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Original Article

Mortalin maintains breast cancer stem cells stemness via activation of Wnt/GSK3β/β-catenin signaling pathway

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Abstract: Previous research indicated that mortalin overexpressed in breast cancer and contributed to carcinogenesis. Mortalin was also demonstrated to promote Epithelial-mesenchymal transition (EMT) and was considered as a factor for maintaining the stemness of the cancer stem cells. However, the underlying mechanisms about mortalin maintaining the stemness of breast cancer stem cells (BCSCs) remain unclear. Here, we identified that increased expression of mortalin in breast cancer was associated with poorer overall survival rate. Mortalin was elevated in breast cancer cell lines and BCSC-enriched populations. Additionally, knockdown of mortalin significantly inhibited the cell proliferation, migration and EMT, as well as sphere forming capacity and stemness genes expression. Further study revealed that mortalin promoted EMT and maintained BCSCs stemness via activating the Wnt/GSK3β/β-catenin signaling pathway *in vivo* and *in vitro*. Taken together, these findings unveiled the mechanism of mortalin in maintaining and regulating the stemness of BCSCs, and may offer novel therapeutic strategies for breast cancer treatment.

Keywords: Mortalin, breast cancer stem cells, stemness, Wnt/GSK3β/β-catenin signaling pathway, EMT

Introduction

Breast cancer is the most common incident cancer and the leading cause of cancer deaths for females worldwide [1]. Although surgery, chemotherapy and radiotherapy are well treatments for breast cancer, the morbidity and mortality of breast cancer are still in a high incidence. The reason for the tumor occurrence was the high degree of heterogeneity in tumor with respect to metastases, prognosis and chemo-resistance. Breast cancer stem cell (BCSC) is one of the groups of breast cancer cells which expresses stem cell marker and has a high capability for generation of breast cancer [2]. BCSCs play a key role in breast cancer tumorigenesis and progression, and are correlated to tumor heterogeneous and therapy-resistant [3, 4]. It has been reported that about 2% BCSCs could be resistant to chemotherapy and radiotherapy and cause treatment failure and disease recurrence [5]. Therefore, recognition of molecular mechanisms involved in stemness of BCSCs could be effective for discovering novel treatment strategies to overcome the relapse of breast cancer.

Mortalin, also named GRP75, mtHsp70 or HSPA9, is a member of Hsp70 family. Its sequence is slightly more homologous to those of the Saccharomyces cerevisiae Ssc1p and Escherichia coli DnaK [6]. Mortalin involves in multiple cellular biological processes such as mitochondria biogenesis, chaperoning, mitochondrial import motor, intracellular trafficking, immune response, neuronal differentiation and contributes to human carcinogenesis [7-9]. Previous studies revealed that mortalin is aberrantly over-expressed in intrahepatic cholangiocarcinoma and thyroid carcinoma, it was proved to promote cancer cell proliferation and might serve as a biomarker for treatment [10, 11]. Mortalin also enriched in breast cancer tissues and contributed to the migration and invasive-

Table 1. Sequences of siRNA

NO.	Target Seq
HSPA9-RNAi #1	cgTGCTCAATTTGAAGGGATT
HSPA9-RNAi #2	gcACATTGTGAAGGAGTTCAA
HSPA9-RNAi #3	ccGAGTCAGATTGGAGCATTT

ness of breast cancer cells [12-14]. A recent study showed that malignant cells overexpressing mortalin were enriched with stemness markers and exhibited resistance to chemotherapeutic drugs [15]. The studies indicated that mortalin is possibly a factor for maintaining the BCSCs stemness. However, the mechanisms about mortalin maintain the stemness in breast cancer remains unexplored.

In this study, we first detected the expression of mortalin in breast cancer tissues and cell lines and constructed over-expression and knockdown vector of mortalin to observe the influence of mortalin for BCSCs-enriched populations. Next, we detected the effect of mortalin on the cell stemness character, EMT and Wnt/β-catenin signaling pathway. To further illustrate the regulation mechanism of mortalin in BCSCs, we treated cells transfected with mortalin vectors with LiCl, a GSK3β inhibitor. Based on the research, we proved that overexpression of mortalin promoted cell EMT and maintained the stemness of BCSCs via activating Wnt/GSK3β/β-catenin signaling pathway. The results indicated that mortalin could be a promising target for treating BCSCs, and anti-mortalin could be a new idea for the breast cancer therapy.

Materials and methods

Human breast cancer tissue samples collection

24 pairs of breast cancer tissues and normal adjacent tissue samples were collected from the Department of Tumor Surgery, the General Hospital of Ningxia Medical University. All samples were approved by the Ethical Committees of the General Hospital of Ningxia Medical University (Ethics No. 2019-175). Written informed consents were obtained from all participants involved in the study.

Cell lines and cell culture

Human breast cancer cell lines MCF-7, MDA-MB-231, BT-474 and SK-BR-3 and a normal epithelial cell line MCF-10A were purchased from the Cell bank of the Chinese Academy of Sciences. MCF-7, MDA-MB-231 and SK-BR-3 cells were maintained in DMEM (Gibco, CA, USA) medium supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA). BT-474 cells were cultured in 1640 medium (Gibco, CA, USA) supplemented with 10% FBS. MCF-10A cells were cultured in Mammary Epithelial Cell Medium (ScienCell, CA, USA). All the cells were cultured at 37°C with 5% CO_2.

Mammosphere formation assay

In order to obtain spherical cells, cells were seeded into ultra-low attachment 6-well plates (Corning) at a density of 1000 cells/well and cultured in low oxygen condition. Mammospheres were grown in a DMEM/F12 medium (Gibco, CA, USA) supplemented with B27 (1:50, Invitrogen), 20 ng/ml EGF (Pepro Tech, New Jersey, USA), 20 ng/ml bFGF (Pepro Tech, New Jersey, USA), 5 μg/ml insulin (Sigma-Aldrich, St. Louis, Missouri, USA). 1.0 mL of culture medium was supplemented every 3 days. After 10 days culture, spheroids larger than 50 μm in diameter were counted and photographed using a microscope.

Lentivirus vector constructed and transfection

Lentivirus vectors for over-expression or knockdown of mortalin were constructed and packaged by Shanghai Genechem Co. Ltd (Shanghai, China). MCF-7 and MDA-MB-231 cells were transfected with the vector according to the manufacturer's instructions respectively. The transfected cells were selected with puromycin (Clontech, San Francisco, USA). Cells with mortalin stable overexpression and knockdown were named as OE-Mot and Si-Mot, respectively. The siRNA sequences for knockdown of mortalin are listed in **Table 1**.

RNA extraction, reverse transcription, and qRT-PCR

Total RNA was extracted from cells with TRIzol reagent (Invitrogen, CA, USA). The RNA reverse-transcribed into cDNA using Quantitect Reverse Transcriptase Kit (TaKaRa, Shanghai, China) following the manufacturer's protocol. Then the cDNA was used to perform qRT-PCR using the SYBR-Green PCR system (Takara Bio, Inc.). The relative expression levels of mRNA

Table 2. Sequences of primer

Name	Seq
HSPA9	F: GTCAGATTGGAGCATTTGTGTT
	R: CGAGTCATTGAAATAAGCTGGG
ALDH1	F: GACAATGCTGTTGAATTTGCAC
	R: AAGGATATACTTCTTAGCCCGC
ABCG2	F: GTCGTACTGGGACTGGTTATAG
	R: TCTCTACCACAAAGAGTTCCAC
OCT4	F: GTGGTCCGAGTGTGGTTCTGTAAC
	R: CCCAGCAGCCTCAAAATCCTCTC
NANAG	F: GATGCAAGAACTCTCCAACATC
	R: CTGGTGGTAGGAAGAGTAAAGG
APC	F: TGGAGAACTCAAATCTTCGACA
	R: ATCTGTCCAGAAGAAGCCATAG
β-catenin	F: TGGATTGATTCGAAATCTTGCC
	R: GAACAAGCAACTGAACTAGTCG
c-myc	F: CGACGAGACCTTCATCAAAAAC
	R: CTTCTCTGAGACGAGCTTGG
cyclin D1	F: GTCCTACTTCAAATGTGTGCAG
	R: GGGATGGTCTCCTTCATCTTAG
GAPDH	F: TGTTGCCATCAATGACCCCTT
	R: CTCCACGACGTACTCAGCG

Table 3. The information of antibodies

Antibodies	Source company	Dilution ration
Anti-mortalin	Santa Cruz	1:1000
Anti-GAPDH	Cell Signaling Technology	1:1000
Anti-ALDH1	Cell Signaling Technology	1:1000
Anti-ABCG2	Santa Cruz	1:500
Anti-OCT4	Cell Signaling Technology	1:1000
Ani-Nanog	Cell Signaling Technology	1:1000
Anti-E-cadherin	Cell Signaling Technology	1:1000
Anti-N-cadherin	Cell Signaling Technology	1:1000
Anti-Vimentin	Cell Signaling Technology	1:1000
Anti-Slug	Cell Signaling Technology	1:1000
Anti-ZEB1	Cell Signaling Technology	1:1000
Anti-APC	Abcam	1:1000
Anti-Axin	Abcam	1:1000
Anti-GSK3β	Abcam	1:5000
Anti-p-GSK3β	Cell Signaling Technology	1:1000
Anti-β-catenin	Cell Signaling Technology	1:1000
Anti-p-β-catenin	Cell Signaling Technology	1:1000
Anti-c-myc	Abcam	1:1000
Anti-cyclin D1	Abcam	1:200
Anti-Histon H3	Cell Signaling Technology	1:1000
Anti-Ki-67	Cell Signaling Technology	1:200

were evaluated by using the $2^{-\Delta\Delta Ct}$ method. Primer sequences are listed in **Table 2**.

Western blotting

Proteins of cells were extracted using Whole Cell Lysis Assay (KeyGEN Bio TECH, Jiangsu, China) and Nuclear and Cytoplasmic Protein Extraction Kit (KeyGEN Bio TECH, Jiangsu, China). The BCA protein reagent kit (Thermofisher, Scientific, Inc.) was used to measure the protein concentration. The protein samples (30-50 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked by 5% milk before incubating with specific primary antibodies overnight at 4°C. Then the membranes were incubated with secondary antibodies for 1 h at room temperature. Finally the protein bands were detected and scanned by Bio Imaging Systems (BIO-RAD, CA, USA). The antibodies are listed in **Table 3**.

Flow cytometric analysis

Cells were digested with trypsin without EDTA and 5×10^5 cells were counted for analysis. After being washed twice with PBS, the cells were collected and stained with allophycocyanin (APC)-conjugated anti-CD44 (BioLegend, CA, USA) and phycoerythrin (PE)-conjugated anti-CD24 (BioLegend, CA, USA) for 30 min following the manufacturer's instructions. After being diluted in moderate PBS, cells were examined using BD flow cytometer and the data were analyzed using FlowJo software.

Colony formation and wound-healing assay

Cells were plated at a density of 1000 cells/well in a 6-well plate and incubated for 10 days. Then the cells were fixed with 100% methanol for 15 min and stained with 1% crystal violet for 10 min. Visible colonies in the wells were counted under a light microscope directly.

For Wound-healing assay, the cells were incubated in 6-well plates until density reached nearly 80-90% confluence. The cell monolayer was wounded by 200 μl pipette tips. The wound areas were micrographed at 0, 24, 48 and 72 h and analyzed by ImageJ software.

Immunohistochemical analysis (IHC)

Human breast cancer tissues, normal adjacent tissues and tumor tissues from mouse xeno-

graft models were paraffin-embedded and cut into 5 μm thick slices. The tissue sections were deparaffinized, rehydrated and preincubated with primary and secondary antibodies. Then the slides were immunostained with diaminobenzidine (DAB) and counterstained with hematoxylin (CWBIO, Beijing, China). Antibodies used for IHC are provided in **Table 3**.

Mouse xenograft model

The procedures of all animal experiments were conducted in accordance with principles and guidelines approved by the Ningxia Medical University Ethics Committee of Animal Research. BALB/c Nude mice (4 to 6 weeks old) were purchased from the Beijing Vital River Laboratory Animal Technology Co. Ltd (Beijing, China) and reared according to the Animal Care guideline. Twenty-four female BALB/C nude mice were randomly divided into four groups as Si-NC group, Si-Mot group, Si-NC+LiCl group and Si-Mot+LiCl group. MCF-7 cells were transfected with lentivirus vectors and disposed with LiCl (30 mmol/L, 12 h), then serum-free culture to form mammospheres. The mammospheres were resuspended in 100 μl PBS and injected 1×10^5 sphere cells into mammary fat pads of the mice. After 24 days, the xenograft tumor models were sacrificed and the tumors were collected. The tumor sizes were measured with caliper and recorded every four days. Tumor volume was calculated as 1/2 (length × width^2). The tumors were also prepared for immunohistochemistry and western blotting analysis.

Statistical analysis

All data were obtained from at least three independent experiments and displayed as mean ± SD. Statistical analyses were performed using SPSS 23.0, GraphPad Prism version 6.0 and Microsoft Office Excel 2013 software. Comparisons between different groups were evaluated by Student's t-test. One-way ANOVA were used for comparing multiple groups. $P<0.05$ was considered statistically significant.

Results

Expression of mortalin in breast cancer

To detect expression of mortalin in breast cancer tissues, qRT-PCR and IHC were performed on 24 pairs of breast cancer tissues and adjacent normal tissues. The results showed that mortalin was over-expressed in breast cancer tissues and localized mainly in the cytoplasm (**Figure 1A** and **1B**). qRT-PCR and western blotting obtained consistent results in breast cancer cell line MCF-7, MDA-MB-231, SKBR-3 and BT-474 and human normal breast epithelial cells MCF-10A (**Figure 1C** and **1D**). Kaplan-Meier Plotter analysis indicated that the tumor with high mortalin expression had a worse overall survival than those with low mortalin expression (**Figure 1E**). Based on the results, we concluded that mortalin is highly expressed in breast cancer cells and might play vital roles in the development of breast cancer.

Expression of mortalin in BCSCs

To examine the relationship between mortalin expression and BCSCs, we conducted mammosphere formation and measured the BCSCs surface marker by flow cytometry. With serum free medium (SFM) and low oxygen condition, we successful cultured mammospheres for three generations. The mammosphere formation capacity of MCF-7 cells was higher than that of MDA-MB-231 (**Figure 2A** and **2B**). Flow cytometry results showed that about 46.6% spheres from MCF-7 cells exhibited $CD44^+/CD24^-$ compared to their counterpart adherent cells (12.5%), while the spheres from MDA-MB-231 cells was 92.9% (**Figure 2C**). We further detected the expression of mortalin, together with stem cell markers ALDH1, ABCG2, OCT4 and Nanog in sphere and adherent cells. The result showed that the mortalin and stem cell markers were all elevated in spherical cells compared to their counterpart adherent cells (**Figure 2D** and **2E**). The results indicated that mortalin was enriched in BCSCs and correlated with the stemness of BCSCs.

The relationship between Mortalin and EMT in breast cancer cells

To assess the role of mortalin in breast cancer progression, lentivirus for over-expressing or knockdown mortalin were constructed and transfected in MCF-7 and MDA-MB-231 cells (Figure S2). Based on qRT-PCR and western blotting results, the expression of mortalin was significantly decreased in 3 siRNA group (**Figure 3A** and **3B**). These results suggested that 3 siRNA lentivirus vectors were all effective and

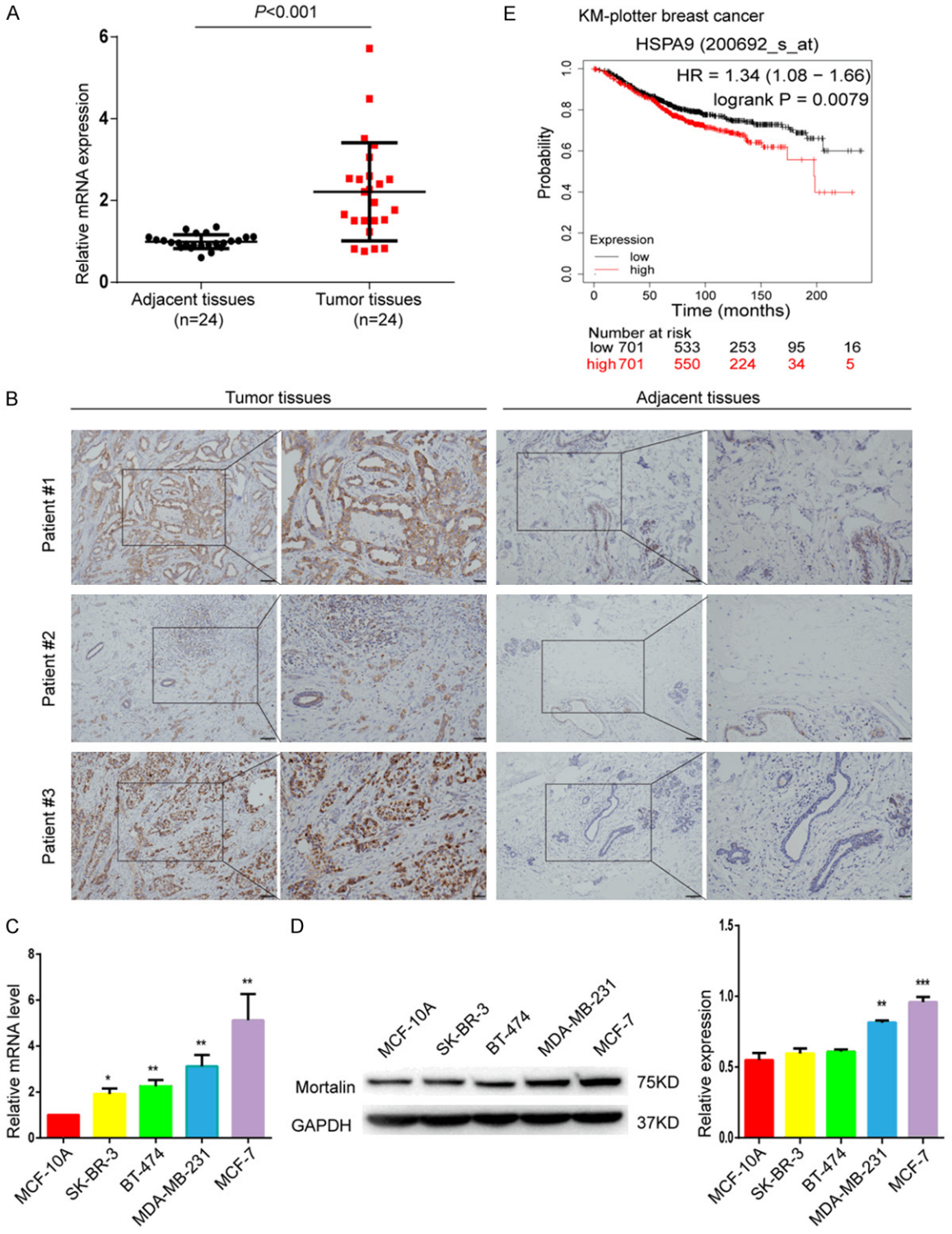
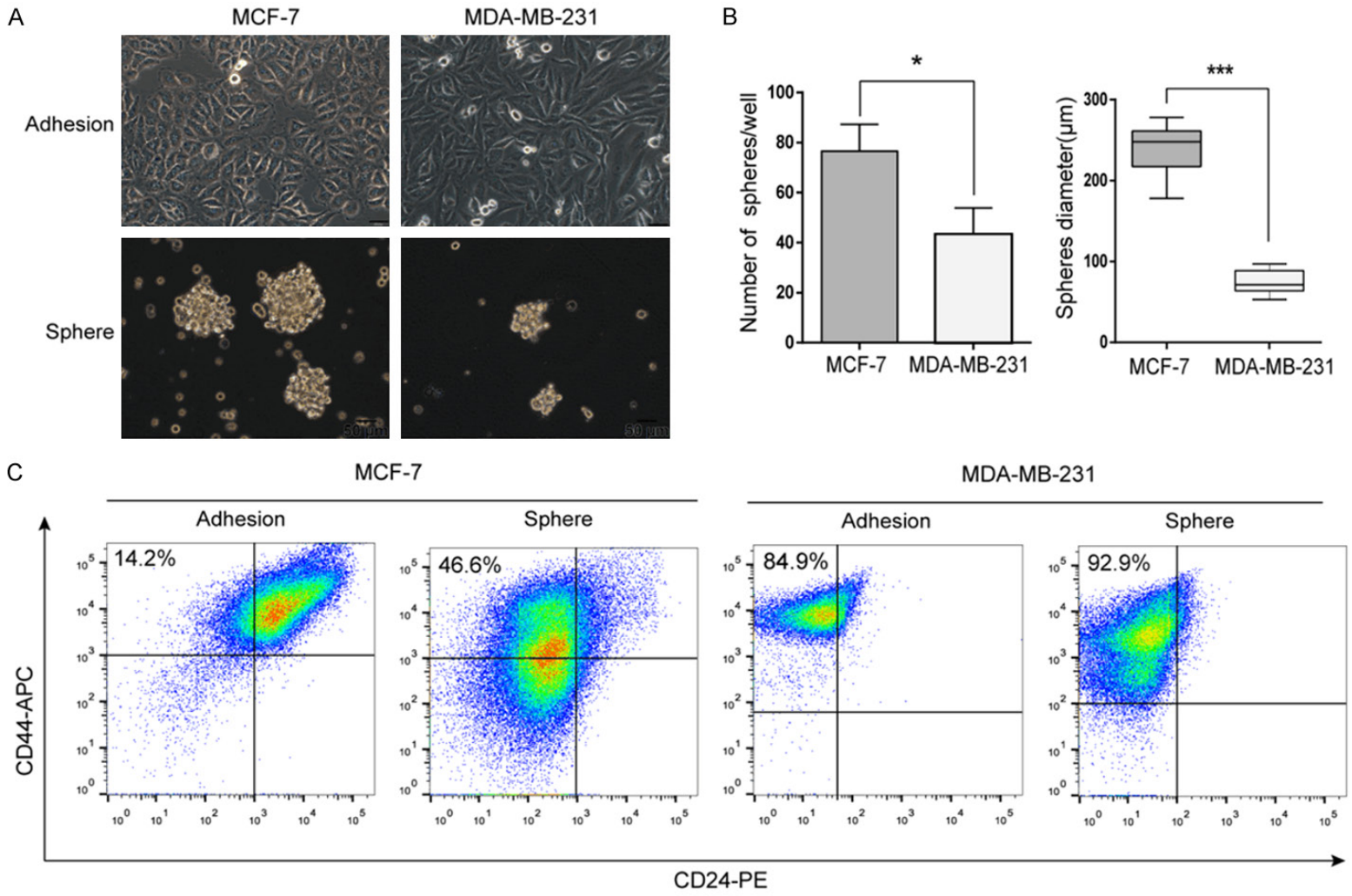


Figure 1. Mortalin is overexpressed in breast cancer and negatively correlated with patient overall survival. A. The expression levels of mortalin in breast cancer tissues and matched adjacent tissues by qRT-PCR. B. Representative pictures of mortalin expression by immunohistochemical staining in breast cancer tissues and matched adjacent tissues (mortalin expression in tumor tissues and adjacent tissues: the left row: ×100; the right row: ×400). C, D. Mortalin expression were detected in human normal breast epithelial cells (MCF-10A) and human breast cancer cells (MCF-7, MDA-MB-231, BT-474 and SK-BR-3) by qRT-PCR and western blotting. E. The correlation between mortalin (HSPA9) expression and the overall survival of breast cancer patients was analyzed by Kaplan-Meier Plotter. GAPDH served as internal control. **P*<0.05, ***P*<0.01, ****P*<0.001.


A
MCF-7
MDA-MB-231
Adhesion
Sphere
B
Number of spheres/well
Spheres diameter(μm)
MCF-7
MDA-MB-231
C
MCF-7
MDA-MB-231
Adhesion
Sphere
14.2%
46.6%
84.9%
92.9%
CD44-APC
CD24-PE

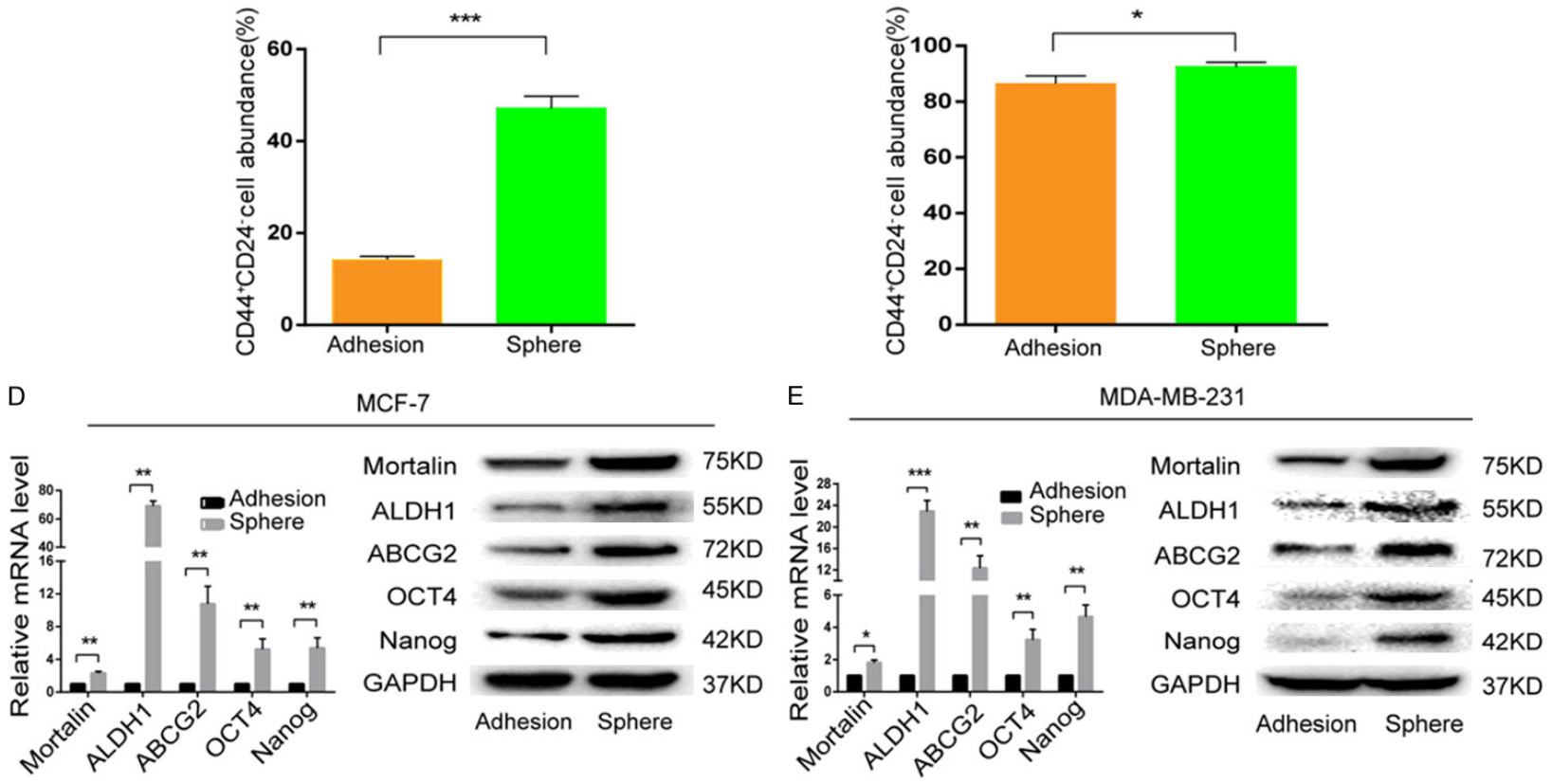
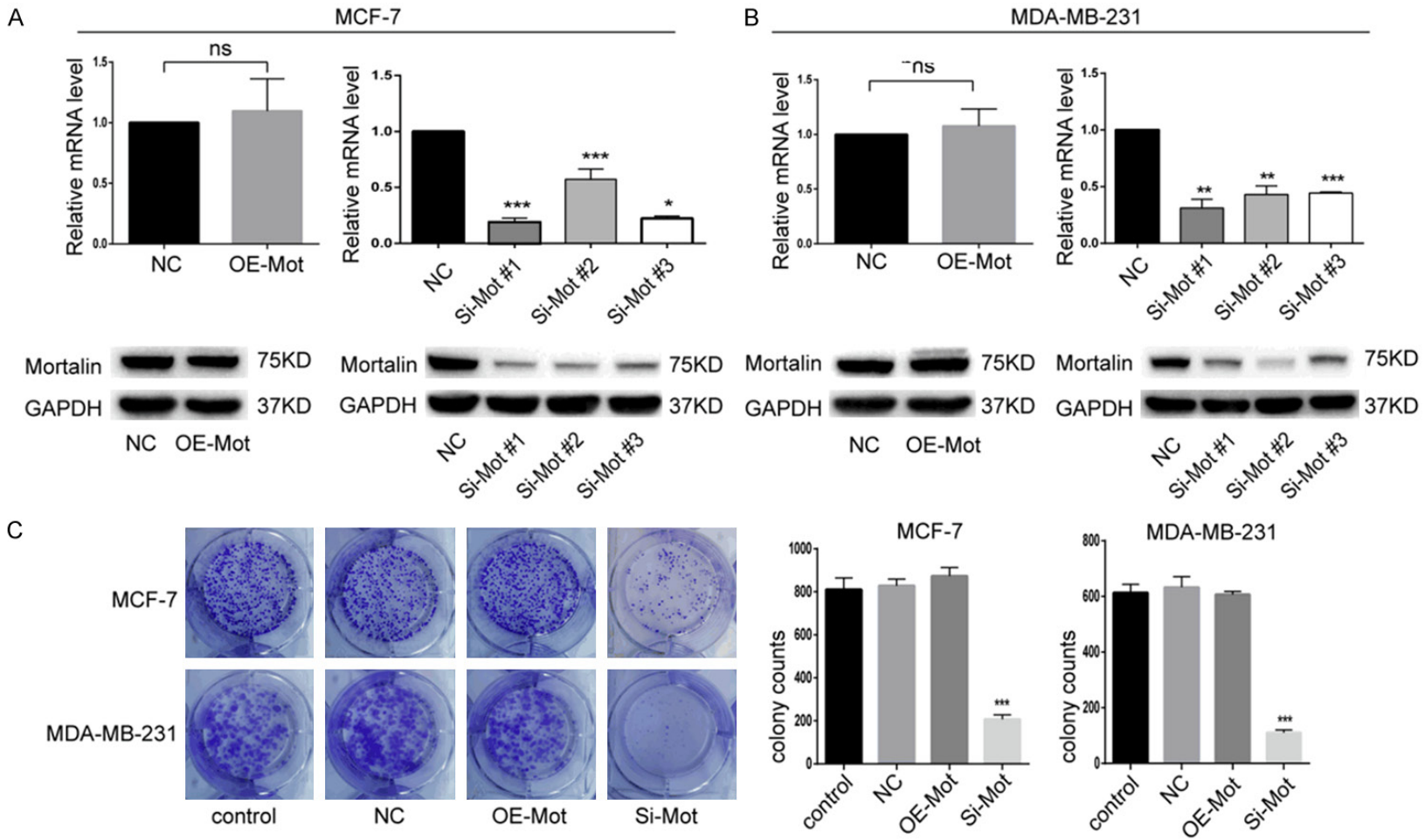


Figure 2. Mortalin is highly expressed in BCSCs. A. BCSCs were enriched by mammosphere formation assay (×200). B. Comparison of mammosphere formation among inoculated cells in MCF-7 and MDA-MB-231 cells. **P*<0.05, ****P*<0.001. C. The $CD44^+/CD24^-$ cell population was assayed by flow cytometry. D, E. The mRNA expression of mortalin, ALDH1, ABCG2, OCT4, Nanog and their protein expression were analyzed by qRT-PCR and western blot, respectively. **P*<0.05, ***P*<0.01, ****P*<0.001.


A
MCF-7
ns
Relative mRNA level
NC
OE-Mot
***
*
Si-Mot #1
Si-Mot #2
Si-Mot #3
Mortalin
75KD
GAPDH
37KD
B
MDA-MB-231
**
***
C
MCF-7
MDA-MB-231
control
NC
OE-Mot
Si-Mot
colony counts

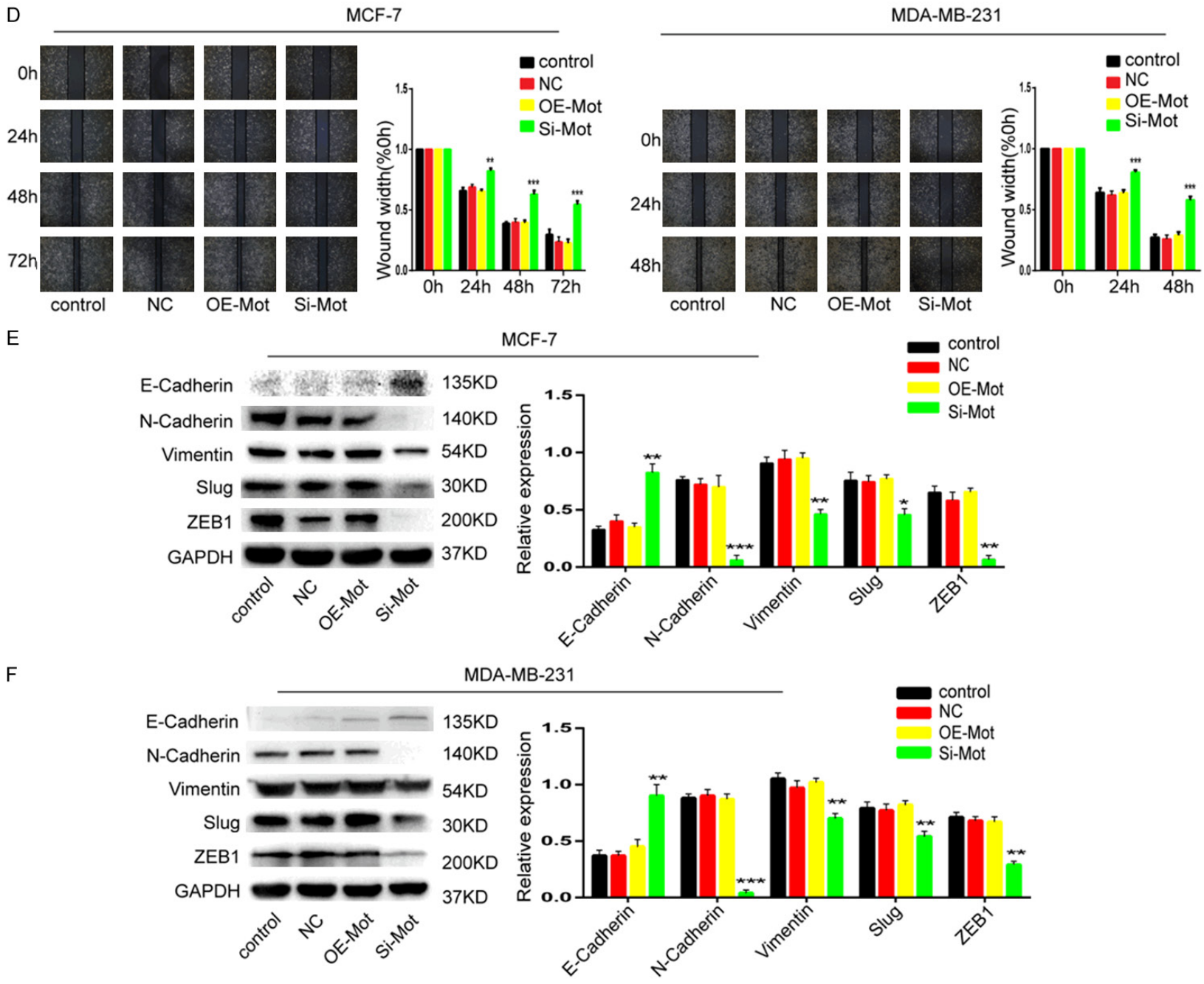

D
MCF-7
MDA-MB-231
0h
24h
48h
72h
control
NC
OE-Mot
Si-Mot
Wound width(%0h)
E
F
E-Cadherin
N-Cadherin
Vimentin
Slug
ZEB1
GAPDH
135KD
140KD
54KD
30KD
200KD
37KD
Relative expression

Figure 3. Effects of mortalin expression on breast cancer cell proliferation, migration and EMT. A, B. Efficiencies for overexpression and knockdown of mortalin were tested by qRT-PCR and western blot analysis in MCF-7 and MDA-MB-231 cells. C. Colony formation assay showed the cell self-renewal in MCF-7 and MDA-MB-231 cells after up-regulation and down-regulation of mortalin. D. The migration assays showed the cell motility in MCF-7 and MDA-MB-231 cells after up-regulation and down-regulation of mortalin. E, F. Protein expression of E-cadherin, N-cadherin, Vimentin, Slug and ZEB1 were analyzed by western blot in MCF-7 and MDA-MB-231 cells. **P*<0.05, ***P*<0.01, ****P*<0.001. ns represents no statistical significance.

stability. We then chose one siRNA vector which has the best interference effect for further experiments (Figure S3). In order to identify the influence of mortalin on the proliferation and migration of breast cancer cells, colony formation and wound healing experiments were performed. The results showed that knockdown of mortalin inhibited proliferation and migration capacity of the breast cancer cells (**Figures 3C**, **3D** and S1). EMT was a biological process for breast cancer tumorigenesis. Here in the study, we examined the EMT-related protein E-cadherin, N-cadherin, Vimentin, Slug and ZEB1 by western blotting. The results proved that knockdown of mortalin could significantly decrease the expression of Vimentin, N-cadherin, Slug and ZEB1 in the breast cancer cells, while increased E-cadherin expression (**Figure 3E** and **3F**). These results suggested that mortalin could promote the proliferation, migration, self-renewal and invasion capacity of breast cancer cells by regulating EMT.

Influence of mortalin on the stemness of BCSCs

In the mammosphere formation assay, knockdown of mortalin significantly inhibited the formation of spheroids (**Figure 4A**). The $CD44^+/CD24^-$ BCSC sub-proportion was evidently decreased in MCF-7 and MDA-MB-231 cells (**Figure 4B**). Meanwhile, ALDH1, ABCG2, OCT4 and Nanog were decreased both in mRNA and protein levels (**Figure 4C** and **4D**). Collectively, these results demonstrated that mortalin could maintain the stemness of BCSCs.

The relationship between mortalin and Wnt/β-catenin signaling pathway

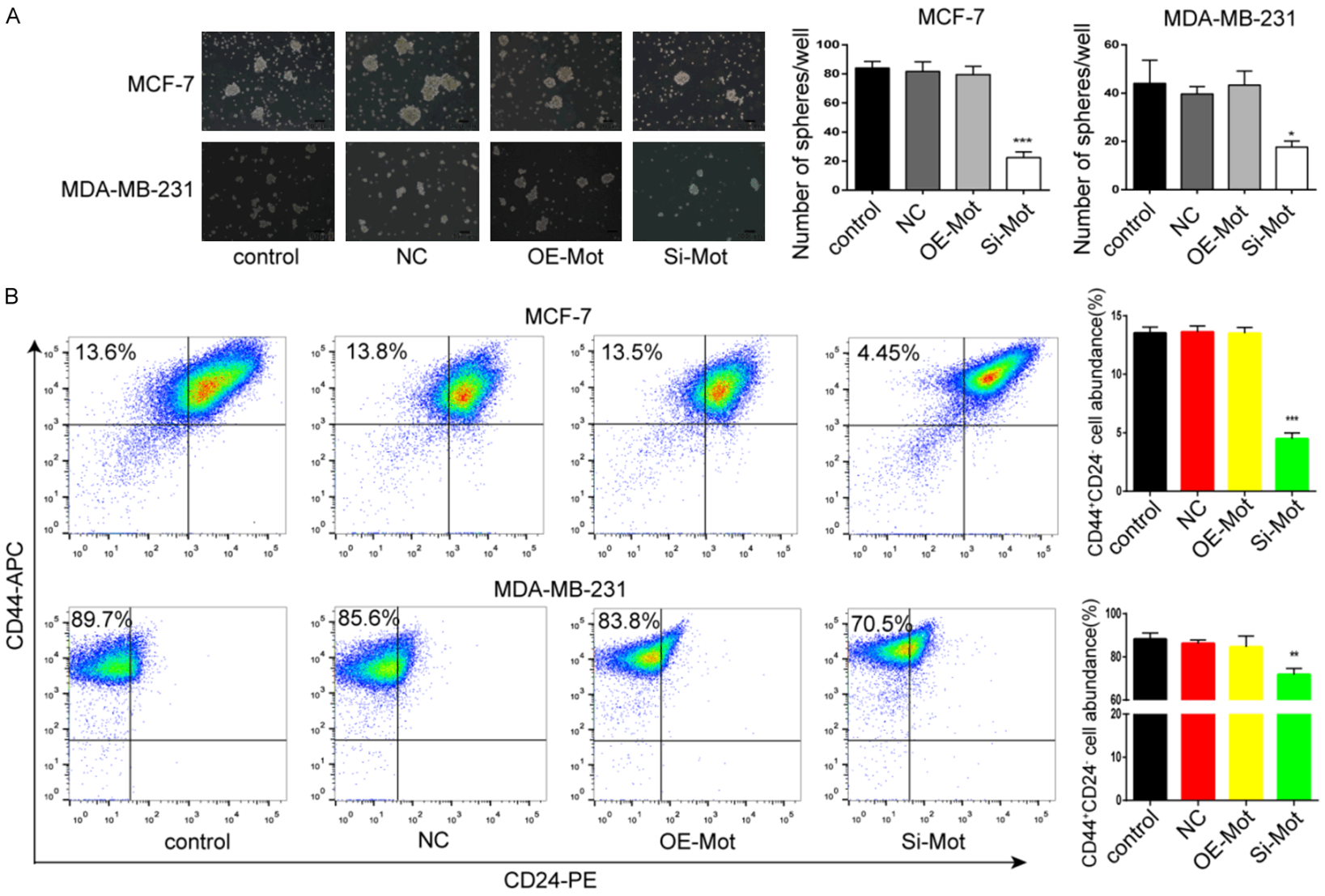
Wnt/β-catenin signaling pathway plays an important role for EMT progression in breast cancer. To identify the relationship between mortalin and Wnt/β-catenin signaling pathway, we examined the expression of the genes in Wnt/β-catenin signaling pathway in MCF-7 and MDA-MB-231 cells, in mRNA and protein level. The result showed that the mRNA level of APC, β-catenin, c-myc and cyclin D1 were significantly decreased in mortalin-KO cells (**Figure 5A** and **5B**). Western blotting proved that knockdown of mortalin significantly inhibited the expression of APC, Axin2, β-catenin, p-GSK3β (S9), cyclin D1 and c-myc, while increased the expression of p-β-catenin in MCF-7 and MDA-MB-231 cells (**Figure 5C** and **5D**). Besides, knockdown of mortalin significantly inhibited the protein expression of nuclear β-catenin and c-myc (**Figure 5E** and **5F**). These results indicated that knockdown of mortalin could inhibit Wnt/β-catenin signaling pathway in breast cancer cells.

Mortalin maintains stemness of BSCSs via the Wnt/GSK3β/β-catenin signaling pathway

In **Figure 5**, we have proved that knockdown of mortalin could inhibit Wnt/β-catenin signaling pathway, but the mechanism was not clear. To further address if mortalin maintains the stemness of BCSCs via Wnt/β-catenin signaling pathway, LiCl, an agonist of Wnt/β-catenin signaling pathway and also a GSK3β inhibitor, was used to determine the association between Wnt/β-catenin signaling pathway and mortalin. In the mammosphere forming assay, knockdown of mortalin significantly decreased the number of the spheres, while LiCl reversed the phenomenon (**Figure 6A** and **6B**). Knockdown of mortalin inhibited p-GSK3β (S9) expression as well as β-catenin (both total protein and nuclear protein), c-myc, cyclin D1 and stem cell markers (ABCG2, OCT4, Nanog) in BCSCs. In addition, LiCl could eliminate the effect of mortalin knockdown on the expression of stem cell markers in MCF-7 and MDA-MB-231 cells (**Figure 6C** and **6D**). The results suggested that mortalin could maintain the stemness of breast cancer cells via activating the Wnt/GSK3β/β-catenin signaling pathway *in vitro*.

Xenograft experiment identified the function of mortalin

Finally, we confirmed the effect of mortalin maintaining stemness in breast cancer *in vivo*. After transfected lentivirus to knock down mor-


A
MCF-7
MDA-MB-231
control
NC
OE-Mot
Si-Mot
MCF-7
MDA-MB-231
Number of spheres/well
B
MCF-7
MDA-MB-231
13.6%
13.8%
13.5%
4.45%
89.7%
85.6%
83.8%
70.5%
CD44-APC
CD24-PE
CD44+CD24- cell abundance(%)

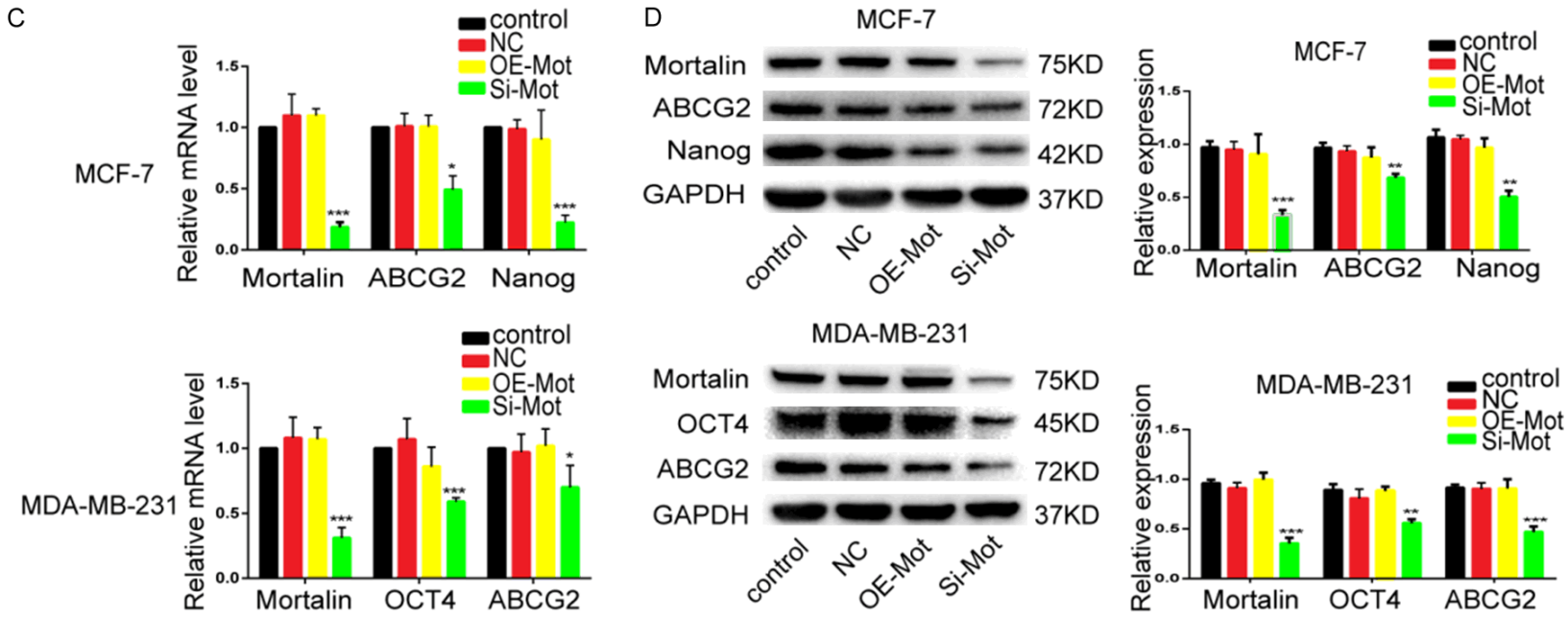


Figure 4. Effects of mortalin expression on BCSCs stemness. A. The spheroid number showed the mammosphere-forming abilities after up-regulation and down-regulation of mortalin. B. Flow cytometry showed the $CD44^{+}/CD24^{-}$ cell population after up-regulation and down-regulation of mortalin. C, D. The mRNA expression of mortalin, ABCG2, Nanog in MCF-7 cells and mortalin, OCT4, ABCG2 in MDA-MB-231 cells and their protein expression were analyzed by qRT-PCR and western blot, respectively. **P*<0.05, ***P*<0.01, ****P*<0.001.

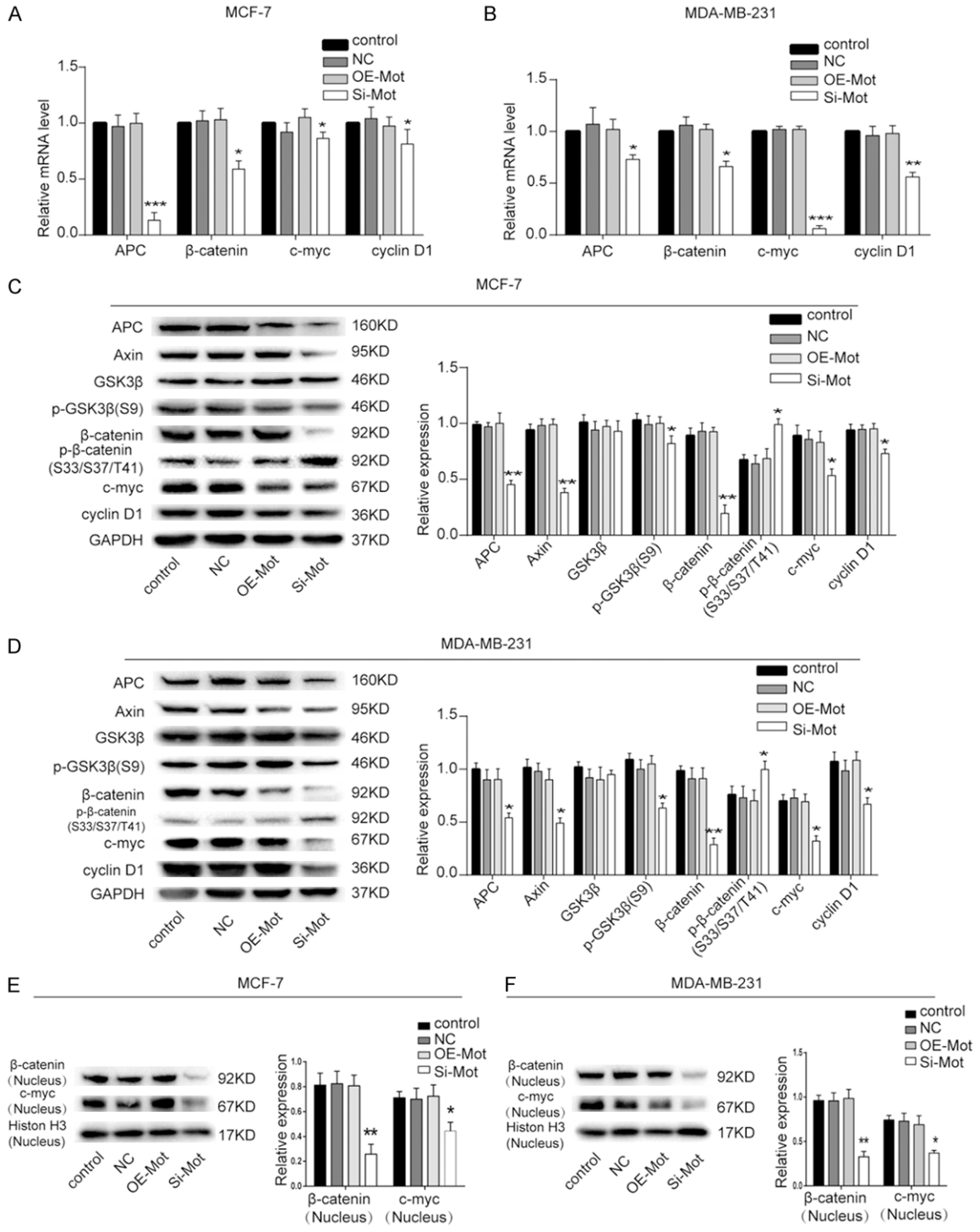


Figure 5. Effects of mortalin expression on Wnt/β-catenin signaling in breast cells. A, B. The mRNA expression of APC, β-catenin, c-myc, cyclin D1 were analyzed by qRT-PCR in MCF-7 and MDA-MB-231 cells after up-regulation and down-regulation of mortalin. C, D. The total protein expression of APC, Axin, GSK3β, p-GSK3β (S9), β-catenin, p-β-catenin (S33/S37/T41), c-myc and cyclin D1 were analyzed by western blot in MCF-7 and MDA-MB-231 cells. E, F. The nuclear protein expression of β-catenin and c-myc were analyzed by western blot in MCF-7 and MDA-MB-231 cells. GAPDH and Histon H3 served as internal controls. **P*<0.05, ***P*<0.01, ****P*<0.001.

talin in LiCl-treated MCF-7 cells, we injected the sphere cells into nude mice for xenograft experiment. The volume and weight of tumor were measured. The results showed that knockdown

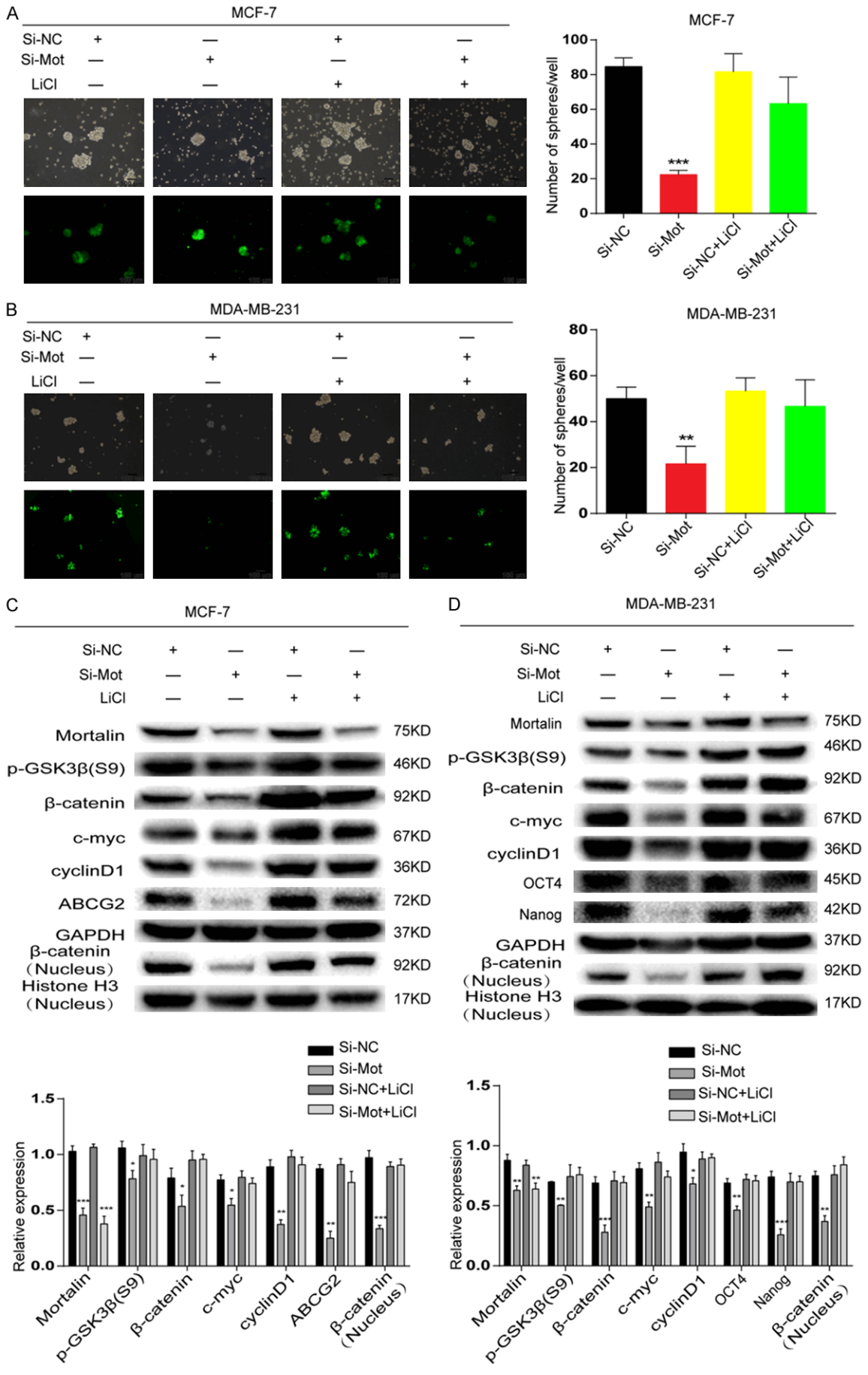

A
MCF-7
Si-NC
Si-Mot
LiCl
MCF-7
Number of spheres/well
Si-NC
Si-Mot
Si-NC+LiCl
Si-Mot+LiCl
B
MDA-MB-231
MDA-MB-231
C
MCF-7
Mortalin
p-GSK3β(S9)
β-catenin
c-myc
cyclinD1
ABCG2
GAPDH
β-catenin (Nucleus)
Histone H3 (Nucleus)
75KD
46KD
92KD
67KD
36KD
72KD
37KD
17KD
Relative expression
D
MDA-MB-231
OCT4
Nanog
45KD
42KD

Figure 6. Mortalin maintain stemness of BCSCs via activating the Wnt/GSK3β/β-catenin signaling pathway *in vitro*. A, B. The spheroid number showed mammosphere-forming abilities. LiCl eliminated the effect of mortalin knockdown on the mammosphere-forming abilities in MCF-7 and MDA-MB-231 cells (×100). C. LiCl eliminated the effect of mortalin knockdown on the total protein expression of ABCG2, β-catenin, p-GSK3β (S9), c-myc, cyclin D1 and the nuclear protein expression of β-catenin, which were analyzed by western blot in MCF-7 cells. D. LiCl eliminated the effect of mortalin knockdown on the total protein expression of OCT4, Nanog, β-catenin, p-GSK3β (S9), c-myc, cyclin D1 and the nuclear protein expression of β-catenin, which were analyzed by western blot in MDA-MB-231 cells. **P*<0.05, ***P*<0.01, ****P*<0.001.

of mortalin could reduce tumor volume and weight. After treated with LiCl, the average volume and weight of xenograft tumor were increased significantly (**Figure 7A-C**). We then collected xenograft tumor tissues and conducted immunohistochemistry and western blot. The results proved that the knockdown of mortalin could inhibit the expression of cell proliferation marker Ki-67, p-GSK3β, β-catenin, c-myc, cyclin D1 and ABCG2, while the inhibition was significantly rescued by treating with LiCl (**Figure 7D**). Western blot was consistent with the results (**Figure 7E**). The findings suggested that mortalin could maintain breast cancer stem-like properties via the Wnt/GSK3β/β-catenin signaling pathway *in vivo* (**Figure 8**).

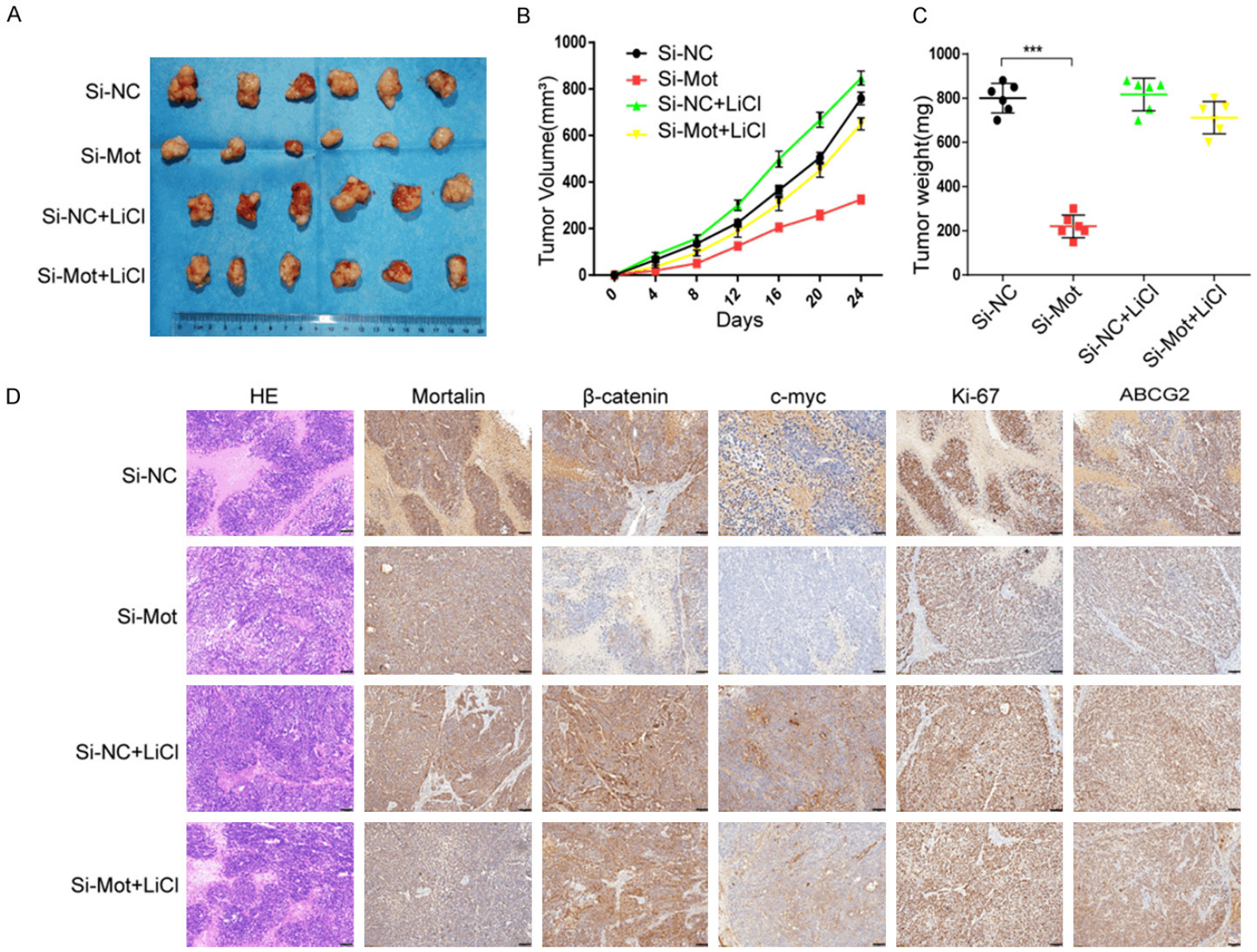
Discussion

Breast cancer was one of the most common malignancies which caused by the development of malignant cells in the breast [16]. Despite of several advanced therapies exist, the occurrence of metastasis and drug-resistance still leads to a high morbidity and mortality for breast cancer [17]. BCSCs is a group of cells which constitute a subpopulation of tumor cells that endowed with stem cell properties and have a high capacity for tumor generation in vivo [5, 18]. BCSCs were initially described as the cells with the surface markers of $CD44^+/CD24^{-/low}$, which displayed self-renewal and tumor-initiating capacity [19, 20]. Later, several other biomarkers that characterize BCSCs have been identified, such as ALDH1, OCT4, Sox2, Nanog, and ABCG2 [21, 22]. The mammosphere formation is often used to estimate the stemness phenotypes of BCSCs, because BCSCs tend to become sphere in serum free medium and under low oxygen condition [23]. In our study, we successfully harvested mammosphere from MCF-7 and MDA-MB-231 cells. The average number and diameter of spheres from MCF-7 cells were bigger than that of MDA-MB-231 cells. The expression of $CD44^+/CD24^-$ subpopulation in MCF-7 and MDA-MB-231 spheres cells was significantly higher than that in counterpart adherent cells. Further detection of ALDH1, ABCG2, OCT4 and Nanog mRNAs and proteins identified that the mammosphere cells displayed the features of stem cells, which could be BCSCs.

BCSCs can be enriched and isolated by culturing of cells in non-differentiating serum-free and low oxygen culture conditions. In breast cancer, the mammosphere culture has been widely used to enrich and isolate BCSCs from breast cancer cell lines. In this study, we successfully harvested mammosphere from MCF-7 and MDA-MB-231 cells by serum-free and low oxygen culture conditions. The mammosphere formation ability and tumorigenic are closely related to the stemness. The stem cells gene and protein of $CD44^+/CD24^-$, ALDH1, ABCG2, OCT4 and Nanog are all high expression in the BCSCs mammosphere. The results proved the mammosphere we got was the BCSCs.

Epithelial-mesenchymal transition (EMT) was a biological process in which polarized epithelial cells are transformed into motile mesenchymal cells with interstitial phenotype through specific procedures [24]. In breast cancer cells, EMT participates in the occurrence and progression of cancer, and promotes the cancer cells invasive, migratory, metastasis and drug-resistance [25, 26]. In BCSCs, the intermediate EMT states generate the properties of stemness which is essential for tumor propagation [27-29].

Mortalin is one of members in Hsp70 family. It was initially found in the saccharomyces cerevisiae, namely Ssc1p [30]. In mitochondria biogenesis, mortalin acts as a chaperoning and influence most of these cellular functions, like regulates cell stress response, cell proliferation and apoptosis. Previous research


A
Si-NC
Si-Mot
Si-NC+LiCl
Si-Mot+LiCl
B
Si-NC
Si-Mot
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Si-Mot+LiCl
Tumor Volume(mm³)
1000
800
600
400
200
0
0
4
8
12
16
20
24
Days
C
***
Tumor weight(mg)
1000
800
600
400
200
0
Si-NC
Si-Mot
Si-NC+LiCl
Si-Mot+LiCl
D
HE
Mortalin
β-catenin
c-myc
Ki-67
ABCG2
Si-NC
Si-Mot
Si-NC+LiCl
Si-Mot+LiCl

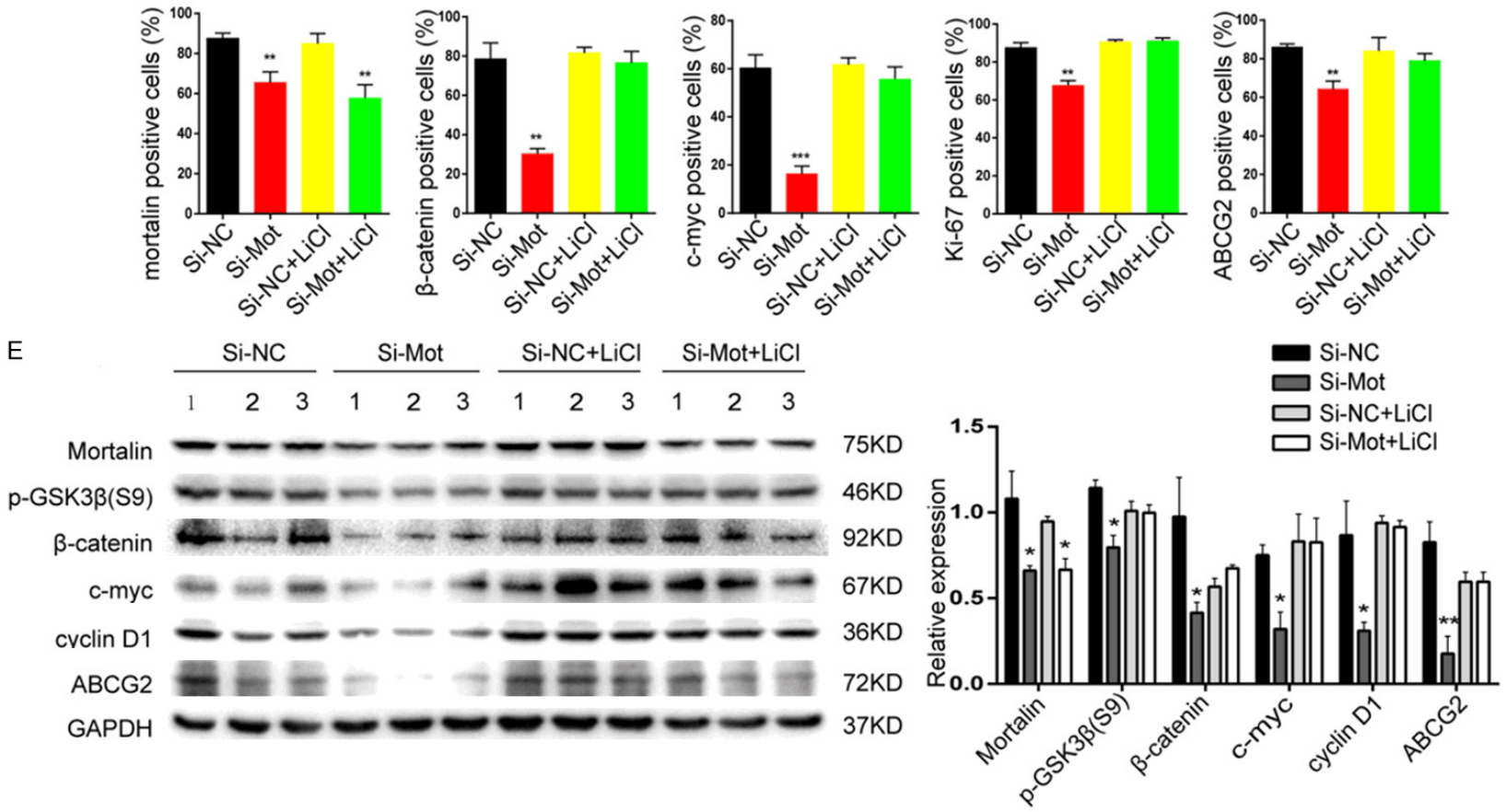

Figure 7. Mortalin maintain stemness of BCSCs via activating the Wnt/GSK3β/β-catenin signaling pathway *in vivo*. A-C. Tumor size is pictured, and tumor volume and weight were measured for detecting tumor proliferation and stemness. D. HE staining and immunohistochemical detection of mortalin, ABCG2, β-catenin, c-myc and Ki-67 in tumor xenografts (×100). E. The expression of mortalin, ABCG2, p-GSK3β, β-catenin, cyclin D1 and c-myc were analysis by western blotting in tumor xenografts. **P*<0.05, ***P*<0.01, ****P*<0.001.

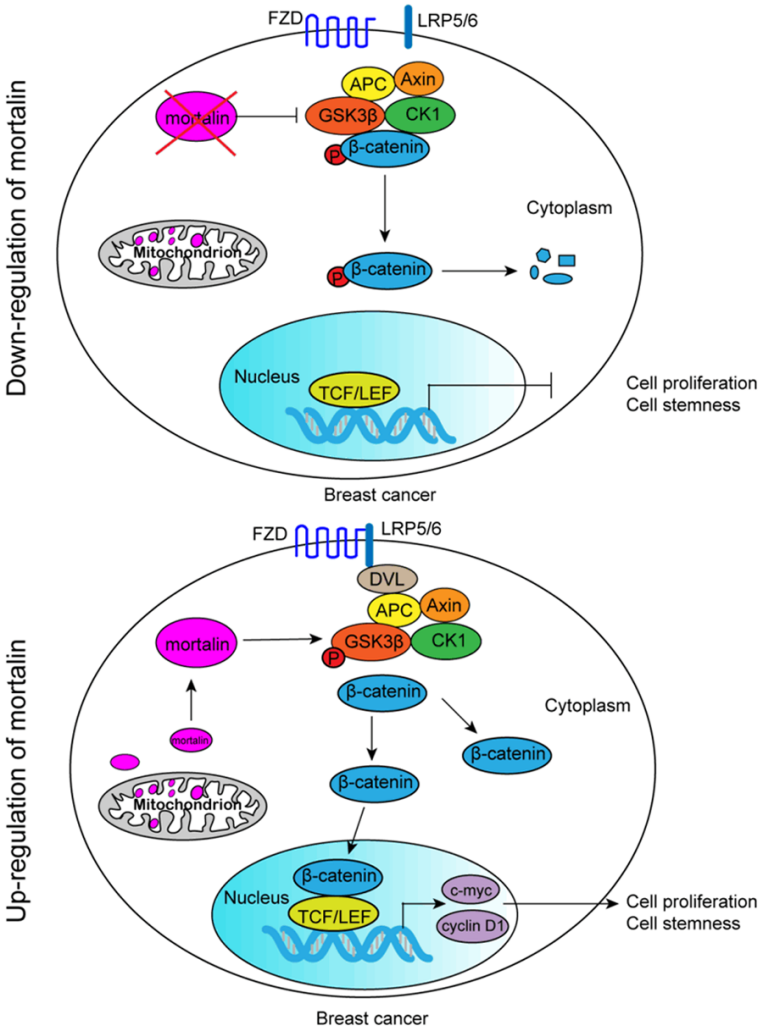

Figure 8. Proposed model to illustrate the mechanisms of mortalin promote cell proliferation and maintain stemness by Wnt/GSK3β/β-catenin signaling pathway. Original Western blotting images are presented in Figure S4.

reported that GRP75 (the gene name of mortalin) acts as an oncogene in many types of cancer and participated in tumorigenesis and tumor progression [11, 31]. In ovarian carcinoma and colorectal cancer cells, the over-expression of mortalin is correlated with Vimentin expression and promotes EMT [32, 33]. In breast cancer research, Jin H. et al. reported that mortalin have a high correlation with metastasis of cancer in invasive ductal carcinoma of breast [12]. Na Y. et al. reported that mortalin contributes to breast cancer cells EMT transition through up-regulating PI3K/Akt and JAK/STAT signaling pathways [14]. In our study, we first demonstrated that mortalin is highly expressed in breast cancer tissues and BCSCs. Moreover, we proved that knockdown of mortalin could inhibit the proliferation, invasion and EMT of breast cancer cells. Based on our research, we verified that mortalin have the capacity of promoting the proliferation, migration and invasion of breast cancer cells by regulate EMT.

Wnt/β-catenin signal pathway is widely reported to play an important role in human cancers [34]. Canonical Wnt/β-catenin pathway components and processes include the Wnt secretory machinery, Wnt co-receptors, components of the β-catenin destruction complex and nuclear co-factors [35]. When the signaling is inactive, β-catenin is phosphorylated and degraded by a destruction complex, which contains the scaffold protein Axin, APC and the kinases GSK3β. When the pathway is activated, the destruction complex is inactivate, leads to a translocation of β-catenin into the nucleus to activate the downstream targets. The role of Wnt/β-catenin pathway in breast cancer development and progression has been studied intensively [36, 37]. The pathway was also known for playing an important role in breast tumor growth and BCSCs maintenance [5, 38, 39]. In the Wnt/β-catenin signaling pathway, GSK3β plays as a key factor and regulates the signaling pathway activities. GSK3β promote the phosphorylation of β-catenin at Ser33 and Ser37, which leads to the degradation of β-catenin and thus inhibited the activation of Wnt/β-catenin signaling pathway. When the phosphorylation site at Ser9 of GSK3β is activated, which led to the accumulation of β-catenin, and finally activated Wnt/β-catenin signaling pathway [40, 41]. This transcriptional switch leads to a change of multiple cellular processes that are associated with breast can-

cer EMT transition, metastasis and stemness and thereby influence breast tumor progression [42-44]. In this study, we found that knockdown of mortalin significantly inhibited mammosphere formation and decreased the expression of stem cell markers by regulating EMT process in BCSCs. Besides, knockdown of mortalin could inhibit Wnt/β-catenin signaling pathway. LiCl, a GSK3β inhibitor, reversed the inhibitory effects of mortalin both *in vivo* and *in vitro*. The results proved that GSK3β could influence the function of mortalin on the activity of Wnt/β-catenin signaling pathway and the stemness of BCSCs.

In our study, we successfully isolated and identified BCSCs from MCF-7 and MDA-MB-231 cells. And we proved for the first time that mortalin is highly expressed in BCSCs. When knockdown of mortalin in MCF-7 and MDA-MB-231 cells, the EMT process was inhibited, as well as cell proliferation, mammosphere formation ability and the expression of stem cell markers. More importantly, we proved that mortalin could activate the Wnt/GSK3β/β-catenin signaling pathway both in vivo and in vitro experiments. The results confirmed that mortalin might maintain BCSCs stemness by regulating EMT process via activating Wnt/GSK3β/β-catenin signaling pathway.

In conclusion, our research elucidated the critical role of mortalin in maintaining the stemness of BCSCs. Over-expression of mortalin promoted EMT and maintain the stemness of BCSCs in breast cancer cells. Mortalin might maintain BCSCs stemness via activating the Wnt/GSK3β/β-catenin signaling pathway both *in vivo* and *in vitro*, and provide a potential target for breast cancer therapy.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

BCSCs, breast cancer stem cells; EMT, Epithelial-mesenchymal transition; Hsp70, heat shock protein 70; IHC, Immunohistochemical; SFM, serum free medium.

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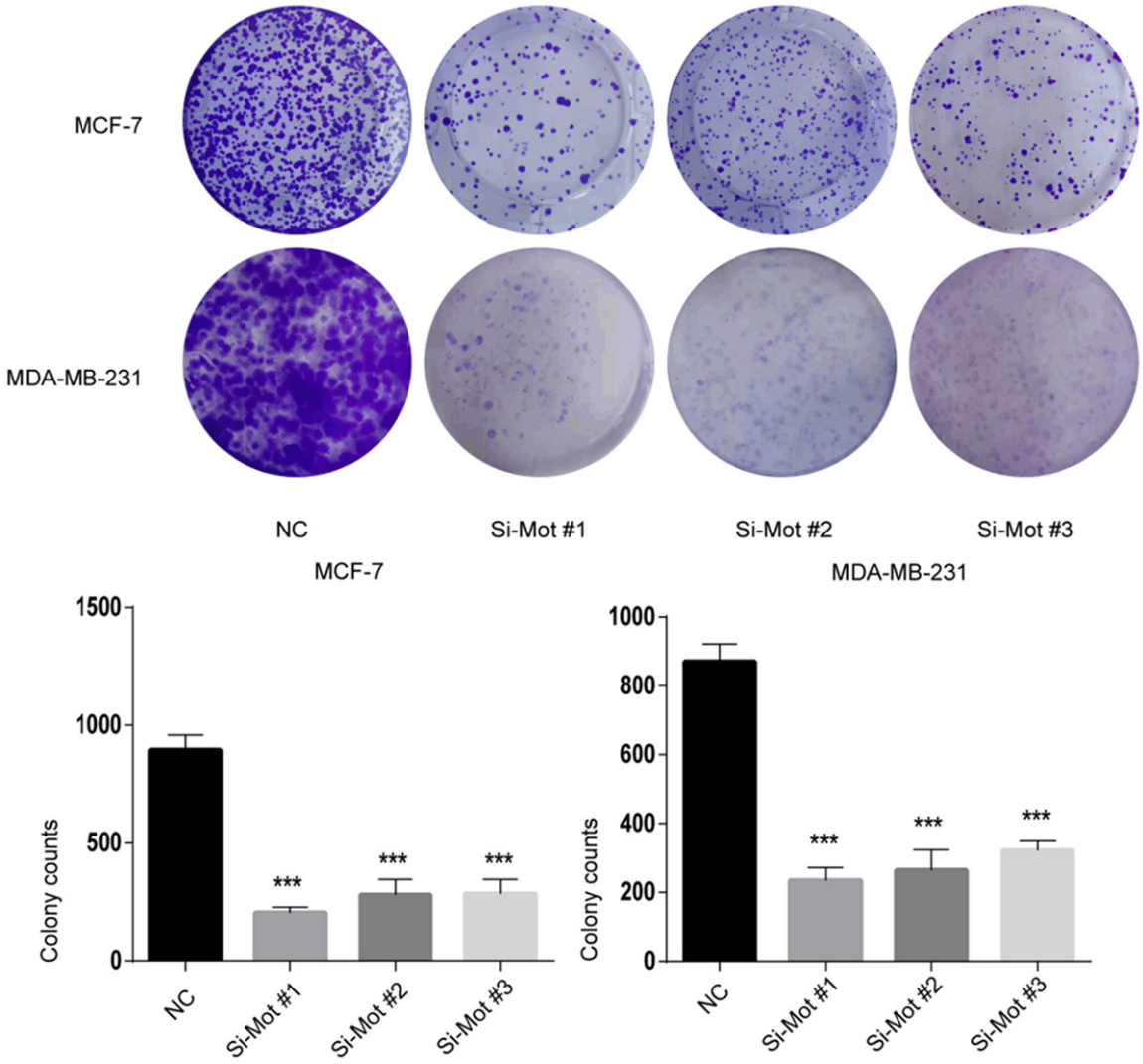

Figure S1. Colony formation assay showed the cell proliferation capacity of MCF-7 and MDA-MB-231 cells after down-regulation of mortalin.

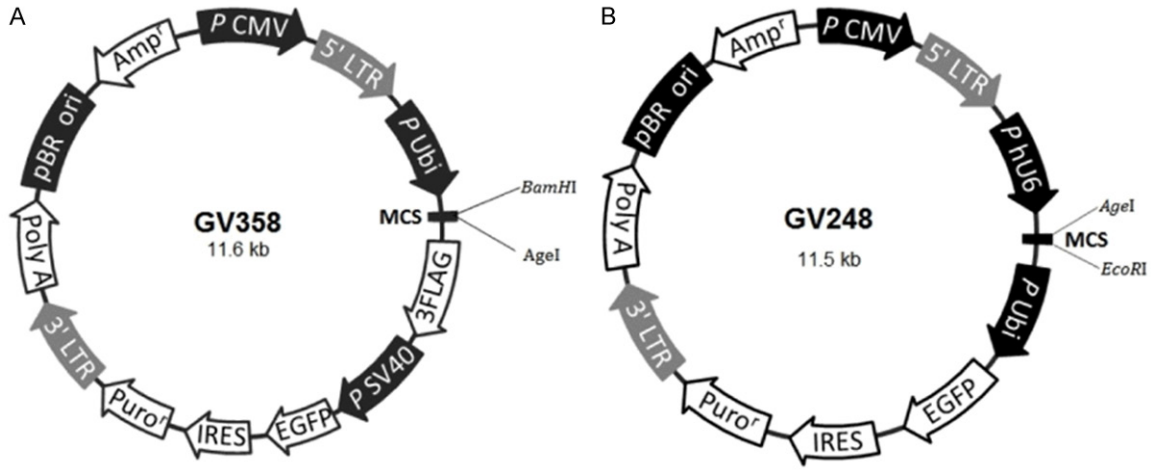

Figure S2. Construction of lentivirus vector maps for over-expression or knockdown of mortalin. A. Construction of lentivirus vector map for over-expression of mortalin. B. Construction of lentivirus vector map for knockdown of mortalin.

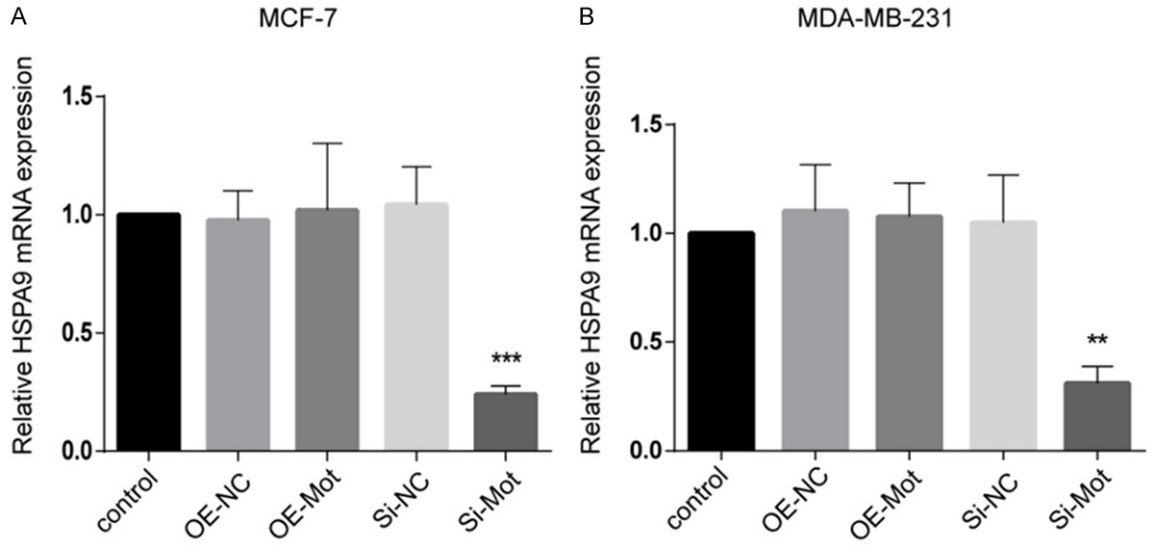

Figure S3. The expression of mortalin was tested by qRT-PCR in breast cancer cells. A. The expression of mortalin was tested by qRT-PCR in MCF-7 cells. B. The expression of mortalin was tested by qRT-PCR in MDA-MB-231 cells.

Figure 1-D

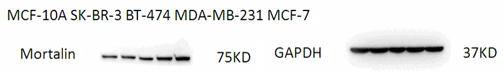

Figure 2-D

Figure 2-E

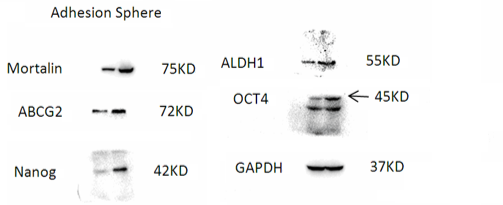

Figure 3-A

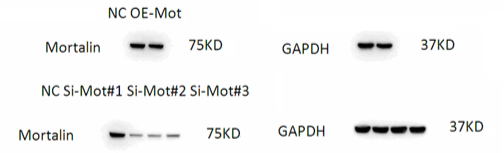

Figure 3-B

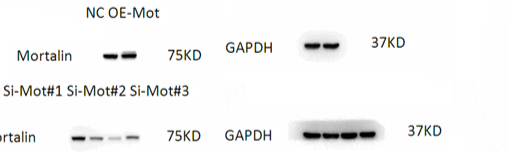

Figure 3-E

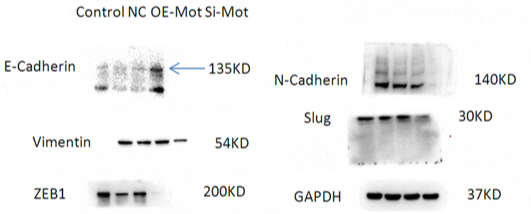

Figure 3-E

Figure 4-D MCF-7

Figure 4-D MDA-MB-231

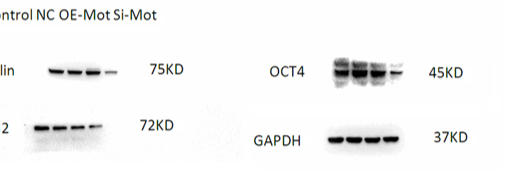

Figure 5-C

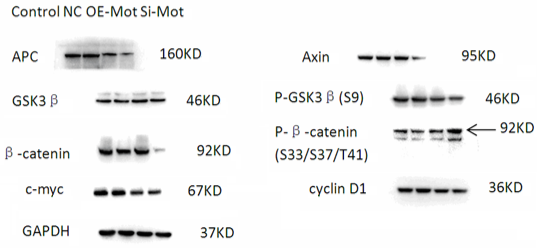

Figure 5-D

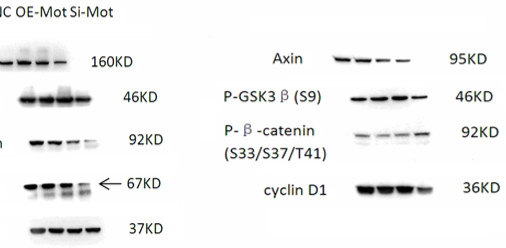

Figure 5-E

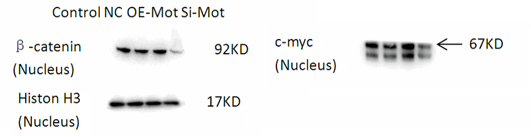

Figure 5-F

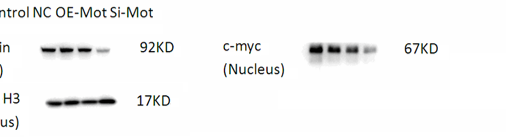

Figure 6-C

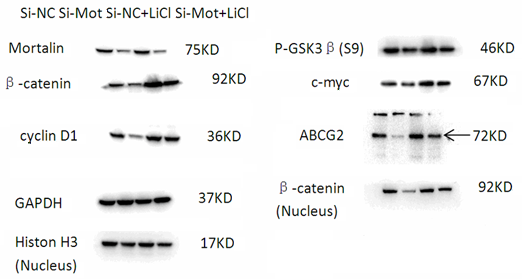

Figure 6-D

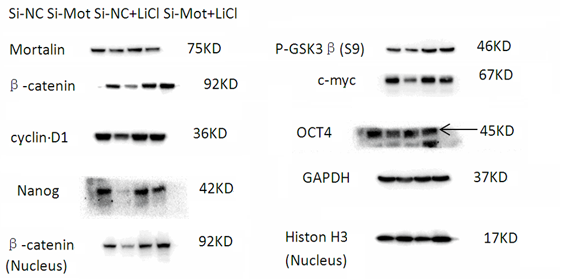

Figure 7-E

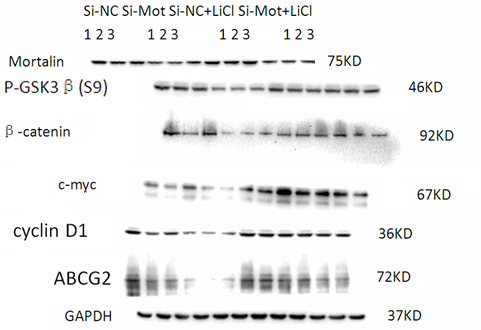

Figure S4. Original Western blotting images of **Figures 1-8**.