TMEM216 inhibits breast cancer lung metastasis by modulating IGF1R-IRS4 signaling pathway

Yingying Wang[1#], Xinyang Bai[1#], Zhiyuan Du[1], Jiaxi Dong[1], Shenglong Xu[1], Xiaopeng Li[1], Junnan Zhou[1], Jiahui Zhou[1], Zirui Xu[1], Xi Kuang[1], Xiaoyang Sun[1], Qianqian Gong[1], Ling Su[1*], Xiangguo Liu[1,2,3*]

[1]Shandong Provincial Key Laboratory of Animal Cell and Developmental Biology, School of Life Sciences, Shandong University, Qingdao, China

[2]Key Laboratory of the Ministry of Education for Experimental Teratology, Shandong University, Jinan, China

Correspondence

Xiangguo Liu, PhD. Shandong University School of Life Sciences, Room N8-108, 72 Binhai Road, Qingdao 266237, P. R. China. Phone: 86-531-88364330; E-mail: xgliu@sdu.edu.cn; Or Ling Su, PhD. E-mail: suling@sdu.edu.cn;

#These authors contributed equally to this work.

Methods

Primers for PCR-based genotyping of transgene mice

Tmem216 forward: 5'-TGTGTTCTGCATCTGTACATTCATC-3'
Tmem216 reverse: 5'-GCTTGGACTCTGCATTTTAGATGT-3'
MMTV-Cre forward: 5'-CTGATCTGAGCTCTGAGTG-3'
MMTV-Cre reverse: 5'-CATCACTCGTTGCATCGACC-3'
MMTV-PyVT forward: 5'-GGAAGCAAGTACTTCACAAGGG-3'
MMTV-PyVT reverse: 5'-GGAAAGTCACTAGGAGCAGGG-3'

Whole-mount staining

Mammary tissue specimens from the inguinal region of mice representing distinct developmental stages were processed for whole-mount staining using established protocols. The adipose-containing mammary complexes were carefully dissected and flattened on microscope slides. Tissue fixation was performed overnight at ambient temperature using Carnoy's solution (75% ethanol and 25% glacial acetic acid). Sequential rehydration was achieved through immersion in decreasing ethanol concentrations (70%, 50%, 20%) for hourly intervals, followed by distilled water rinsing before chromatic staining. The carmine-based staining solution was prepared by dissolving 1g carmine (Cat. no. C1022; Sigma-Aldrich) and 2.5g aluminum potassium sulfate (Cat. no. A7167; Sigma-Aldrich) in distilled water, followed by 20-minute boiling and filtration, with final volume adjustment to 500mL. Tissues were immersed in this staining solution overnight at room temperature. Post-staining processing involved progressive dehydration through ascending ethanol concentrations (70%, 90%, 100%) with 60-minute incubations at each concentration, followed by tissue clarification using methyl salicylate (Cat. no. M2047; Sigma-Aldrich). Morphological examinations were conducted using a Nikon SMZ1500 stereomicroscope equipped with brightfield illumination.

Immunohistochemistry (IHC) and immunofluorescence (IF) staining

For histological analysis, mammary gland tissues, mouse xenografts, and lungs were processed by immersion in 4% formaldehyde solution at room temperature overnight to immobilize cellular structures. Paraffin sections (4 μm thick) were prepared for routine H&E staining and immunohistochemical analysis. Samples were baked at 60 °C for 4h, then de-paraffinized by three 10-min extractions in 100% xylene, followed by 5-min each of descending grade of alcohol (100%, 95%, 80%, and 70%). Samples were then washed briefly with phosphate-buffered saline (PBS) before transferring to boiling 10 mM sodium citrate buffer (pH 6.0) for 30 min. For immunohistochemistry, endogenous peroxidase activity was quenched by treating the sections with 3% hydrogen peroxide for 10 minutes. A blocking step was implemented using 5% normal goat serum in PBS for 30 minutes at room temperature to minimize nonspecific binding. The sections were subsequently exposed overnight at 4°C to the primary antibody of interest. Detection utilized the ABC peroxidase system (Cat. no. PK-6105, Vector Labs) with DAB (Cat. no. SK-4105, Vector Labs) as the chromogenic substrate, following the manufacturer's protocol. ImageJ software was employed for quantitative analysis of the immunohistochemical staining. Immunofluorescence studies involved MCF7 cells labeled with Alexa DyLight-488 and DyLight-594-conjugated secondary antibodies (Boster). Nuclei were counterstained with 1 μg/ml DAPI (Beyotime), and the samples were imaged using a ZEISS LSM900 confocal microscope equipped with a laser scanning system.

Western blots

Protein samples were subjected to 8%-12% SDS-PAGE for separation and subsequently transferred onto PVDF membranes. The membranes were then incubated with primary antibodies. Secondary antibodies, which were peroxidase-conjugated anti-mouse or anti-rabbit IgG from Bio-Rad, were applied for detection. Visual detection of the antigen-antibody complexes was achieved using an enhanced chemiluminescence assay.

Immunoprecipitation

Cells were lysed using lysis buffer on ice for 30 minutes, followed by centrifugation at 4°C for 15 minutes to isolate the supernatants. The obtained supernatants were incubated with the specific antibody at 4°C for 60 minutes. Subsequently, the mixture was incubated with protein A/G beads (Biolinkedin) at 4°C for 120

minutes. After incubation, the beads were washed twice with 1 ml of lysis buffer, and the bound proteins were eluted using 20 μl of 2× SDS buffer (100°C) for 10 minutes. Finally, the samples were centrifuged before undergoing Western blot analysis.

CCK-8 assay

Cellular suspensions were plated in 96-well microplates at a density of 5,000 viable cells per well and maintained under standard culture conditions for 24 hours. Following the incubation period, the growth medium was carefully aspirated and replaced with 100 μL of freshly prepared CCK-8 working solution (10% v/v CCK-8 reagent [Meilunbio MA0218] in complete medium). The cell-incorporated plates were subsequently subjected to 90-minute incubation at 37°C in a humidified atmosphere. Optical density measurements were performed using a microplate spectrophotometer with primary detection at 450 nm. To ensure experimental reproducibility, this protocol was executed with three independent biological replicates, each containing technical triplicates.

Cell migration and invasion assay

Transwell migration assays were conducted using 8-μm pore polyethylene terephthalate membrane inserts (Cat. no. 353097, BD), while invasion assessments employed Matrigel-coated counterparts from the same manufacturer. Following enzymatic detachment, cell suspensions containing 1.5×10^4 to 1×10^5 cells were aliquoted in 200 μL serum-free DMEM into upper compartments. The lower chambers contained DMEM supplemented with 10% fetal bovine serum as a chemotactic stimulus. After 14-hour incubation under standard culture conditions, migratory/invasive cells were quantified. Transmembrane cells were fixed with 4% formaldehyde (30 min, RT) and stained with 0.1% crystal violet (30 min) before microscopic enumeration. Values for cell migration or invasion were expressed as the mean number of cells per microscopic field over five fields per one insert for triplicate experiments. Experimental validation included three independent biological replicates with technical triplicates.

Fig. S1

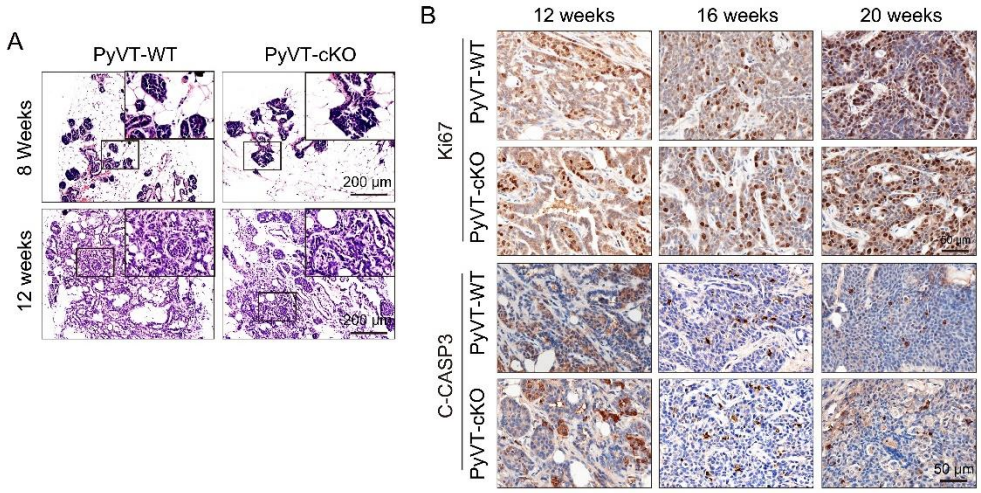

Fig. S1. TMEM216 does not affect the proliferation of primary tumors in spontaneous BrCa mouse models. (A) H&E staining of the mammary glands of PyVT-WT and PyVT-cKO mice at 8 weeks and 12 weeks. (B) Immunohistochemical staining of Ki67 and C-CASP3 in mammary tumors of PyVT-WT and PyVT-cKO mice at 12 weeks, 16 weeks, and 20 weeks.

Fig. S2

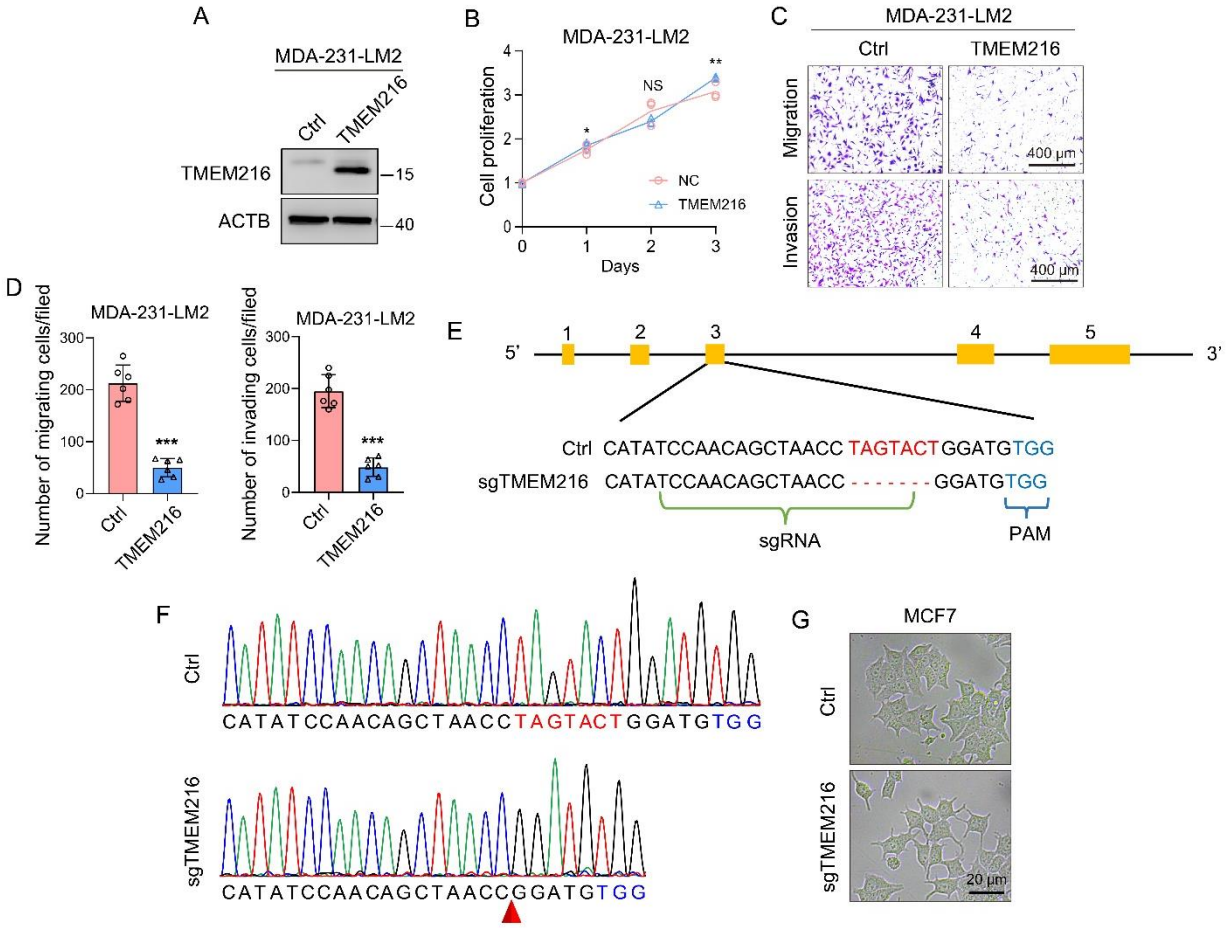

Fig. S2. TMEM216 does not affect the proliferation of BrCa cells but inhibits their migration. (A) Western blot assays of MDA-231-LM2 cells with overexpression of TMEM216. (B) The CCK-8 assays demonstrate that overexpression of TMEM216 does not affect the proliferative capacity of MDA-231-LM2 cells. Data are shown as mean ± SEM. NS, not significant; *$P < 0.05$, **$P < 0.01$, RM ANOVA followed by post hoc LSD test. (C) Migration and invasion assays of MDA-231-LM2 cells after TMEM216 overexpression. (D) The histograms represent the statistical analysis of migrating and invading cells depicted in (C). Data are shown as mean ± SEM. ***$P < 0.001$, P values were obtained using two-tailed Student's t-tests. (E) Schematic diagram showing the human *TMEM216* gene locus. The sgRNA was designed to delete a portion of the third exon of *TMEM216*. The red letters indicate the deleted base sequence in $MCF7^{sgTMEM216}$ cells. (F) Sanger sequencing was performed on $MCF7^{Ctrl}$ and $MCF7^{sgTMEM216}$ cells, revealing that the TAGTACT bases were deleted in the $MCF7^{sgTMEM216}$ cell. (G) Typical cell morphology images of $MCF7^{Ctrl}$ and $MCF7^{sgTMEM216}$ cells show an increase in cell protrusions following TMEM216 deletion.

Fig. S3

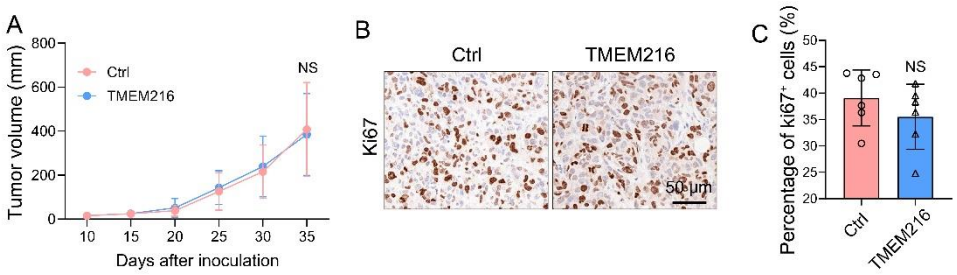

Fig. S3. TMEM216 does not affect the proliferation of breast orthotopic xenograft. (A) Control and TMEM216 overexpression MDA-231-LM2 cells were injected into the fourth mammary fat pads on the left and right sides of the same female BALB/c nude mouse, respectively. Tumor volumes were measured with calipers at the indicated time points. Data are shown as mean ± SD. NS, not significant; RM ANOVA followed by post hoc LSD test. (B) Representative immunohistochemical staining for Ki67 in tumor tissues was obtained from each experimental group. (C) The histograms show the mean value for the percentage of Ki67-positive cells with statistical evaluation ($n = 6$). Data are shown as mean ± SEM. NS, not significant; P values were obtained using two-tailed Student's t-tests.