The circRNA DENND4C/miR-145-5p/HOXA9 Pathway Regulates Tumor Growth and Bone Metastasis in Breast Cancer

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Primary research

Keywords: breast cancer bone metastasis, circRNA DENND4C, HOXA9

Posted Date: October 23rd, 2020

DOI: https://doi.org/10.21203/rs.3.rs-95054/v1

Abstract

Background

Circular RNAs (circRNAs) are involved in the occurrence and development of breast cancer bone metastasis. This study aims to identify whether the circRNA DENND4C/ miR145-5p/HOXA9 axis is involved in the regulation of cell invasion and migration and breast cancer progression.

Methods

circRNA DENND4C, miR-145-5p and HOXA9 expression was measured in serum samples from healthy volunteers, breast cancer patients without bone metastasis and breast cancer patients with bone metastasis. Moreover, we analyzed the levels of circRNA DENND4C, miR-145-5p and HOXA9 according to different conditions of differentiation, tumor volume and lymph node metastasis. The online software starBases and the dual-luciferase reporter gene assay were used to predict the relationship miR-145-5p, circRNA DENND4C and HOXA9 mRNA. MTT assays were performed to assess the effect of circRNA DENND4C on proliferation. To assess the proliferation of breast cancer cells among different groups. Statistical significance was determined byStudent's t-test which was used for comparisons between two groups and one-way analysis of variance followed by Tukey's post hoc test for comparisons between more than two groups.

Results

The expression patterns of circRNA DENND4C, miR145-5p and HOXA9 were altered in patients with breast cancer bone metastasis. Notably, the stimulatory effects of circRNA DENND4C overexpression on HOXA9 were eliminated by miR-145-5p upregulation. circRNA DENND4C overexpression promotes proliferation, migration and invasiveness by regulating the miR-145-5p/HOXA9 axis. circRNA DENND4C downregulation suppresses proliferation, cell viability and invasiveness by regulating the miR-145-5p/HOXA9 axis.

Conclusion

Our study suggest that circRNA DENND4C/miR-145-5p/HOXA9 pathway was involved in tumor growth and bone metastasis in breast cancer. This findings may facilitate the development of potential therapeutic agents to improve the prognosis of patients with breast cancer bone metastasis.

1. Background

Breast cancer is the second leading cause of cancer-related deaths among women worldwide(1, 2), and with 50–70% of patients experience bone metastasis, which is more common in patients with end-stage disease and is associated with significant morbidity(3-6). To improve the unsatisfactory outcome of breast cancer bone metastasis, further research is urgently needed to explore the underlying mechanisms involved in breast cancer stemness properties, such as proliferation, invasion, and metastasis.

Circular RNAs (circRNAs) are a class of single-stranded noncoding RNA molecules that involved in the occurrence and development of human diseases including cancers(7, 8). Chen et al. reported that circRNA FECR1 promotes metastasis by regulating TET1 and DNMT1 in breast cancer(9). Moreover, Karedath, T et al. suggested that circRNA NKRD12 (ankyrin repeat domain 12) can promote invasion and migration as well as alter the metabolism in cancer and can induce a strong phenotypic change by significantly regulating the cell cycle in breast cancer(10). Notably, circRNA domain containing 4C (circRNA DENND4C) has been reported to be related to cell proliferation and invasion under hypoxic conditions(11, 12). However, the role and underlying mechanism of circRNA DENND4C in breast cancer progression has rarely been reported in the literature.

CircRNAs typically exert their biological functions by binding to miRNAs and affecting their normal function in human cancers(13-15). Zhao et al. reported that circ RNA CDR1as acts as an RNA sponge of miR-641 and that miR-641 can bring circ RNA CDR1as and HOXA9 together(16). Similarly, many studies have reported the relationship between miRNAs and circRNAs in breast cancer(17-19). Previous work has demonstrated that miR-145-5p regulates the proliferation of pulmonary microvascular endothelial cells(20), renal cell carcinoma cells(21), ovarian cancer cells(22), non-small cell lung cancer cells(NSCLC) (16), and breast cancercells(23). Notably, the complementary sites between miR-145-5p and circRNA DENND4C were predicted by a bioinformatics analysis using starBase. In addition, Homeobox protein Hox-A9 (HOXA9) was reported to be related to the prognosis of colon cancer(24), and to regulate head and neck squamous cell carcinoma progression(25). Furthermore, HOXA9 can be negatively regulated by miR-145-5p in osteosarcoma cells(26), and the online prediction of the binding sites between miR-145-5p and the 3' untranslated regions (UTRs) of HOXA9 mRNA was determined using starBase software. All the above information prompted us to hypothesize that circRNA DENND4C positively regulates HOXA9 by sponging miR-145-5p.

In this study, we measured the expression levels of circRNA DENND4C, miR145-5p and HOXA9 in patients with breast cancer bone metastasis. In addition, we investigated the biological role of these factors in the migration and invasiveness of breast cancer cells. Moreover, we explored whether the circRNA DENND4C/miR-145-5p/HOXA9 axis is involved in regulating cell invasion and migration and breast cancer progression, which will contribute to improvement of the prognosis of patients with breast cancer bone metastasis.

2. Results

2.1 Ectopic expression of circRNA DENND4C, miR-145-5p and HOXA9 in patients with breast cancer bone metastasis

To identify the relevance of circRNA DENND4C, miR-145-5p and HOXA9 in tumor growth and bone metastasis, we investigated whether the expression patterns of circRNA DENND4C, miR-145-5p and HOXA9 were altered in cases of breast cancer bone metastasis. To achieve this, circRNA DENND4C, miR-145-5p and HOXA9 expression was measured in serum samples from healthy volunteers (n = 59), breast

cancer patients without bone metastasis (n=56) and breast cancer patients with bone metastasis (n =19). The data from the qPCR analysis indicated that significant upregulation of circRNA DENND4C (Fig. 1A), the substantial overexpression of miR-145-5p(Fig. 1B), and accordingly, an obvious reduction in HOXA9 (Fig. 1C) in breast cancer patients with bone metastasis compared with healthy volunteers and breast cancer patients without bone metastasis. In addition, Western blot revealed that HOXA9 was highly expressed at the protein level in patients with bone metastasis (Fig. 1D, E). Moreover, we analyzed the levels of circRNA DENND4C, miR-145-5p and HOXA9 according to different conditions of differentiation, tumor volume and lymph node metastasis. Significant upregulation of circRNA DENND4C expression (Fig. 1F), distinct downregulation of miR-145-5p expression (Fig. 1G) and substantial upregulation of of HOXA9 expression (Fig. 1H) were observed in patients with poor differentiation, larger tumor volume and lymph node metastasis compared with patients with well-differentiated tumors, smaller tumor volumes and no lymph node metastasis. All these data indicated that the expression patterns of circRNA DENND4C, miR145-5p and HOXA9 were altered in patients with breast cancer bone metastasis.

2.2 CircRNA DENND4C positively regulated HOXA9 by spongingmiR-145-5p in breast cancer cells

Previous data have implied that underlying molecular mechanisms exist among circRNA DENND4C, miR-145-5p and HOXA9, and these mechanisms were validated in our experiments. Initially, the online software starBases predicted that miR-145-5p can bind to circRNA DENND4C (Fig. 2A) and the 3' UTR regions of HOXA9 mRNA (Fig. 2D).In addition, previous studies supported he finding that circRNA DENND4C acts as an RNA sponge of miR-145-5p, and that HOXA9 is a downstream target of miR-145-5p, which indicates that miR-145-5p might serve as a “bridge” that links circRNA DENND4C and HOXA9. The above hypothesis was validated by the experiments described below: we found that circRNA DENND4C promoted HOXA9 expression in breast cancer cells by targeting miR-145-5p (Fig. 2). Furthermore, the dual-luciferase reporter gene assay results showed that miR-145-5p could bind to both circRNA DENND4C (Fig. 2B) and the 3' UTR regions of HOXA9 mRNA (Fig. 2E) in breast cancer cells, while the RNA pull-down assay validated that miR-145-5p was enriched by both circRNA DENND4C (Fig. 2C) and HOXA9 (Fig. 2F) probes in breast cancer cells. Moreover, small interfering RNA (siRNA) against circRNA DENND4C was successfully transfected into breast cancer cells, which caused DENND4C downregulation (Fig. 2G), miR-145-5p overexpresion (Fig. 2I) and HOXA9 downregulation. Our results showed that circRNA DENND4C negatively regulates miR-145-5p and positively regulates HOXA9 mRNA levels in breast cancer cells. Notably, the stimulatory effects of circRNA DENND4C overexpression on HOXA9 were eliminated by miR-145-5p upregulation.

2.3. Upregulation of circRNA DENND4C promotedthe proliferation, migration and invasionofbreast cancer cells by targeting the miR-145-5p/HOXA9 axis

Since circRNA DENND4C can regulate HOXA9 through miR-145-5p, we next investigated whether the circRNA DENND4C/miR-145-5p/HOXA9 pathway regulates the proliferation, migration and invasiveness of breast cancer cells. To achieve this, circRNA DENND4C overexpression vectors (Fig. 3A), a miR-145-5p mimic (Fig. 3B) and short hairpin RNA (shRNA) for HOXA9 knockdown (KD) (Fig. 3C) were pre-transfected

into breast cancer cells. Then, MTT assays were performed, and the results revealed that circRNA DENND4C overexpression promoted breast cancer cells proliferation, which was reversed by both miR-145-5p overexpression and HOXA9 silencing (Fig. 3D). In addition, upregulation of circRNA DENND4C also increased the migration (Fig. 3E, F) and invasiveness (Fig. 3G, H) of breast cancer cells, and these effects were abrogated by miR-145-5p overexpression and HOXA9 silencing. These observations indicated that circRNA DENND4C overexpression promotes proliferation, migration and invasiveness by regulating the miR-145-5p/HOXA9 axis

2.4 Downregulation of circRNA DENND4C suppressed the proliferation, migration and invasiveness of breast cancer cells by targeting the miR-145-5p/HOXA9 axis

To investigate whether suppression of circRNA DENND4C may lead to a growth disadvantage in vivo, circRNA DENND4C KD vectors (Fig. 4A), a miR-145-5p inhibitor (Fig. 4B) and short hairpin RNA (shRNA) for overexpression of HOXA9 (Fig. 4C) were pretransfected into breast cancer cells. Then, the above cells were subjected to MTT (Fig. 4D) and cell viability (Fig. 4E) assays, and the results showed that circRNA DENND4C downregulation suppressed the proliferation and viability of breast cancer cells. These effects were reversed by both miR-145-5p overexpression and HOXA9 silencing. In addition, circRNA DENND4C downregulation also attenuated the wound healing ability of breast cancer cells, and this effect was reversed by miR-145-5p downregulation and HOXA9 overexpression (Fig. 4F, G). These observations indicated that circRNA DENND4C downregulation suppresses proliferation, cell viability and invasiveness by regulating the miR-145-5p/HOXA9 axis.

2.5 Effect ofcircRNA DENND4C on tumor growth as a result oftargeting the miR-145-5p/HOXA9 axisin vivo

To further investigate the role of circRNA DENND4C, MDA-MB-231 cells (5 × 106 in 20 μl PBS) were stably transfected with a negative control (NC), KD-circRNA DENND4C, KD-DENND4C+miR145-5p inhibitor or KD-DENND4C+High-HOXA9 and were injected into the bone marrow space of BALB/c-nu/nu mice to establish an in vivo xenograft model. Tumor volume was measured 10 days after injection. According to the data, tumor volume (Fig. 5A) and weight (Fig. 5B) were significantly reduced in the KD-DENND4C group, but miR145-5p and HOXA9 weakened the suppressive effect of circRNA DENND4C downregulation. To assess the proliferation of breast cancer cells among the different groups, tumor sections were stained for Ki67 using immunohistochemistry. Notably, the results showed that circRNA DENND4C downregulation suppresses cell proliferation, while miR-145-5p and HOXA9 expression restrains the suppressive effects, which suggested that reduced DENND4C inhibits tumor growth by targeting the miR-145-5p/HOXA9 axis.

2.6. The circRNA DENND4C/miR-145-5p/HOXA9 axis regulated the stemness of breast cancer cells

These findings suggest that the circRNA DENND4C/miR-145-5p/HOXA9 pathway regulates cell stemness. We found that circRNA DENND4C downregulation suppressed the stemness of breast cancer cells by targeting the miR-145-5p/HOXA9 axis (Fig. 5). Specifically, we cultured primary cells isolated from tumor

tissue of the different groups under standard conditions, and the results showed that the cells from the circRNA DENND4C group contained a lower percentage of CD44+/CD24− cells by flow cytometry, while the KD-DENND4C+miR-145-5p inhibitor and KD-DENND4C+High-HOXA9 groups showed the opposite trend (Fig. 5A, B). In addition, we found that cells from the circRNA DENND4C group had reduced SOX2 and OCT4 protein levels and increased CK 14 and CK18 expression, while the KD-DENND4C+miR-145-5p and KD-DENND4C+High-HOXA9 groups exhibited the opposite trend (Fig. 5C, D). Similarly, further experiments validated that KD of circRNA DENND4C suppressed proliferation and that the suppressive effects of circRNA DENND4C ablation on stemness were abolished by miR-145-5p knockdown and HOXA9 overexpression, as indicated by the MTT assay results (Fig. 5E). Moreover, annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining assays were performed to examine apoptosis. The proportion of annexin V-PI-positive cells was increased due to KD of circRNA DENND4C, but the miR-145-5p inhibitor and High-HOXA9 groups exhibited an inverse trend, which suggested that the circRNA DENND4C/miR-145-5p/HOXA9 axis can regulate cell apoptosis. These results indicated that the circRNA DENND4C/miR-145-5p/HOXA9 axis regulates the stemness of breast cancer cells.

3. Discussion

Accumulating evidence has demonstrated that circRNAs are involved in cell proliferation, migration, and invasion in breast cancer bone metastasis(27-29). A better understanding of the molecular mechanism involved in the development and progression of breast cancer bone metastasis may allow us to explore the underlying mechanisms of this disease aspect. Here, we report the effects of the ectopic expression of circRNA DENND4C, miR145-5p and HOXA9 in breast cancer bone metastasis. Furthermore, we identified their roles in breast cancer and that miR145-5p combines with circRNA DENND4C and HOXA9. We also confirmed that circRNA DENND4C positively regulates HOXA9 by sponging miR-145-5p in breast cancer bone metastasis.

Recently, the regulatory potential of circRNAs has been frequently reported, and they are known to participate in the development and progression of breast cancer(30-32). Previous studies have suggested that circ-TFF1 overexpression can contribute to breast cancer progression by targeting miR-326/TFF1 signaling(33). In addition, circFBXW7 was reported to regulate proliferation and metastasis in breast cancer(34). Interestingly, a previous study suggested that circRNA DENND4C regulates hepatocellular carcinoma cell proliferation and apoptosis(35), and maintains permeability of the blood- tumor barrier(36). In addition, circRNA DENND4C was reported to be overexpressed in breast cancer and that KD of circRNA DENND4C suppresses the proliferation of breast cancer cells in hypoxic conditions(37). In our experiments, we identified circRNA DENND4C as a significantly upregulated circRNA that is correlated with tumor progression in breast cancer bone metastasis.

Moreover, miR-145-5p was predicted to be downstream target of circRNA DENND4C. Previously, miR-145-5p was reported to be involved in NSCLC proliferation, metastasis, and immune evasion(38). miR-145-5p acts as a sponge of circRNA DUSP16 and promotes tumorigenesis and invasion of gastric cancer(39). In our study, miR-145-5p overexpression repressed breast cancer cell proliferation, migration and invasion.

In addition, miR-145-5p eliminated the function of circ RNADENND4C. A direct correlation between miR-145-5p and circRNA DENND4C was also confirmed. Our results suggest that miR-145-5p can be negatively regulated by circRNA DENND4C.

Furthermore, we usedmiRNA response element (MRE) and TargetScan analysis to predict the targets of miRNA-binding sites. Among the candidates, HOXA9 was verified as a downstream target of miR145-5p by luciferase reporter assays. Many studies reported that HOXA9 is involved in the cell proliferation(40-43). A previous study demonstrated that HOXA9 regulates myeloid cells and further validated that miR-708 can directly target Meis1, which strongly impedes HOXA9- mediated transformation and homing capacity as well as the induction of myeloid differentiation. Notably, it was reported that HOXA9 is a downstream regulatory target of the ten–eleven translocation (TET) family of methylcytosine dioxygenases, which regulate breast cancer growth and metastasis(44). Here, our results demonstrated that HOXA9 was overexpressed in breast cancer bone metastasis samples compared with control samples and that miR-145-5p suppressed the proliferation, migration and invasiveness of breast cancer cells. Furthermore, circRNA DENND4C downregulation suppressed both the proliferation and viability of breast cancer cells, which were reversed by both miR-145-5p overexpression and HOXA9 silencing. Additionally, using flow cytometry, we examined the percentage of CD44+/CD24− cells derived from primary cultures of different tumor tissues. Our results indicated that the circRNA DENND4C/miR-145-5p/HOXA9 axis regulates the stemness of breast cancer cells.

In summary, our data suggested that circRNA DENND4C is highly expressed in breast cancer bone metastasis where it promotes cell proliferation, invasion, and migration. Theoretically, circRNA DENND4C competitively binds miR-145-5p to attenuate the suppressive effect of miR-145-5p on HOXA9. These findings provide insight into the mechanism of breast cancer bone metastasis, and provide a potential target for the treatment of this disease.

4. Methods

4.1. Patients

In this study, serum samples from healthy volunteers (n = 59), breast cancer patients without bone metastasis (n=56) and breast cancer patients with bone metastasis (n =19) were tested. All patients were treated at The First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China), and the study was approved by its Institutional Review Boards. The Ethics Committee of The First Affiliated Hospital of Sun Yat-sen University approved the procedures. Written informed consent for the use of human materials was approved according to The First Affiliated Hospital of Sun Yat-sen University's ethical guidelines, and all experiments were performed in accordance with the relevant guidelines and regulations.

4.2. Cell culture and transfection

The breast cancer cell lines MDA-MB-231, MCF-7 and SK-BR3 were purchased from the Chinese Academy Sciences Cell Bank and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal

bovine serum (FBS; HyClone, Marlborough, MA, USA) at at 37°C in a humidified-atmosphere containing 5% CO2. The overexpression vectors(Roche, Switzerland) for circRNA DENND4C (High- DENND4C) and HOXA9 (High-HOXA9), the miR-145-5p mimic and inhibitor (Invitrogen, CA, USA) and the short harpin RNA (shRNA)(Bioss, Beijing, China) for circRNA DENND4C (KD- DENND4C) and the small interfering RNA (siRNA)(Sigma-Aldrich; Merck KGaA) for HOXA9 (KD-HOXA9) were each pre-transfected into breast cancer cells. The above vectors were transfected into breast cancer cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

4.3. RNA isolation and qRT-PCR amplification

Total RNA was extracted using TRIzol® reagent (Thermo) and cDNA was obtained using the SuperScript® cDNA Synthesis Kit (Thermo) according to the manufacturer's instructions. Next, cDNA was transferred to a 20-μl PCR mixture composed of 2× UltraSYBR Mixture (Guang zhou, China). The primers were as follows: circRNA DENND4C forward: 5'-GGGGCAGCAGTATTGTGAAA-3' and reverse, 5'-AAGACTGTGTGCTCCCCATT-3'); miR-145-5p forward 5'- CTGATGGTGGAGAGCTCACA-3' and reverse 5'-GTGCAGGGTCCGAGGT-3'; U6 forward: 5'-GACTATCATATGCTTACCGT-3' and reverse: 5'-GGGCAGGAAGAGGGCCTAT-3'; HOXA9 forward: 5'-CTTACCCAAGCTTCACTCACC-3' and reverse: 5'-AAGAGGCCTGGTGCTACTAC-3'; β-actin forward: 5'-TCATGAAGTGTGACGTGGACATC-3' and reverse: 5'-CAGGAGGAGCAATGATCTTGATCT-3'. U6 or β-actin was used as an internal control to normalize target gene transcripts.

4.4. Luciferase reporter assay

The amplified miR-145-5p inhibitor sequence and miR-NC(Thermo Fisher Scientific, Inc) were transfected into breast cancer cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

To identify the binding sequences and uniform resource locator, a luciferase reporter assay was performed. The miR-145-5p inhibitor or miR-NC and the pRL-TK vector (Promega Corporation) carrying the mutant (mut) or wild-type (wt) DENND4C and HOXA9 3' untranslated region (3' UTR) were co-transfected into breast cancer cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Three days later, the cells were lysed with Dual-Glo®Reagent (Promega Corporation), and luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega Corporation). The firefly luciferase activity was normalized to Renilla luciferase activity

4.5. RNA pull-down assay

RNA pull-down assays were performed to identify the binding sites between miR-145-5p and circRNA DENND4C and the 3'UTR regions of HOXA9 mRNA. The biotin-labeled circRNA DENND4C and HOXA9 probes(Invitrogen, USA) were designed and constructed. Then, the above-mentioned probe-streptavidin Dynabeads were incubated with breast cancer cell lysates at 37 °C for 12 hours. Finally, to assess miR-145-5p enrichment, real -time qPCR was performed according to the manufacturer's instructions .

4.6. Western blot analysis

Proteins extracted from cell lysates or cell culture supernatant were subjected to electrophoresis. After the proteins were transferred and blocked, the membranes were incubated with rabbit anti-β-actin (1:1000; ab8227, Abcam, Cambridge, UK), anti-HOXA9 (1:1000; EPR3655(2), ab140631) anti-OCT4(1:1000; EPR17980, ab200834) anti-SOX2 (1:1000; ab97959) anti-Ck 14(1:1000; EP1612Y, ab51054), and anti-Ck18(1:1000; EPR1626, ab133263) overnight at 4°C.

4.7. *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay*

An MTT assay was performed to evaluate the role of the circRNA DENND4C/miR-145-5p/HOXA9 axis in breast cancer cells. MDA-MB-231, MCF-7 and SK-BR3 cells were stably transfected with NC or KD-circ RNA DENND4C or KD-DENND4C+miR145-5p inhibitor or KD-DENND4C+High-HOXA9 and were seeded into 96-well plates at a density of 5×10^3 cells/well, after which they were incubated for 24 hours. Then, 20 μl of MTT solution (5 mg/ml) was added into each well and incubated for an additional 4 hours. After 160 μl DMSO was added to each well, the optical density (OD) value of each well was measured at 450 nm in a spectrophotometer (Philips, China).

4.8. Migration and invasion assays

Migration assays were performed in 48-well Transwell® plates (8.0-μm pore size; Costar, Corning Incorporated, USA). After pre-transfection with the overexpression vectors for circRNA DENND4C (High-DENND4C), High-DENND4C +miR-145-5p mimics, High- DENND4C +KD-HOXA9(siRNA) and NC-vectors, breast cancer cells were seeded into the upper chamber of a Transwell system at a concentration of 6×10^3 cells/well. After 24 hours of incubation, cells that had migrated to the bottom side of the Transwell membrane inserts were stained with crystal violet.

Invasion assays were performed using a Transwell chamber coated with Matrigel® (Becton Dickinson, USA). Briefly, cells from different groups were added into the upper chamber. After the Transwell system was incubated for 24 hours, cells that invaded to the bottom side of the membrane were fixed and stained with crystal violet.

4.9. Wound-healing assay

To further assess cell invasion, a wound-healing assay was performed in 6-well plates for 24 hours. After pretransfection with the siRNA for circRNA DENND4C silencing (KD-DENND4C), KD-DENND4C+miR-145-5p inhibitor, KD- DENND4C+High-HOXA9 (overexpression vectors for HOXA9) or NC-vectors, artificial wounds were generated with a sterile 10-μl pipette tip. Images were obtained under 100× magnification.

4.10. Experimental murine models of breast carcinoma bone metastasis

BALB/c-nu/nu mice (6-10 weeks of ages, 60-100 g, purchased from The Guangdong Medical Laboratory Animal Center) were maintained in a 12:12 hour light/dark cycle at 23˚C and 50-70% humidity. Animals

experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of The First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China), and in all experiments, the mice were euthanized with 50 mg/kg 1% sodium pentobarbital according to IACUC guidelines. We made an incision along the right femur of each mouse. A surgical scalpel was used to drill a tiny hole in the cortex, and MDA-MB-231 cells transfected with the KD-DENND4C vector, KD-DENND4C+miR-145-5p inhibitor, KD- DENND4C+High-HOXA9 or NC-vectors were injected into the bone marrow through the hole at a density of 5×10^6 cells per mouse. Then, we repaired the hole with bone wax and sutured the patellar tendon and wound. Tumor growth was monitored daily. After the animals were euthanized, we obtained tumor tissue under aseptic conditions. Then, tumor tissues were cut into small pieces and were subjected to enzymatic dissociation (0.2% collagenase, Abcam; Cambridge, UK) at 37°C for 90 min. The cell suspension was filtered through a 40-μm filter (Biosharp Life Sciences, China). After dissociation, the cells were cultured in DMEM (HyClone; USA) supplemented with 10% FBS (Gibco;CA, USA.)

4.11. Immunohistochemistry (IHC)

Tissues were fixed in 10% neutral-buffered formalin and then paraffin-embedded. Paraffin-embedded tissues were deparaffinized and rehydrated for further 3,3'-diaminobenzidine peroxidase (DAB)-base immunohistochemical staining. Then, after proteolytic digestion and peroxidase blocking, the slides were incubated with a Ki67 antibody (1:100, ab15580, Abcam, USA) overnight at 4°C.

4.12. Flow cytometry assay

To assess the stem cell phenotype conversion, the cells were resuspended in 200 μl PBS and incubated with anti-CD44-APC (4 μl/ ml) and anti-CD24-BV510 (6 μl/ml) (BD Bioscience, USA) at 4°C for 1 hour. After the cells were washed three times with PBS and suspended in 100 μl PBS, the analysis was performed using a Flow CytoFLEX(Beckman Coulter, USA).

Cell apoptosis was examined using an annexin V-FITC/PI apoptosis detection kit (BD Bioscience, USA) according to the manufacturer's protocol. After the cells were incubated with annexin V-FITC and PI at room temperature for 60 min in the dark, a flow cytometerFlow (Beckman Coulter, USA) was used to measure percentage of apoptotic cells.

4.13. Statistical analysis

Statistical significance was determined byStudent's t-test which was used for comparisons between two groups and one-way analysis of variance followed by Tukey's post hoc test for comparisons between more than two groups; analyses were performed using the Prism software package version 5.03 (GraphPad Software, La Jolla, CA, USA). Each experiment was repeated at least three times. All data are expressed as the mean ± SEM. A difference was considered statistically significant at a level of $P < 0.05$.

Abbreviations

DENND4C: Domain containing 4C; FBS: Fetal bovine serum; HOXA9: Homeobox protein; IHC: Immunohistochemistry; miRNA: MicroRNA; NC: Negative control; MT: Mutant; PBS: Phosphate-buffered saline; PI: Propidium iodide; qPCR: Quantitative real-time PCR; siRNA: Small interfering RNA; WT: Wild-type.

Declarations

Author Contributions: Menghai Zhu conceived and designed the study. Peng Zou, Chong Lian, Bengang Qin performed the study. Gang Chen analysed the data and wrote the paper.

Acknowledgements

Not applicable.

Funding: This study was supported by The Natural Science Foundation of Guangdong Province (grant nos. 2018A030310254 and 2015A030310350).

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The study was approved by the Ethics Review Committee of the First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China). *The animal study followed the Guidelines for the Animal Care and Use approved by* the First Affiliated Hospital of Sun Yat-sen University.

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Tables

Table. Clinical and sociodemographic characteristics of the population studied

Clinicopathological characteristics	Healthy volunteers(Num)	Breast cancer without bone metastasis (Num)	Breast cancer with bone metastasis (Num)
Age	24	19	8
<65	35	37	11
≥ 65			
Sex	28	5	2
Male	31	51	17
Female			
TNM stage		56	19
Ⅰ-Ⅱ			
Ⅲ-Ⅳ			
Lymph node metastasis		38	15
Positive		18	4
Negative			
Tumor Size		29	6
≤4 cm		27	13
>4 ㎝			
Differentiation		11	5
Well		45	14
Poor			

Figures

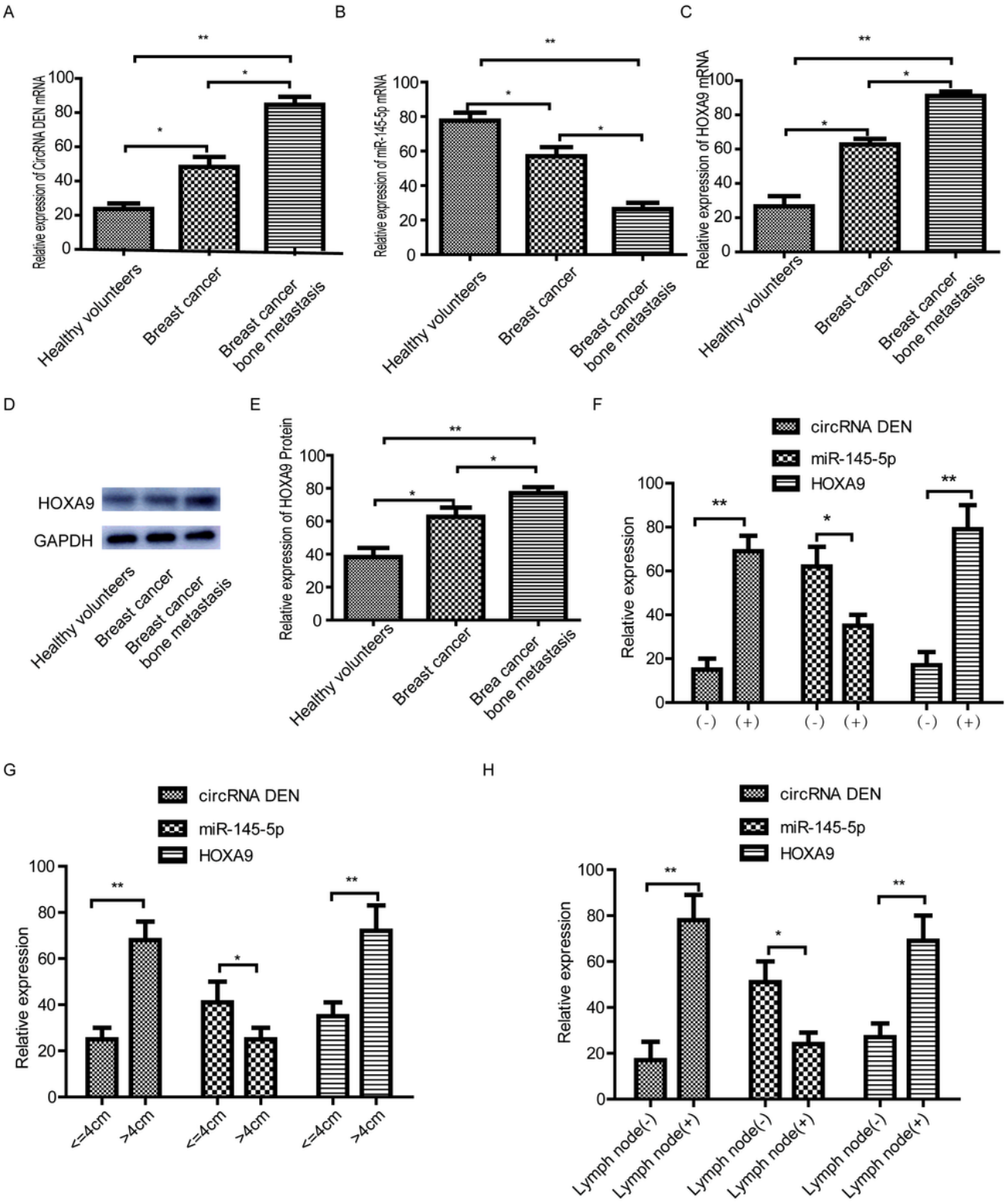

Figure 1

Expression of circRNA DENND4C, miR 145-5p and HOXA9 in patients with breast cancer bone metastasis. (A) The expression of circRNA DENND4C in the plasma of healthy volunteers, patients with breast cancer and breast cancer bone metastasis. (B) The expression of miR-145-5p in the above three groups.(C) The expression of HOXA9 miRNA in the above three groups.(D) The expression of HOXA9 protein (E) Quantification of HOXA9 in the plasma of the above three groups.(F) Expression of circRNA

DENND4C,miR-145-5p and HOXA9 with well-differentiated or poorly-differentiated cancer. (G)Expression of circRNA DENND4C, miR-145-5p and HOXA9 in patients with tumor volumes less than or greater than 4 cm3. (H) Expression of circRNA DENND4C, miR-145-5p and HOXA9 in patients with or without lymphatic metastasis. Each bar represents the mean ± SEM. “DEN” represents “circRNA DENND4C”, *P < 0.05, **P < 0.01.

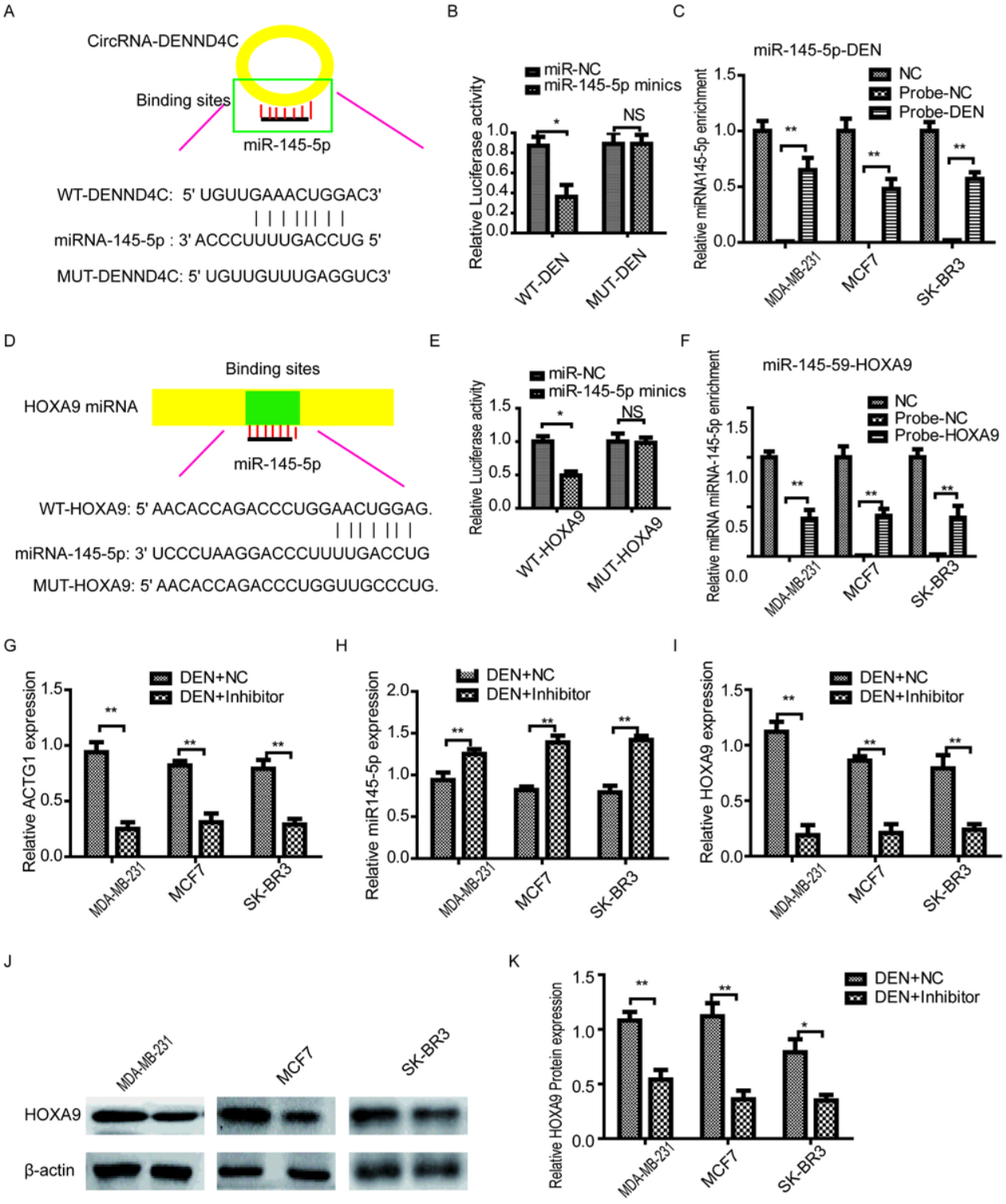

Figure 2

CircRNA DENND4C positively regulated HOXA9 by sponging miR-145-5p in breast cancer cells. The online software starBase predicted the binding sites of miR-145-5p with (A) circRNA DENND4C and (D) the 3' UTR of HOXA9 mRNA. The dual-luciferase reporter gene system validated the targeting sites of miR-145-5p with (B) circRNA DENND4C and (E) the 3' UTR of HOXA9 mRNA. RNA pull-down assay results indicated that miR-145-5p could be enriched by (C) circRNA DENND4C and (F) HOXA9 mRNA probes, respectively. (G) CircRNA DENND4C was silenced in breast cancer cells, as determined by real time qPCR. CircRNA DENND4C negatively regulated the expression of (H) miR-145-5p and positively regulated (I) HOXA9 mRNA in breast cancer cells. (J) Western blot analysis revealed that circRNA DENND4C downregulation inhibited HOXA9 protein expression in breast cancer cells. (K) Quantification of HOXA9 was normalized to β-actin.Each bar represents the mean ± SEM. “DEN” represents “circRNA DENND4C”, *P < 0.05, **P < 0.01. All experiments were performed at least three times and each sample was run in duplicate in each individual experiment.

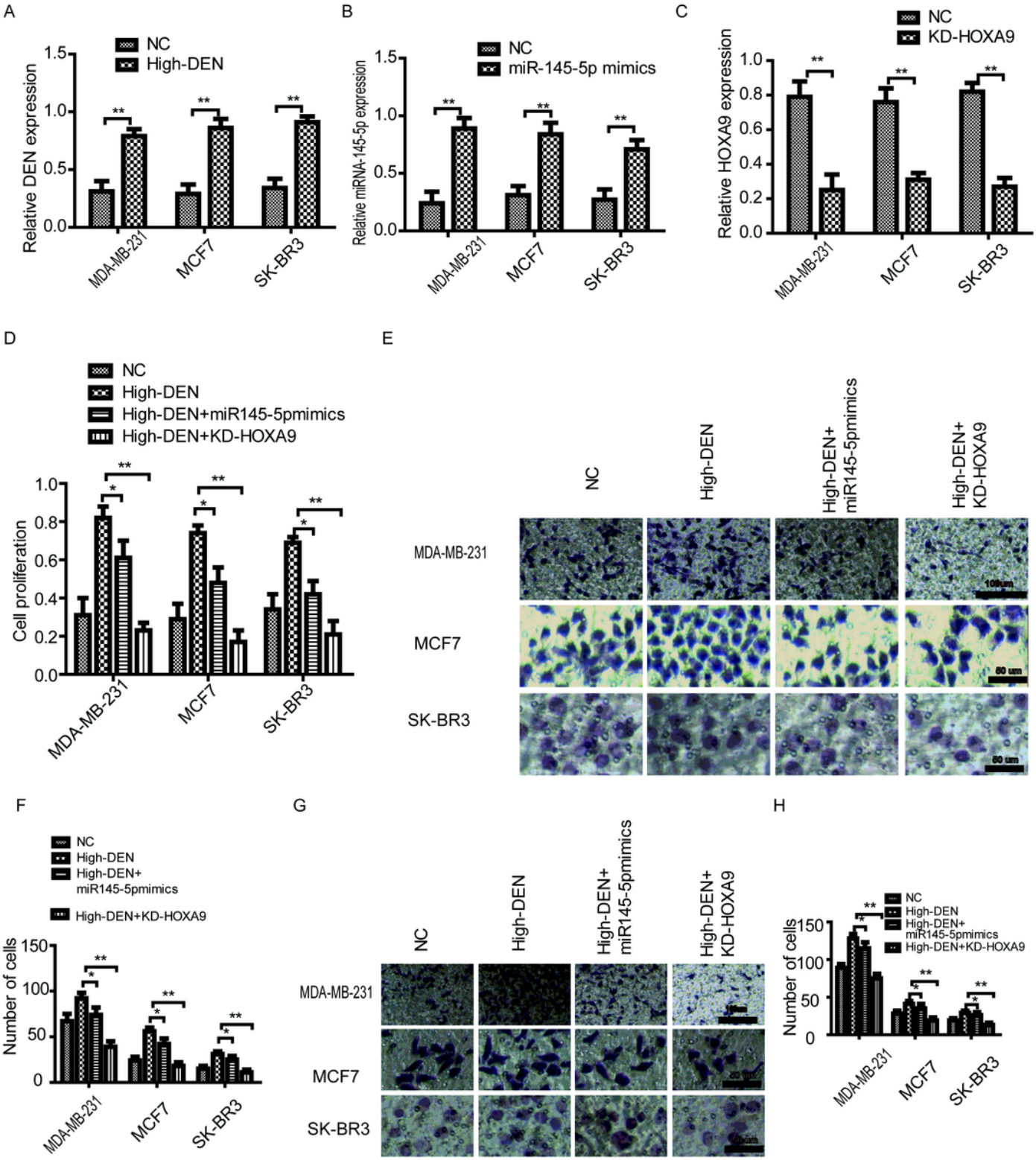

Figure 3

Upregulation of circRNA DENND4C promoted the proliferation, migration and invasiveness of breast cancer cells by targeting the miR-145-5p/HOXA9 axis. The (A) circRNA DENND4C overexpression vectors, (B) miR-145-5p mimic and (C) vectors for HOXA9 ablation were transfected into breast cancer cells, and their expression was examined by real time qPCR. Breast cancer cells (MDA-MB-231, MCF7 and SK-BR3) were subjected to (D)an MTT assay, (E, F) migration assay and (G, H) invasion assay and the results

indicated that upregulated circRNA DENND4C promoted the proliferation, migration and invasiveness of breast cancer cells by regulating the miR-641/HOXA9 axis. Each bar represents the mean ± SEM. “DEN” represents “circRNA DENND4C”, *P < 0.05, **P < 0.01. All experiments were performed at least three times and each sample was run in duplicate in each individual experiment.

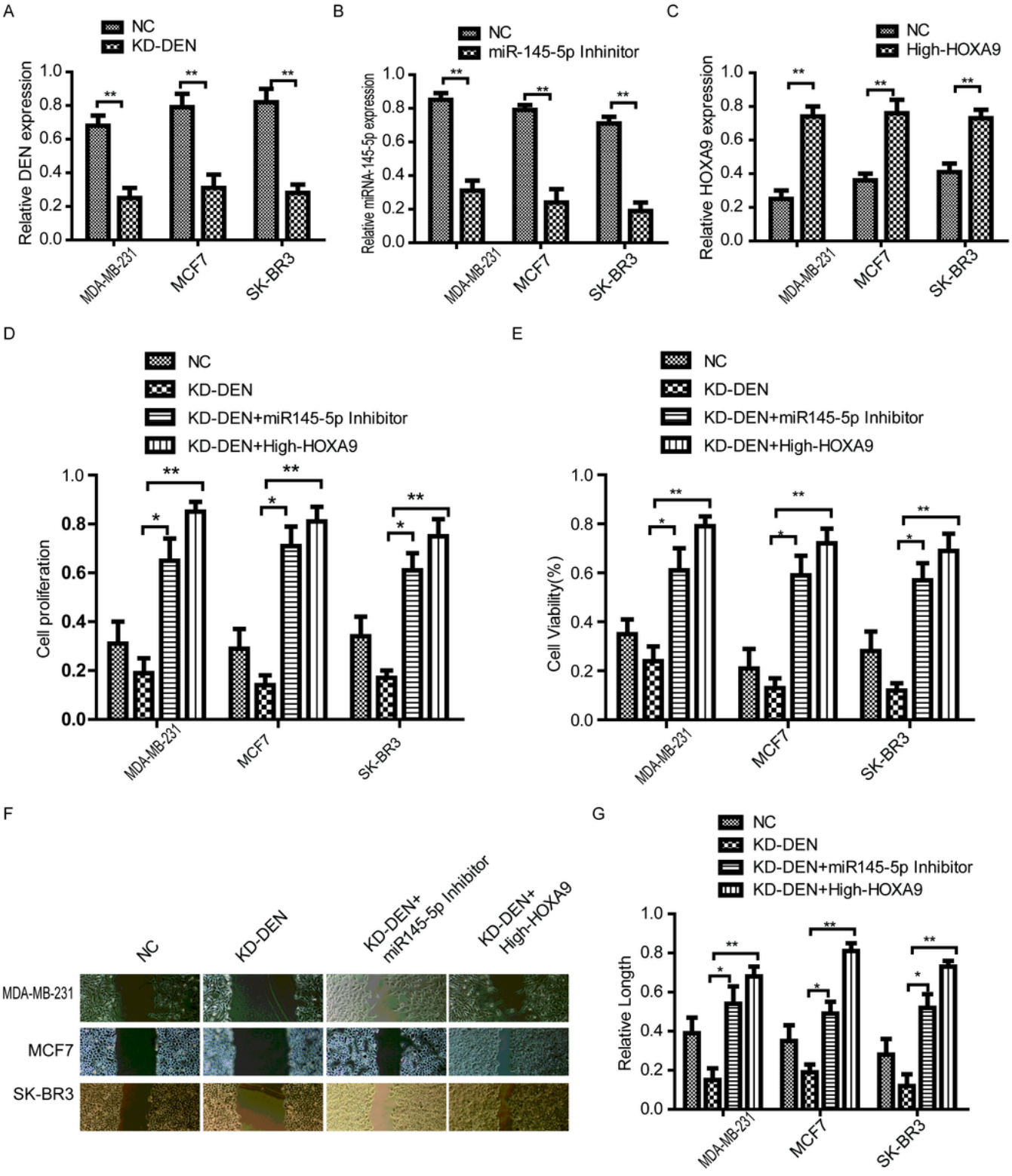

Figure 4

Downregulation of circRNA DENND4C suppressed the proliferation, migration and invasiveness of breast cancer cells by targeting the miR-145-5p/HOXA9 axis. The (A) circRNA DENND4C downregulation vectors, (B) miR-145-5p inhibitor and (C) High-HOXA9 vectors were transfected into breast cancer cells, and thentheir expression in these cells was examined by real- time qPCR. Breast cancer cells (MDA-MB-231, MCF7 and SK-BR3) were subjected to (D) an MTT assay and (E) a cell viability assay and the results indicated that upregulated circRNA DENND4C promoted the proliferation of breast cancer cells by regulating the miR-641/HOXA9 axis. (F, G) The wound-healing assay results suggested that circRNA DENND4C downregulation attenuated healing. Each bar represents the mean ± SEM. “DEN” represents “circRNA DENND4C”, *P < 0.05, **P < 0.01. All experiments were performed at least three times, and each sample was run in duplicate in each individual experiment.

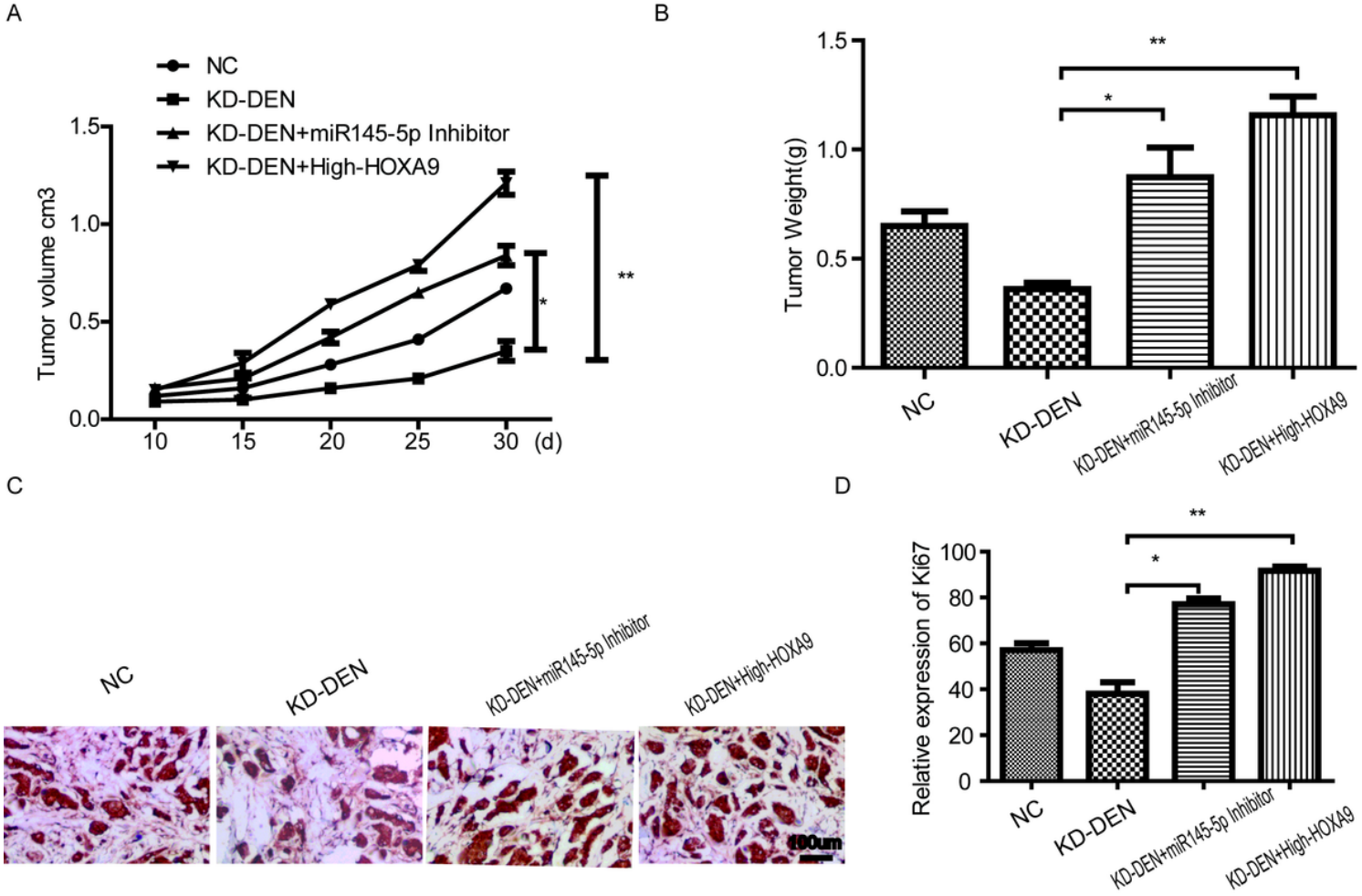

Figure 5

CircRNA DENND4C affected tumor growth by targeting the miR-145-5p/HOXA9 axis in vivo. A breast cancer xenograft model was established using MDA-MB-231 cells. (A) Tumor size was measured using a Vernier caliper once a week until the animals were sacrificed after 40 days of treatment. (B) Tumor weight was measured at the last time point. (C) Representative IHC for Ki 67 in tumor sections from the different groups. (D) Quantification of Ki67. Each bar represents the mean ± SEM.“DEN” represents “circRNA DENND4C”, *P < 0.05, **P < 0.01.

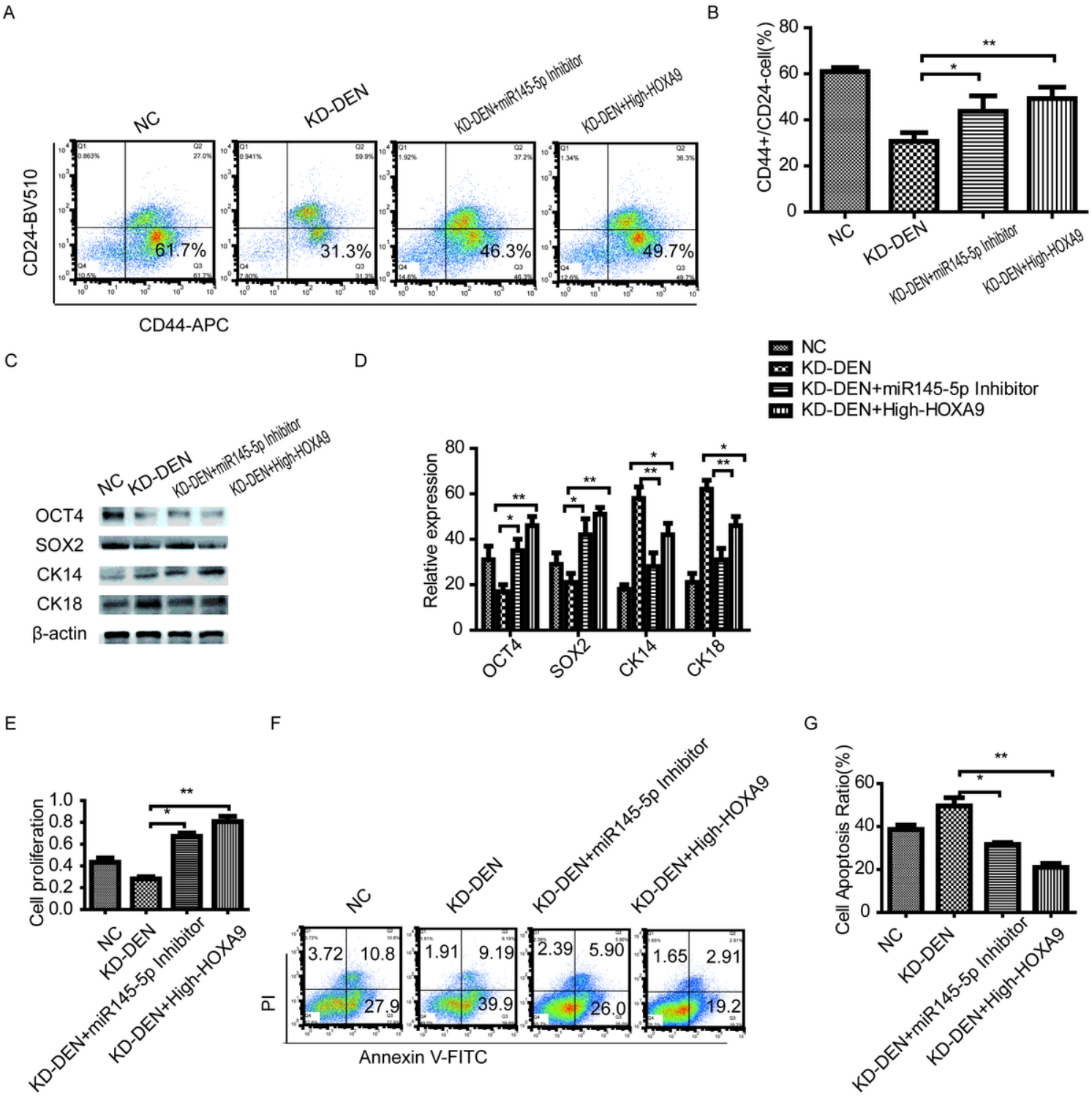

Figure 6

The circRNA DENND4C/miR-145-5p/HOXA9 axis regulated the stemness of breast cancer cells in vitro. Primary cell cultures were obtained from tumor tissues from mice in each group. (A, B) The percentage of CD44+/CD24− cells in the different groups according to flow cytometry. (C)Western blot analysis shows the protein expression levels of OCT4, SOX2, CK14, and CK18 in the different groups. (D) Quantification of HOXA9 was normalized to β-actin. (E) MTT assay shows cell proliferation in the different groups. (F) Annexin V -FITC vs. propidium iodide (PI) staining analysis shows the percentage of apoptotic cells in the

different groups. (G) Quantification of the apoptosis ratio indicated that circRNA DENND4C suppressed apoptosis through the miR-145-5p/HOXA9 axis. “DEN” represents “circRNA DENND4C”, *P < 0.05, **P < 0.01. All experiments were performed at least three times, and each sample was run in duplicate in each individual experiment.