Original Article
Runx2 is required for activity of CD44+/CD24-/low breast cancer stem cell in breast cancer development

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Abstract: Runx2, a master regulator of osteogenesis, is abnormally expressed in advanced breast cancer. Here we addressed Runx2 contribution to breast cancer cell growth and metastasis. We found that CD44 and Runx2 were both elevated in breast cancer tissues compared with the adjacent normal tissues in breast cancer patients. Runx2 expression was significantly correlated with tumor TNM stage, metastasis and poor prognosis. We then screened several breast cancer cell lines and found that Runx2 expression level was positively related to the malignant level of the cells screened. Knockdown of Runx2 in high metastatic cell line MDA-MB-231 could inhibit breast cancer cell vitality, invasion and clone formation capacity, while overexpression of Runx2 in low metastatic cell line MCF-7 could increase those malignant behaviors. The mechanism might be due to Runx2 positively regulating cancer stem cell properties, as CD44 expression level and CD44+/CD24-/low breast cancer stem cell population were both significantly decreased in Runx2 knockdown cells. Cancer stem cell renewal ability such as soft agar clone formation, mammospheres formation and tumor formation ability in null mice were all decreased after knockdown of Runx2. On the contrary, overexpression of Runx2 could enhance all above stem cell renewal ability. Lastly, we explored how Runx2 changes cancer stem cell population. We found it could affect epithelial mesenchymal transition (EMT). Runx2 could regulate mesenchymal marker and epithelial marker expression and affect activation of Wnt/β-catenin signaling pathway. These results together strongly suggest that Runx2 can promote CD44+/CD24-/low breast cancer stem cell properties and breast cancer tumorigenesis through EMT process.

Keywords: Runx2, breast cancer stem cell, breast cancer, epithelial-mesenchymal transition

Introduction

Breast cancer is the most common form of cancer diagnosed in women and it affects an estimated 10% of women worldwide [1]. An understanding of the molecular mechanisms of breast cancer proliferation and metastasis is very important to promote novel therapeutic strategies for breast cancer [2]. The cancer stem cell theory poses that cancers develop from a subset of malignant cells that possess stem cell characteristics, and it may account for the development of a variety of malignancies, including breast cancer [3]. Many studies confirmed that BCSCs (breast cancer stem cells) exist in cells with a cell surface profile of CD44+/CD24-/low phenotype. The distinctive subtypes were extended to ESA+ and ALDH-1+ (aldehyde dehydrogenase-1) [4, 5]. The CD44+/CD24-/low phenotype is the most widely studied marker of BCSCs. Many studies showed that BCSCs were major factors of breast cancer tumorigenesis, especially in the initial stage and metastasis process [6]. For example, most (70%) of the cells in early bone marrow metastases of breast cancer patients contain CD44+/CD24-/low cells [7], but the mechanism of how BCSCs are regulated is poorly understood.

Runt-related transcription factor 2 (Runx2) is a scaffolding protein that promotes bone formation by interacting with regulators of cell

growth or mediators of signaling cascades that are upregulated in tumor cells, especially in breast cancer and metastatic breast cancer. Many well-characterized Runx2 target genes, e.g., vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs), bone sialoprotein (BSP) and osteopontin (OPN), are associated with tumor growth, invasion and metastasis [8-11]. Our previous studies documented an important role for Runx2 in the support of breast cancer cell growth and activation of a variety of genes, including Sox-9, Col10a1, and MMP9 [12-14]. Notably, we recently found that Runx2 was highly expressed in the CD44+/CD24-/low population and in metastatic breast cancer cell such as MDA-MB-231, Sum149. All these might suggest that Runx2 could promote tumorigenesis via the regulation of breast cancer stem cells. Therefore, in this study we investigated whether and how Runx2 promoted the growth and metastasis of breast cancer via CD44+/CD24-/low breast cancer stem cells.

Materials and methods

Cell culture and transfection

Human breast cancer cell lines MCF-7, MDA-MB-231 and a normal human breast epithelial cell line MCF-10A were purchased from Shanghai Institute of Biochemistry and Cell Biology (SIBCB; Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) and maintained at 37°C in a 5% CO_2-humidified incubator. Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used for cell transfection according to the manufacturer's protocol with empty pc-DNA3.1 (blank) vector or pc-DNA3.1-RUNX2 plasmid (100 nM; Yearthbio, Changsha, China), respectively. Cells were used for subsequent experimentation 48 h post-transfection.

Clinical sample collection

Tissue samples were obtained from 75 patients (age range, 31-69 years; mean age, 52.5 years) diagnosed with breast cancer in the Department of Breast Surgery, the First Affiliated Hospital of Anhui Medical University (Hefei, China) and the Department of Emergency Surgery, Fuyang Hospital of Anhui Medical University (Fuyang, China) from September 2014 to March 2016. None radiotherapy and chemotherapy were performed before surgery. A series of demographic and clinical data were collected including tumor characteristics and pathologic stages. Survival and follow-up data were acquired by telephone interviews. The distributions by postoperative stage were 52% Stage I and II and 48% Stage III and IV. Primary breast cancer tissue and adjacent healthy tissue were collected and stored at -80°C following histopathological evaluation. The follow-up period was 5 years. The current study was performed with approval from the Ethics Committee at Anhui Medical University. Written informed consent was obtained from all patients.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from tissues or cell lines using TRIzol ® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Total RNA (1 μg) was reverse transcribed into cDNA using the miScript Reverse Transcription kit (Qiagen, Inc., Valencia, CA, USA), according to the manufacturer's protocol. qPCR was subsequently performed in an ABI 7500 PCR machine (Thermo Fisher Scientific, Inc.) using the miScript SYBR Green PCR kit (Qiagen, Inc.) according to the manufacturer's protocol. The following primers were used: Runx2 forward primer (5'-CCGGAAT-GCCTCTGCTGTTATGA-3' and reverse primer (5'-ACTGAGGCGGTCAGAGAACAAACT-3'); β-actin forward primer (5'-ACTGGTCTCAGTCAGTG-TACAGC-3') and reverse primer (5'-ACAGG-AAGTCCCTTGCCATC-3').

Western blot

Western blotting was performed according to a standard procedure. Briefly, protein lysates were boiled in the sample loading buffer, resolved using 8% sodium dodecyl sulfate-PAGE (SDS-PAGE) and transferred onto a PVDF membrane. Membranes were blocked with 5% skim milk powder, probed with primary antibodies at 4°C overnight, and incubated with corresponding secondary antibodies for 1 hour at room temperature.

Unbound antibodies in each step were washed with TBST four times. The immune complexes were detected using an enhanced chemiluminescence kit. Antibodies: anti-Runx2 (Abcam, ab76956), anti-CD24 (GTX37755), anti-CD44 (Abcam, ab51037).

Immunohistochemical staining

The tissue sections were deparaffinized at 60°C for 10 min followed by a 15 min immersion in xylene. The sections were rehydrated via sequential incubation in 100%, 90%, and 70% ethanol. Samples were rinsed with PBS followed by distilled water and incubated for 30 min in H_2O_2. Antigen retrieval was performed via microwave irradiation for 10 min. The sections were incubated with anti-Runx2 (Abcam, ab76956), anti-CD44 (Abcam, ab51037), anti-TGF-β (CST #3711) and anti-CD24 (GTX-37755) antibodies at 4°C overnight. The slides were washed 4 times in TBS/saponin and incubated with biotinylated secondary antibodies for 30 min. Avidin-biotin-peroxidase reagents were added, and the slides were incubated with a 0.5 mg/mL HRP substrate solution (DAB+H_2O_2 prepared in distilled water) to expose the resulting peroxidase activity. Slides were washed four times in PBS and counterstained for 1 min with hematoxylin. The slides were sealed and observed under optical microscopy.

Cell viability assay

The cells were plated into 96-well plates at 3000 cells per well and cultured at 37°C, 5% CO_2 for 0, 24, 48, and 72 h. MTT (50 μl) was added to each well and incubated for 4 h. The supernatant was discarded, and 150 μl of dimethyl sulfoxide was added to each well with agitation for 15 min. The dissolved reaction mixture was placed in a microplate reader and read at the absorbance of 490 nm.

Transwell assay

For invasion assays, chamber inserts were coated with Matrigel (BD Biosciences). Cells were resuspended in serum-free medium and added to the upper chamber. After 48 h of incubation, the cells on the upper surface were removed mechanically, and the filters were fixed and stained with crystal violet for 20 min. Filters were washed with PBS to re-move excess staining, and cells were counted under an inverted microscope.

Colony formation assay

500 cells/well were seeded in 6-well plates. Cells were incubated for 14 days and stained with 0.2% crystal violet for 20 min at room temperature. The number of colonies in each well were counted.

Soft agar colony formation assays

A 24-well plate was coated with a 1:1 ratio of 1.2% agarose and 2× DMEM and solidified for 30 min. A top layer of 0.7% agarose was placed on top of the bottom layer, and 2× DMEM with the cells at a density of 3000 cells/ml were added. Plates were incubated for 3 weeks and photographed under a microscope and the colonies were counted.

Flow cytometry and FACS analysis

The expression of cell surface markers (CD44 and CD24) was analyzed using flow cytometry. Briefly, cells were suspended in PBS containing 2% BSA (106 cells/100 μl). Combinations of FITC-CD44 and PE-CD24 or their respective isotype controls were added to the cell suspension at the concentrations recommended by the manufacturer and incubated at 4°C in the dark for 30 min. The labeled cells were washed with PBS and analyzed on a flow cytometer (BD Biosciences, Bedford, MD, USA).

Mammosphere formation assays

Single cell suspensions of adherent cultures were plated in 6-well ultralow attachment plates (Falcon, Corning Life Science) at $2×10^3$ cells/well. Mammosphere cultures were maintained in serum-free DMEM/F12 media (1:1), B27 (1:50), EGF (20 ng/ml) and bFGF (20 ng/ml), insulin (5 μg/ml), and hydrocortisone (5 μg/ml). After 10 days, mammospheres (sphere-like structures with diameter of ~50 μm) were clearly detected using an optical phase contrast microscope (Nikon-Eclipse TS100, 4× magnification). Cultures were pipetted up and down to dissociate spheres into single cells, which were reseeded for mammosphere formation. The experiment ended at the third generation of mammosphere formation. The

number of mammospheres in each well that were 60 µm or larger in size were counted according to the manufacturer's protocol (MammoCult™ Human Medium, Stemcell Technologies).

In vivo tumourigenicity assay

Animal experiments were performed according to procedures approved by the Nanjing Model Animal Institute. To examine the effect of Runx2 on tumorigenesis, female BALB/c nude mice (6-8 wks of age) were injected with 1×10^7 exogenous Runx2-knockdown MDA-MB-231 cells on the left upper limb and vector control cells on the right limb. Tumor formation was examined periodically. Tumor sizes and weights were measured every 2~4 days for 4 weeks. Mice were sacrificed, and the tumors were isolated at 50 days post-injection. Upon tumor removal, tumor volumes were calculated using the equation tumor volume = (length × width)/2. The tumor samples were fixed in 10% neutral-buffered formalin for further analyses.

Statistical analysis

The data are presented as the means ± SD. Student's t-test was performed to evaluate statistical significance. Correlation analysis of clinical pathological characteristics was performed using the Chi-squared test, and Kaplan-Meier survival curves were used to analyze survival differences. *indicates P<0.05, **indicates P<0.01. Data were analyzed using the SPSS 16.0 software.

Results

Runx2 is positively related to malignant level of breast cancer both in vivo and in vitro

We detected the expression of Runx2 in six breast cancer cell lines and one normal breast epithelial cell line. The result showed its level is generally higher in all cancer cell lines than in normal cells. But the elevated degree of Runx2 is quite different in those breast cancer cells. It's much higher in the aggressive cell line such as triple negative breast cancer cell lines, MB-231, Sum149 and Sum159 than other non-metastatic cell lines such as Bt474, skb3 and MCF-7 (**Figure 1A**). This result suggests the expression level

of Runx2 is related to the metastatic degree of cell lines. Because one of the known characteristics of metastasis is the predominance of cells with BCSC attributes, we then examined CD44, CD24 and Runx2 expression levels in cells and tissues. In three different aggressive cell lines (MCF-10A, MCF-7 and MB-231), CD44 level positively correlated with Runx2 , on the contrary, CD24 level negatively correlated with Runx2 (**Figure 1B**). Patients with breast cancer were divided into two groups according to the mean Runx2 expression value (0.872): high Runx2 expression and low Runx2 expression. The expression level of Runx2 was higher in breast cancer tissue samples than adjacent tissue samples from patients with breast cancer (**Figure 1C**). Survival analysis showed patients with breast cancer with high Runx2 expression demonstrated a poorer prognosis (**Figure 1D**). Immunohistochemistry showed the expression levels of CD44 correlated with Runx2 in breast cancer tissues and adjacent tissues (**Figure 1E**). The clinical significance of Runx2 expression in breast cancer was examined. The high expression group was associated with advanced clinical staging, lymphatic metastasis and Ki67 expression. However, no association of expression with age, tumor size, ER\PR\HER expression, or differentiation was identified (**Table 1**). These data showed that Runx2 was positively related to the malignancy level of breast cancer both *in vivo* and *in vitro*.

Runx2 promotes breast cell growth and metastasis directly

To clarify the precise effect of Runx2 on breast cancer cell malignant behavior, MCF-7 cells, which have a relatively low endogenous Runx2 level, were transfectedwith LV-Runx2 to upregulate Runx2. The MDA-MB-231 cells were transfected with LV-Runx2-RNAi to knock down Runx2 expression. The mRNA and protein expression of Runx2 were measured using RT-PCR and Western blotting, respectively (**Figure 2A**). We investigated the proliferation and metastatic ability of these cells. The results showed that overexpression of Runx2 increased the viability of MCF-7, and knockdown of Runx2 inhibited MB231 cell viability directly (**Figure 2B**). Similarly, overexpression of Runx2 increased the invasive ability of MCF-7, and knockdown of Runx2 inhibited
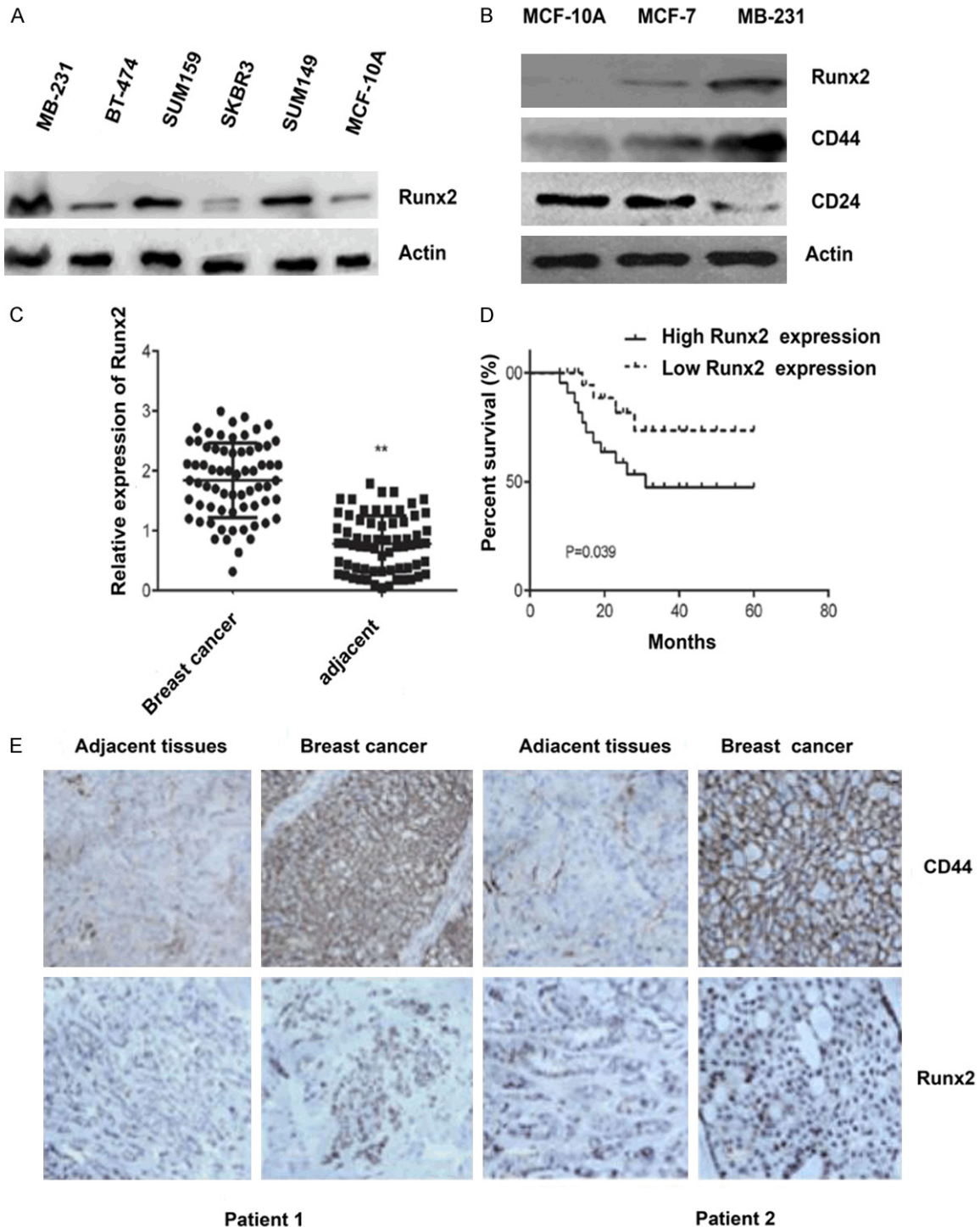
Figure 1. Runx2 is positively related to malignant level of breast cancer both *in vivo* and *in vitro*. A. Screen the difference expression of Runx2 by western blot in a series of breast cancer cell lines. B. The correlation of CD44 and Runx2 expression in breast cancer cells. C. The expression of Runx2 in clinical breast cancer patients tumor and adjacent tissues (**P<0.01). D. The correalation between Runx2 expression level and patients overall survival at five years after diagnosis. E. ICH of Runx2 and CD44 in adjacent and carcinoma tissues.

MDA-MB-231 invasion ability (**Figure 2C**). Overexpression of Runx2 increased the plate colony formation of MCF-7, and knockdown of Runx2 inhibited MDA-MB-231 plate colony for-

Table 1. The relationship between Runx2 expression and clinical characteristics in breast cancer patients

Clinical Characteristic	RunX2 Expression		χ^2	P value
	High	Low		
Age, years			7.82	0. 530
<50/≥50	16/28	11/20		
Pathological type			11.46	0. 082
Catheterinfiltration/non-catheter infiltration	38/6	15/16		
Postoperative staging			16.63	0. 226
I-II/III-IV	21/23	18/13		
Lymphatic			14.76	0. 038
Metastasis/without metastasis	25/19	17/14		
Tumor size, cm			5.89	0. 892
≤20/<20-50/>50	21/15/8	13/10/8		
ER status			12.51	0. 067
ER positive/ER negative	16/28	18/13		
PR status			12.29	0. 076
PR positive/PR negative	20/24	16/15		
HER2 status			8.98	0. 227
HER2 positive/HER2 negative	21/23	18/13		
Ki67 expression			17.47	0. 019
Low/high	8/36	21/10		

mation directly (**Figure 2D**). All these data suggest that Runx2 works as an oncogene and promotes breast cancer cell growth and invasion directly.

Runx2 promoted CD44⁺/CD24⁻ breast cancer stem cells renewal and properties directly

The same elevated level of CD44 and Runx2 expression in high metastatic breast cancer lines and patient breast tumor samples prompted us to investigate whether Runx2 regulated CD44⁺ breast cancer stem cells directly. First, we checked the CD44⁺/CD24$^{-/low}$ cancer stem cell populations in the above-mentioned cell lines. As show in **Figure 3A** and **3B**, knockdown of Runx2 reduced CD44 protein levels, increased CD24 levels and reduced the CD44⁺/CD24$^{-/low}$ cell population in all three ShRNA-Runx2-transfected MB231 cell lines (ShRNA194, ShRNA195, and ShRNA196), especially the shRNA-196 group, which was chosen for subsequent experiments. Overexpression of Runx2 increased CD44 protein levels, decreased CD24 levels and increased the CD44⁺/CD24$^{-/low}$ cell population. We checked stem cell properties, such as soft agar colony formation, self-renewal and

tumorigenesis ability in null mice. As expected, the overexpression of Runx2 significantly increased soft agar colonies formation and mammosphere generation of MCF-7 cells, and knockdown of Runx2 expression significantly reduced the mammospheres formation of MB-231 cells (**Figure 3C**, **3D**). *In vivo* tumor xenograft experiments showed that Runx2 knockdown (MB-231 group) had slower tumor growth speed and smaller tumor volume compared to normal MB-231 cells (**Figure 3E**). Immunohistochemistry in mice tumor samples examined the expression of Runx2 and CD44. Consistent with the analyses of cell lines, knockdown of Runx2 expression caused a significant decrease in CD44 expression (**Figure 3F**) in tumor tissues. These data demonstrated that Runx2 promoted CD44⁺/CD24⁻ breast cancer stem cell renewal and properties directly.

The mechanism of how Runx2 regulates breast cancer stem cell may through EMT process and Wnt/β-catenin signal pathway

EMT generates cells that are less differentiated and give rise to cancer stem cells. During cell culturing, we found that Runx2 expression
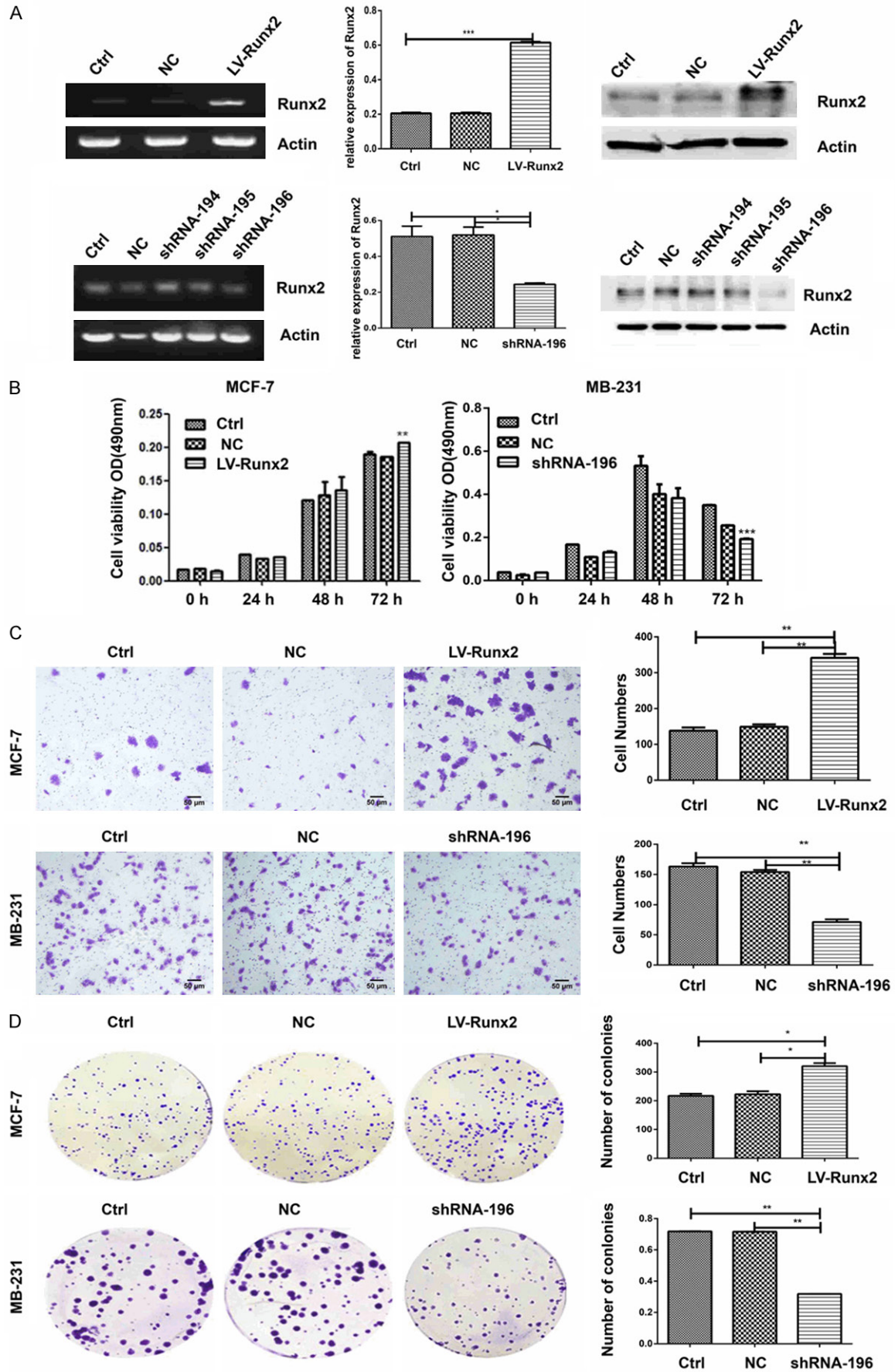
Figure 2. Runx2 promotes breast cell growth and metastasis directly. A. The mRNA and protein expression of Runx2 in MB-231 and MCF-7 cells with or without LV-Runx2 or Sh-RNA-Runx2 transfected was measured using RT-PCR and Western blotting. B. Cell viability was detected using the MTT assay in Runx2 overexpression and inhibition cell lines (**P<0.05, ***P<0.01 compared to Ctrl and NC). C. Cell invasion abilities were detected in Runx2 overexpression or inhibition cell lines (**P<0.05 compared to Ctrl and NC). Original magnification = 10×. Scale bar = 50 μm. D. Colony formation abilities in Runx2 overexpression or inhibition cell lines (*P<0.05, **P<0.01 compared to Ctrl and NC). Ctrl: cells without transfection; NC: cells transfected with blank vector. All the data were expressed as mean ± SEM.
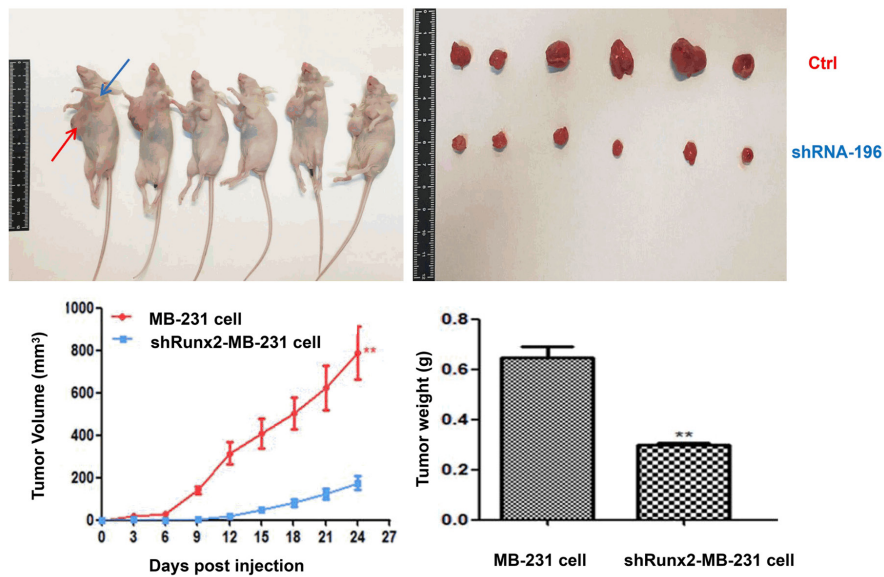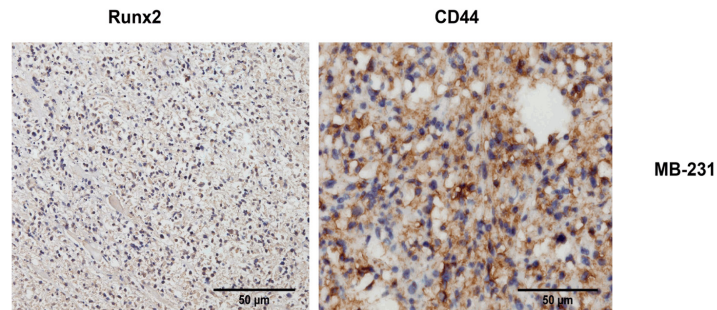
shRunx2-MB-231
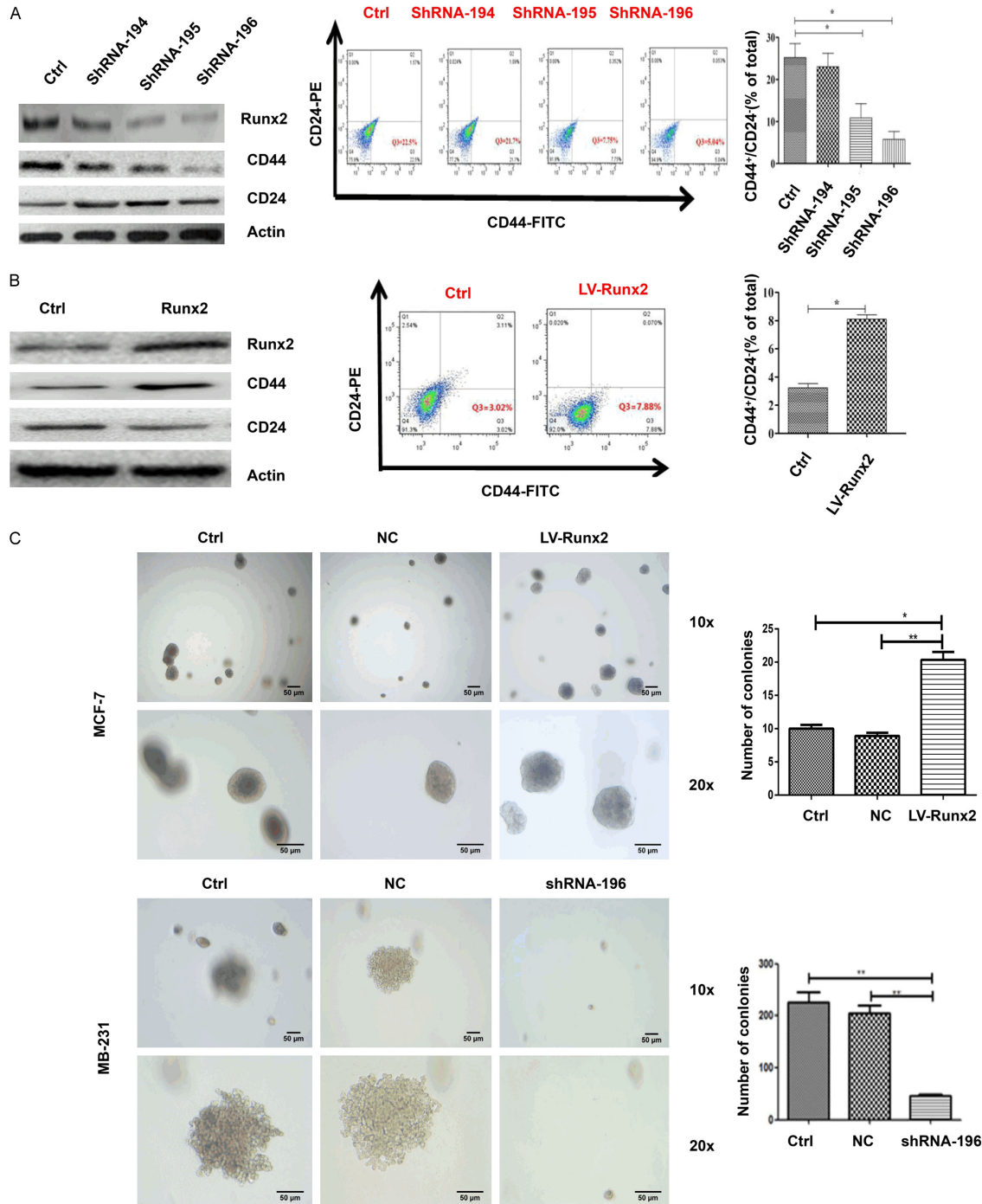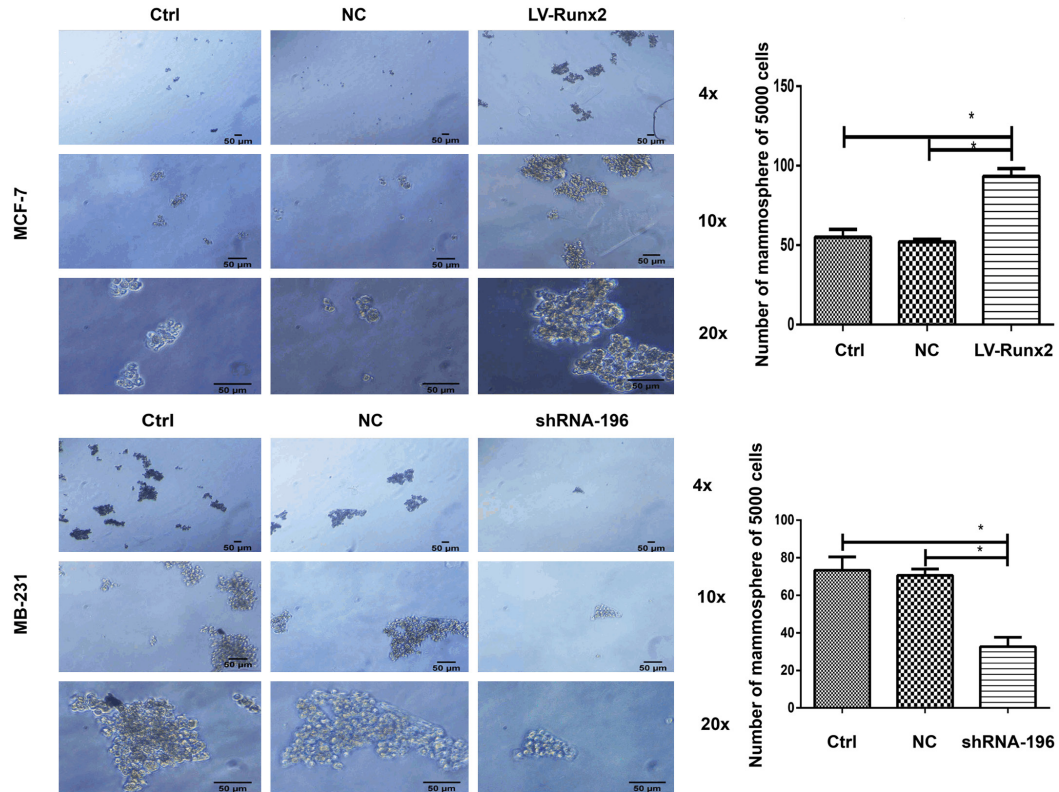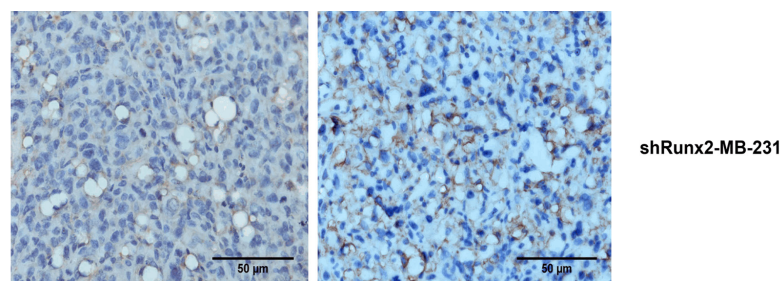
Figure 3. Runx2 promoted CD44+/CD24- breast cancer stem cell renewal and properties. A. The expression of CD44 and CD24 and the population of CD44+/CD24-/low were detected using Western blotting and flow cytometry in MDA-MB-231. B. The expression of CD44 and CD24 and the population of CD44+/CD24-/low were detected using Western blotting and flow cytometry in MCF-7. C. Non-anchor growth ability using soft agar colony formation assays. (two-tailed Student's t-test, *P<0.05, **P<0.01 ,compared to Ctrl and NC). D. Stem cell self-renewal ability assessed using the mammosphere formation assay (two-tailed Studen's t-test, *P<0.05, compared to Ctrl and NC). E. Tumor xenograft experiments: MB-231 cells or MB-231-Sh-RNA-Runx2 cells were injected subcutaneously into BALB/c mice. The red arrow represents Ctrl group, the blue arrow represents shRNA-196 group. The growth of breast tumors was monitored every 3 days after injection. Tumor sizes and weights were measured and recorded. n = 6. Data are presented as the means ± SEM from six mice. (**P<0.01, compared to the MB-231 transfected group). F. Expression of Runx2 and CD44 was analyzed using ICH in tumor tissues. Original magnification: 40×. Scale bar = 50 μm.

affected cell morphology typically. In Runx2 overexpression cells, the polarized epithelial cells became loose, and the shapes became oval, like mesenchymal cells (**Figure 4A**). The Runx2 knockdown cells became more tightly connected with each other and showed more polarization, similar to epithelial cell characteristics (**Figure 4B**). These phenomena suggest that Runx2 expression positively regulates the EMT process. To confirm this hypothesis, we examined EMT markers using Western blotting. The results showed that expression of E-cadherin in Runx2 overexpression cells was significantly lower than normal cells, and the expression of N-cadherin and MMP-3/9 was much higher, which demonstrated that an increase in Runx2 resulted in EMT changes (**Figure 4C**). In contrast, reduced Runx2 resulted in MET (**Figure 4D**). The Wnt/β-catenin signal pathway is one of the most important regulatory mechanisms during EMT. Therefore, we further examined two key molecules in the Wnt pathway, β-catenin and p-GSK-3β. Both of these proteins were changed with Runx2 expression (**Figure 4C**, **4D**). These data suggest that Runx2 regulates the EMT process via the Wnt/β-catenin signaling pathway.

Discussion

The Runx family of mammalian transcription factors plays fundamental roles in the differentiation of osteoblasts and chondrocytes (Runx2) [15], hematopoietic cells (Runx1) [16,

17] and neurons (Runx3) [18]. Runx proteins are also increasingly implicated in cancer progression, both positively and negatively [19, 20]. Contrasting their pro-metastatic role, which was mostly studied in advanced breast and prostate cancer [21, 22], most Runx proteins are well known for their tumor suppressor properties. For example, Runx3 is a bona fide tumor suppressor gene, and its methylation contributes to gastric cancer [23], and ablation of Runx1 activity leads to leukemia [24]. However, the function of Runx2 is very complicated, especially in breast cells. Runx2 is expressed at low levels in the murine mammary gland, and its levels fluctuate during the cycles of pregnancy, lactation and involution suggesting it plays multiple roles in the normal regulation of in mammary. Detailed analyses of murine mammary cells showed that Runx2 is specifically expressed in the basal/myoepithelial (CD24-/low, Sca1-) subpopulation [25], which is the same compartment where the mammary epithelial stem cells are found [26, 27]. However, Runx2 was upregulated in several breast cancer cell lines compared to normal mammary epithelial cells [28]. Furthermore, the expression of Runx2 in these cells is important for cell growth and invasion [29, 30]. Because the functioning of Runx2 begins in mammary stem cells, which generate progenitors and develop into luminal and basal mammary lineages [31, 32], it is very possible that Runx2 affects cancer stem cells and the development of breast cancer.
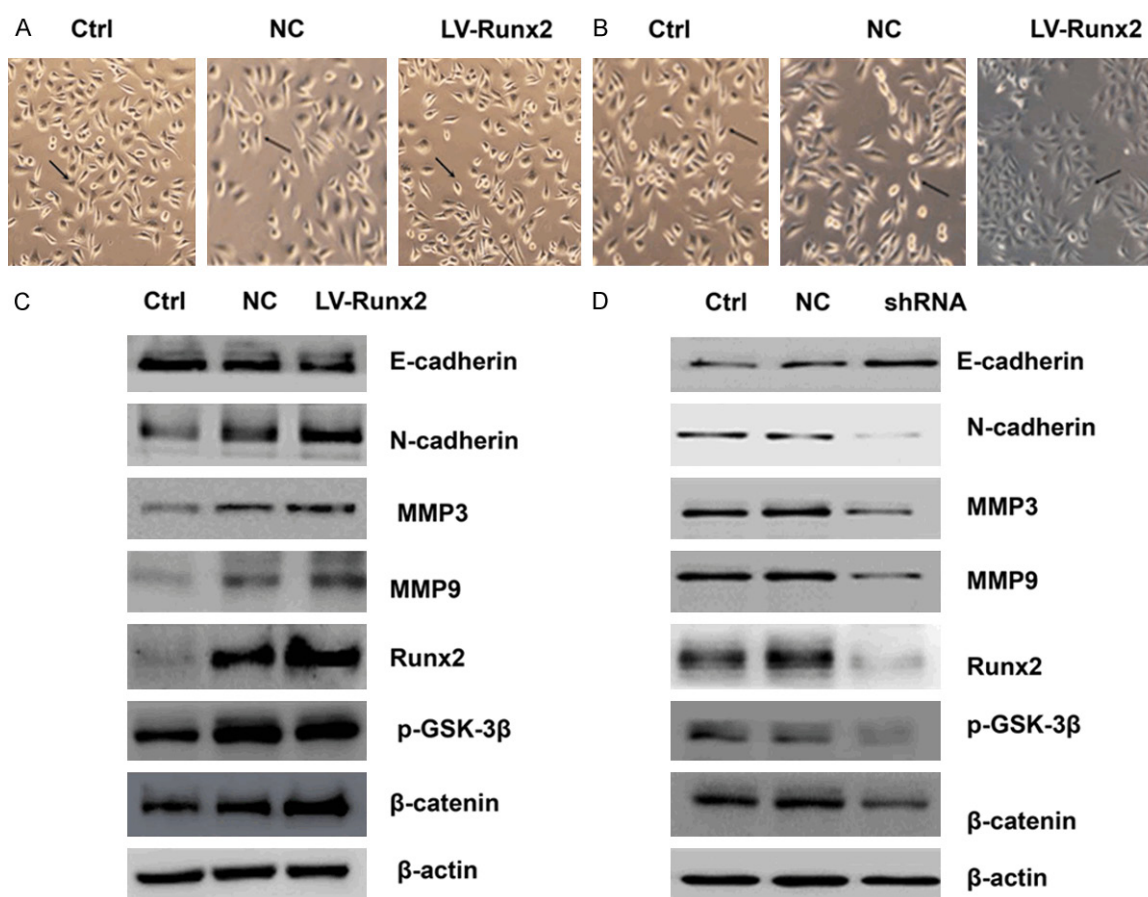
Figure 4. Runx2 affect EMT process through Wnt/β-catenin signal pathway. A, B. Cell morphological changes were observed under reverted microscope. C, D. Relative protein expression of the EMT markers (E-cadherin, N-cadherin, and MMP-3 and 9) and the Wnt/β-catenin signal markers (p-GSK-3β and β-catenin) in different Runx2 level cells by Western blotting.

In this study, there are three novel findings that reveal the important contribution of Runx2 in breast cancer and breast cancer stem cells. First, Runx2 levels were increased in many breast cancer cells, what's more, the degree of Runx2 increasing was quite different between breast cancer cells. It was much higher in the aggressive triple-negative breast cancer cell lines, MB-231, Sum49 and Sum159, than the other non-metastatic cell lines, Bt474, skb3 and MCF-7, which suggests that the expression level of Runx2 correlated to the metastatic degree of the breast cancer. In patients, the Runx2 high expression group was also associated with advanced clinical staging and lymphatic metastasis, which may be used as an indicator of malignancy level and the auxiliary diagnosis of breast cancer development. Second, our study showed that Runx2 regulated the CD44$^+$/CD24$^{-/low}$ breast cancer stem cell population directly. The expression level of Runx2 was positively linked to the percentage of cancer stem cells. Runx2 regulated cancer stem cell renewal abilities, such as soft agar clone formation, mammospheres formation and tumor formation ability in null mice. These results explained why a high level of Runx2 leading to a more aggressive cell phenotype and poorer pathological stage and prognosis. Last, we found the possible mechanism of how Runx2 increase BCSCs population might refer to EMT via the Wnt/β-catenin signaling pathway. Many studies have demonstrated that EMT was tightly linked to BCSCs formation and biology [34, 35]. Differentiated mammary epithelial cells that have undergone EMT give rise to tumorigenic and highly metastatic CD44$^+$/CD24^{-low} cells, which are akin to breast cancer stem cells, and stem cells isolated from breast carcinomas

express a number of canonical EMT markers [35, 36]. A number of signaling pathways are responsible for inducing and maintaining CSC properties, including the Wnt, Notch, and TGFβ1 pathways [37-39]. But the precise molecular mechanism is still very unclear. Our data showed a new mechanism for the regulation of EMT in breast cancer stem cell formation by the Wnt-Runx2 pathway.

But the more detailed signal involved in this process still need be investigated. One of our other studies found that the miR205-Runx2 axis played a vital role in triple-negative breast cancer (TNBC) via the regulation of stemness and EMT. We will further examine whether miR-205 is also involved in the wnt-RunX2 pathway and identify the downstream target genes of Runx2 in the regulation of stemness and EMT.

Disclosure of conflict of interest

None.

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