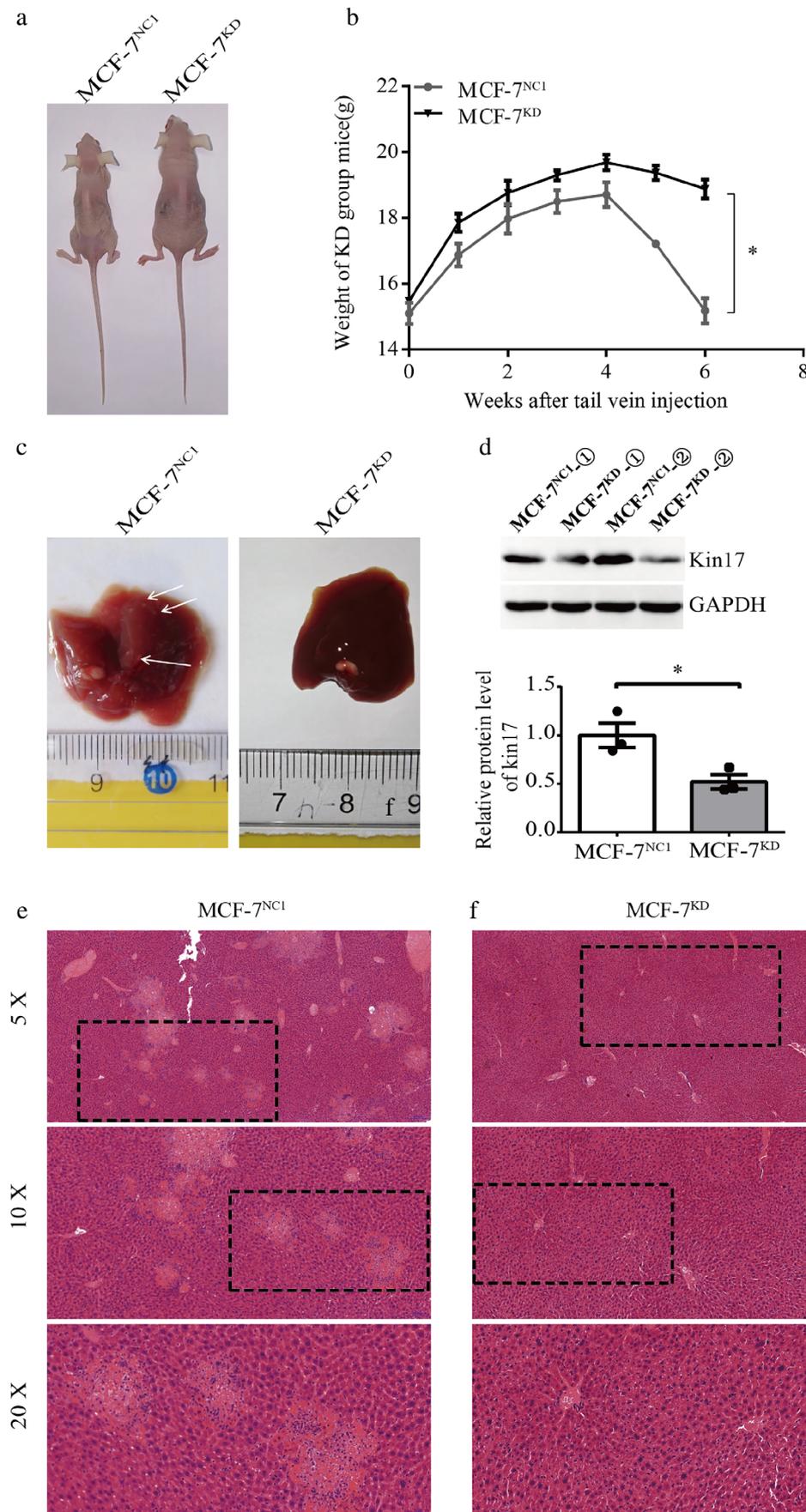
Based on our findings that KIN17 plays a crucial role in controlling migration and invasion of Lum-A MCF-7 cells, we were interested in investigating the underlying molecular mechanisms. In this line, we checked the activation of different prosurvival and epithelial-to-mesenchymal transition (EMT) markers already known to be involved in breast cancer metastasis including AKT, TCF-8, N-cadherin,  $\beta$ -catenin, vimentin, Snail, and Claudin-1. We found no changes in activation of prosurvival and prometastatic markers AKT upon

**FIGURE 4** KIN17 hyperactivates EMT-associated signaling pathways in luminal-A (Lum-A) breast cancer cells *in vitro*. (a–d) Western blots (left) and their quantitative analyses (right) showing change in expression of protein involved in with AKT (a and b) and EMT-associated signaling pathway (c and d) in MCF-7<sup>KD</sup> (a and c), MCF-7<sup>OE</sup> (b and d) cells compared with respective controls. Mock, untransfected MCF-7 cells, blank control; NC, negative control; KD, KIN17 knockdown; OE, KIN17 overexpression. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ,  $n = 3–6$



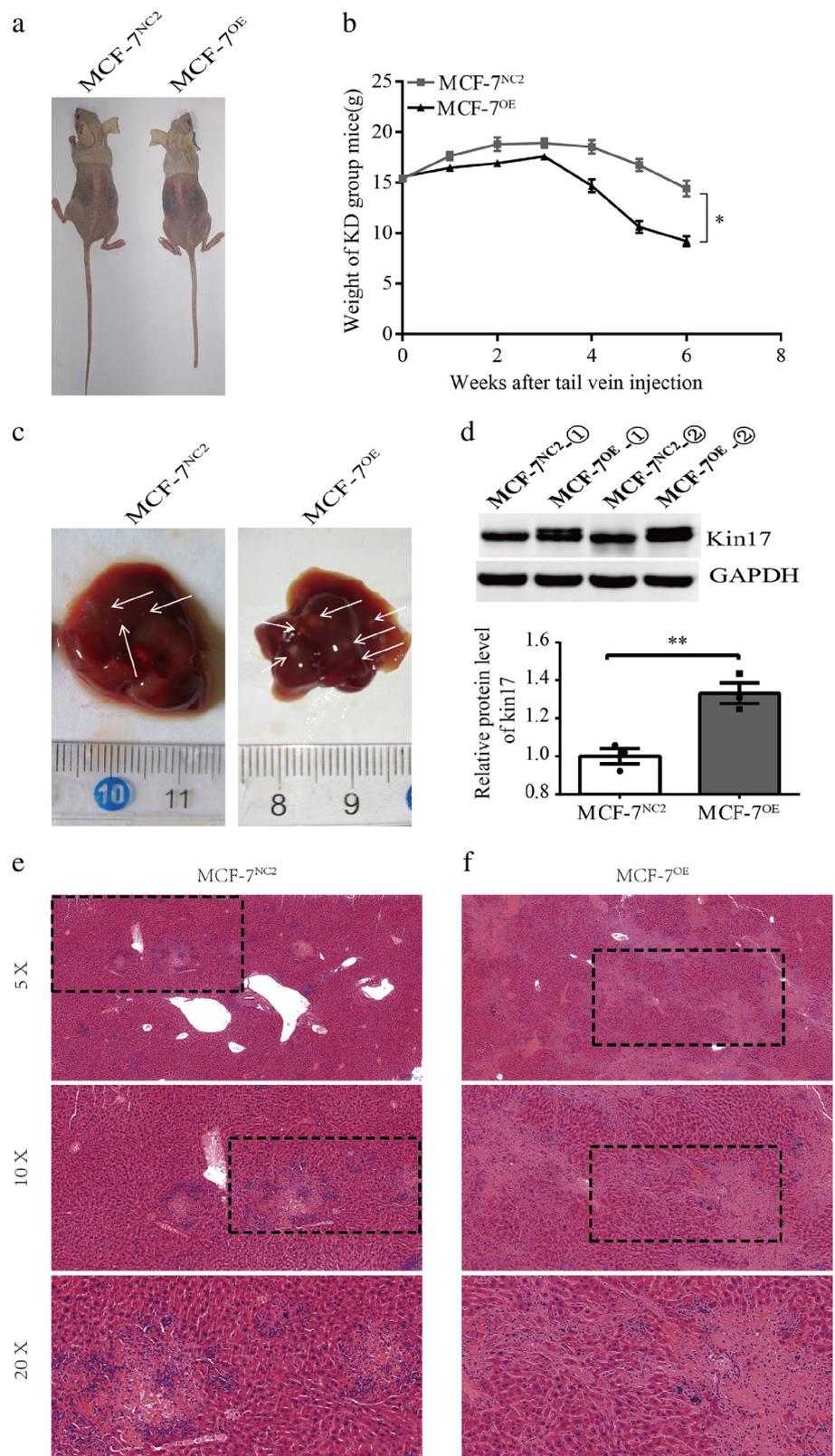
knockdown and overexpression of regulation the KIN17 protein levels in MCF-7 cells compared to respective controls (Figure 4(a), (b)). Interestingly, protein expression of EMT-associated  $\beta$ -catenin, Vimentin, and claudin-1 were significantly decreased in MCF-7<sup>KD</sup> cells compared with MCF-7<sup>NC1</sup> and MCF-7<sup>Mock</sup> cells (Figure 4 (c)), whereas expression of the same EMT-associated markers were significantly upregulated in MCF-7<sup>OE</sup> cells

compared with MCF-7<sup>NC2</sup> and MCF-7<sup>Mock</sup> cells (Figure 4(d)). Moreover, TCF-8, N-cadherin, and Snail, the other EMT-associated proteins, were also slightly changed upon KIN17 knockdown and overexpression (Figure 4(c), (d)). Together, these results confirm that high KIN17 promotes metastatic Lum-A breast cancer MCF-7 cells by hyperactivating EMT-associated signaling pathways.



**FIGURE 5** KIN17 knockdown inhibits metastasis of luminal-A (Lum-A) breast cancer cells *in vivo*. (a) Photograph of representative mice six weeks after tail vein injection of MCF-7<sup>NCI</sup> and MCF-7<sup>KD</sup> cells. (b) Graph showing change in bodyweight of mice six weeks after tail vein injection of MCF-7<sup>NCI</sup> and MCF-7<sup>KD</sup> cells. (c) Image showing representative liver from mice which received MCF-7<sup>NCI</sup> and MCF-7<sup>KD</sup> through tail-vein injection. Mice were sacrificed and livers were collected six weeks after injection. (d) Western blot (top) and their quantitative analysis (bottom) showing KIN17 expression in metastatic nodules from the livers of mice injected with MCF-7<sup>NCI</sup> and MCF-7<sup>KD</sup> cells. (e and f) Representative HE staining images of liver tissues from mice injected with MCF-7<sup>NCI</sup> (e), and MCF-7<sup>KD</sup> (f) cells. NC, negative control; KD: knockdown. \**p* < 0.05, \*\**p* < 0.01 and \*\*\**p* < 0.001, *n* = 3–4

**FIGURE 6** KIN17 overexpression promotes metastasis of luminal-A (Lum-A) breast cancer cells *in vivo*. (a) Photograph of representative mice six weeks after tail vein injection of MCF-7<sup>NC2</sup> and MCF-7<sup>OE</sup> cells. (b) Graph showing change in bodyweight of mice six weeks after tail vein injection of MCF-7<sup>NC2</sup> and MCF-7<sup>OE</sup> cells. (c) Image showing representative liver from mice which received MCF-7<sup>NC2</sup> and MCF-7<sup>OE</sup> cells through tail-vein injection. Mice were sacrificed and livers were collected six weeks after injection. (d) Western blot (top) and their quantitative analysis (bottom) showing KIN17 expression in metastatic nodules from the livers of mice injected with MCF-7<sup>NC2</sup> and MCF-7<sup>OE</sup> cells. (e and f) Representative hematoxylin & eosin (H&E) staining images of liver tissues from mice injected with MCF-7<sup>NC2</sup> (e), and MCF-7<sup>OE</sup> (f) cells. NC, negative control; KD, knockdown. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ,  $n = 3-4$



### KIN17 promotes Lum-A breast cancer metastasis *in vivo*

After establishing an important role for KIN17 in Lum-A breast cancer metastasis *in vitro*, we aimed to validate our

findings *in vivo*. In this line, we performed a tail-vein metastasis assay using MCF-7<sup>KD</sup> and MCF-7<sup>OE</sup> cells together with respective controls *in vivo*. We found that the mice injected with MCF-7<sup>KD</sup> cells remained healthy compared to those injected with control MCF-7<sup>NC1</sup> cells which became stressed

and began to lose bodyweight after four weeks of tail vein injection of cells (Figure 5(a), (b)). The liver of mice injected with MCF-7<sup>KD</sup> cells showed a lower number of metastatic nodules at the liver surface compared to those injected with control MCF-7<sup>NC1</sup> cells (Figure 5(c)). KIN17 protein expression was consistently decreased in metastatic nodules isolated from the livers of mice injected with MCF-7<sup>KD</sup> mice compared to those injected with control MCF-7<sup>NC1</sup> cells (Figure 5(d)). In addition, KIN17 knockdown was accompanied by reduced levels of inflammatory infiltration and lesser degeneration of liver tissue (Figure 5(e), (f)). In contrast to this, we found that the mice injected with MCF-7<sup>OE</sup> cells became stressed and began to lose bodyweight after four weeks of tail vein injection of cells compared to those injected with control MCF-7<sup>NC2</sup> cells (Figure 6(a), (b)). The liver of mice injected with MCF-7<sup>OE</sup> cells showed a higher number of metastatic nodules at the liver surface compared to those injected with control MCF-7<sup>NC2</sup> cells (Figure 6(c)). KIN17 protein expression was consistently increased in metastatic nodules isolated from livers of mice injected with MCF-7<sup>OE</sup> mice compared to those injected with control MCF-7<sup>NC1</sup> cells (Figure 6(d)). In addition, KIN17 overexpression was accompanied by increased levels of inflammatory infiltration and the higher degeneration of liver tissue (Figure 6 (e), (f)). These findings suggest that higher KIN17 expression actively promotes, whereas its downregulation demotes the metastatic spread of Lum-A breast cancer MCF-7 cells *in vivo*.

## DISCUSSION

Breast cancer is classified into four major subtypes namely luminal-A (ER+, PR+, HER2-), luminal-B (ER+, PR+, HER2+), HER2+ (ER-, PR-, and HER2+) and basal-like (ER-, PR-, HER2-, cytokeratin 5/6 positive, and/or HER1+). Each subtype exhibits a varying response to anticancer drugs.<sup>22</sup> Almost 70% of breast cancer patients are confirmed to be the luminal-A subtype. These patients usually show a good response towards hormonal therapy. However, nearly 25% of cases experience recurrent and metastatic disease.<sup>23</sup> Although many new molecular targets have been identified and proposed to be targeted in the clinical to treat breast cancer, in general, and in Lum-A breast cancer, in particular, very few have been fruitful in tackling metastatic disease.<sup>24</sup> Here, using *in silico*, *in vitro* and *in vivo* approaches, we show that KIN17 is a key player involved in the metastatic spread of Lum-A breast cancer MCF-7 cells. Previous studies have shown that KIN17 plays an oncogenic role in certain tumor types via promoting tumors growth and cell proliferation and by demoting apoptotic cell death.<sup>13–15,25</sup>

The last two decades have witnessed gene expression and proteomic profiling as powerful tools in gene-to-disease associations and in the identification of biomarkers of disease onset and progression.<sup>26</sup> In our study, we also established a link between Lum-A breast cancer and KIN17

gene expression through *in silico* approaches. The same data also suggested KIN17 plays a role in the metastatic spread of Lum-A breast cancer MCF-7 cells (Figure 1), which we later validated through *in vitro* and *in vivo* approaches.

Metastasis is a multistep process in which selected cancer cells initially dissociate from their primary site, migrate out, undergo EMT, infiltrate into and survive in circulation and later extravasate from the circulation to develop microtumor niches at a distant site leading to metastatic tumor.<sup>27</sup> Although EMT is an essential step during development, aggressive cancer cells adapt EMT-like characteristics to gain fibroblast-like properties such as reduced intracellular adhesion and increased motility.<sup>28,29</sup> This tightly regulated process is associated with a number of cellular and molecular events and involves downregulation of adhesive epithelial markers and upregulation of mesenchymal markers.<sup>30–33</sup> For instance, E-cadherin, being an epithelial marker, is considered an active suppressor of invasion and growth of many cancer types of epithelial origin,<sup>34</sup> whereas N-cadherin has been found to be associated with increased EMT and metastatic spread in solid cancers.<sup>35</sup> We could not detect E-cadherin in our *in vitro* experiments, or find any significant difference in N-cadherin expression between KIN17 knockdown/overexpression cells respective control (Figure 4). This led us to investigate other EMT associated pathways to confirm the involvement of KIN17 in EMT. Vimentin is another important player in EMT whose dynamic structural changes and spatial reorganization in response to extracellular stimuli assists in coordinating various signaling pathways to promote metastasis.<sup>36</sup> Whilst Vimentin was found to be decreased upon KIN17 knockdown, it was upregulated upon KIN17 overexpression, suggesting Vimentin is an important player of EMT downstream of KIN17. A similar pattern was also observed for  $\beta$ -catenin which is a key downstream effector in the Wnt signaling pathway and has been implicated in the metastatic spread of cancer cells.<sup>37</sup> How the mesenchymal markers are regulated by KIN17 at molecular level needs further investigation.

In conclusion, we found that KIN17 was associated with poor survival in Lum-A breast cancer patients *in silico* for which our *in vitro* data provide evidence that KIN17 is involved in regulating migration and invasion of Lum-A breast cancer MCF-7 cells through promoting acquisition of EMT like phenotype, which we also validated *in vivo*. Overall, these results suggest that it is important to target KIN17 in metastatic Lum-A breast cancer patients.

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## CONFLICT OF INTEREST

The authors declare no competing financial interests.

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## SUPPORTING INFORMATION

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

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