

# DNMT1 facilitates proliferation and metastasis of breast cancer by inducing MEG3 promoter methylation in MEG3/miR-494-3p/OTUD4 regulatory axis

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## Primary research

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## Abstract

**Background:** To explore the mechanism by which DNMT1 potentiates proliferation and metastasis of breast cancer by inducing MEG3 promoter methylation in MEG3/miR-494-3p/OTUD4 regulatory axis.

**Methods:** Human breast cancer cell lines (MCF-7, MDA-MB-231, SKBR3) and human breast epithelial cell line MCF10A were selected for the experiments. The expression levels of DNMT1, MEG3, miR-494-3p and OTUD4 were detected by qRT-PCR. Western blot was used to detect the protein expression levels of DNMT1 and OTUD4. ChIP assay was adopted to verify the binding relationship between DNMT1 and MEG3 promoter region. MethPrimer software was applied to identify MEG3 promoter methylation while methylation-specific PCR was conducted to examine its methylation level. The targeted binding sites of miR-494-3p on MEG3/OTUD4 were predicted by bioinformatics and further verified by RNA binding protein immunoprecipitation assay plus dual-luciferase reporter gene assay. Cell proliferative, migratory and invasive abilities were measured by CCK-8, wound healing and Transwell assays.

**Results:** DNMT1 was highly expressed while MEG3 was poorly expressed in breast cancer cells. Silencing DNMT1 inhibited the proliferation, migration and invasion of breast cancer cells by increasing gene expression of MEG3 through demethylation. MEG3 was identified as a ceRNA that regulated miR-494-3p expression via RNA sponging in breast cancer. In addition, miR-494-3p could bind to the 3'-UTR of OTUD4 mRNA, thus negatively regulating OTUD4 expression. A regulator axis formed by MEG3/miR-494-3p/OTUD4 was thus established and identified to have an impact on proliferation, invasion and migration of breast cancer. Moreover, overexpression of MEG3 could suppress tumor growth of breast cancer *in vivo*.

**Conclusion:** Silencing DNMT1 induced demethylation of MEG3 promoter to promote MEG3 gene expression, in turn inhibiting the expression of miR-494-3p to elevate the expression of its downstream target OTUD4, thus weakening the proliferative, migratory and invasive abilities of breast cancer cells.

## Background

As one of the most common aggressive malignancies, breast cancer has the highest incidence and remains the first cause of cancer death among women worldwide, leading to 522,000 deaths since 2008[1]. Like other solid tumors, distant metastasis (especially lung metastasis) is a major cause of breast cancer-related death and resistance to various treatments[2].

DNA hypermethylation is a major epigenetic feature that distinguishes cancer cells from normal cells, which causes insensitivity of cancer cells to signals indicating growth inhibition and evades programmed cell death by inhibiting tumor suppressor genes[3]. DNA hypermethylation is involved in the occurrence and cell survival of breast cancer, and its initiation mechanism is the abnormal expression of DNA methyltransferases (DNMTs), including DNMT1, DNMT3a and DNMT3b[4-6]. DNMT1 is an important methyltransferase with aberrantly high expression in dividing cells differing from nondividing cells and has become a considerable target aiming for methylation inhibition in cancer therapy[7]. The

downregulation of DNMT1 has been reported to inhibit the proliferation and invasion of breast cancer cells[8], and DNMT1 also can downregulate maternally expressed gene 3 (MEG3) expression through increasing methylation level of MEG3 in breast cancer[9]. MEG3 is identified as an imprinted gene expressed according to the maternal origin and encodes a long non-coding RNA (lncRNA)[10]. As reported, MEG3 is closely related to the progression of breast cancer, and studies have shown that high-expression of MEG3 can stagnate cell growth and increase apoptosis of breast cancer cells[11]. Meanwhile, high-expression of MEG3 suppresses breast cancer cell proliferation, invasion and angiogenesis through AKT pathway[12].

In addition, many studies show that lncRNAs function as a competing endogenous RNA (ceRNA) sponging miRNA and regulating the expression of miRNA targets[13, 14]. It has been reported that lncRNA MEG3 inhibits cell epithelial-mesenchymal transition (EMT) by targeting miR-421 and regulating E-cadherin in breast cancer[15]. High-expression of miR-494-3p has a promoting effect on breast cancer by targeting and down-regulating TRIM21. Nevertheless, there is no report on MEG3 as a ceRNA regulating the expression of miR-494-3p in breast cancer. In addition, the targets of miR-494-3p were also studied in the present research.

In this study, we investigated the function of DNMT1 during MEG3 methylation process and the effect of MEG3/miR-494-3p/OTUD4 regulatory axis on the proliferation, migration and invasion of breast cancer, which could help develop treatment strategies for breast cancer.

## Methods

### Bioinformatics analysis

RAID database and starBase database were used to obtain downstream regulatory miRNAs of MEG3 and the potential targeted binding sites of lncRNA-miRNA. GSE70905 dataset of breast cancer was obtained from GEO database, including 45 normal samples and 45 tumor samples. Normal samples were taken as control and differential analysis was conducted by using “limma” package in R. *P*-value was corrected by using FDR method. Differentially expressed genes (DEGs) were screened with  $|logFC|>1$  and *p* value $<0.05$ . The downstream target genes of miR-494-3p were predicted by TargetScan, mirDIP and starBase databases. Targeted binding sites of miRNA-mRNA were consulted on the TargetScan database.

### Cell culture

Human breast epithelial cell line MCF10A (3111C0001CCC000406), human breast cancer cell lines MCF-7 (3142C0001000001079), MDA-MB-231 (3111C0001CCC000014), SKBR3 (3142C0001000000313), and human embryonic kidney cell line HEK-293 (3111C0001CCC000010) were selected for this study. MCF10A, MDA-MB-231 and HEK-293 cell lines were purchased from the cell resource center of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. MCF-7 and SKBR3 cell lines were purchased from cell bank of China Center for Type Culture Collection. MCF10A cell line was cultured in DMEM-F12 medium. MCF-7 and HEK-293 cell lines were incubated in MEM-EBSS (MEM Eagles with

Earle's Balanced Salts) medium. MDA-MB-231 cell line was placed in L15 medium (Leibovitz Medium). SKBR3 cell line was cultured in McCoy's 5A Media medium (Modified with Tricine). The mediums were all purchased from Hyclone and contained 10% fetal bovine serum (FBS).

### Lentivirus vector construction

MEG3 cDNA was cloned into pcDNA4 vector, while the short hairpin RNAs (shRNAs) targeting DNMT1, MEG3 and OTUD4 were cloned into PLKO.1 vectors. pPAX2 and pVSVG along with target vectors were co-transfected into 293T cells to construct lentiviral vectors. Supernatant was harvested at 24 h and 48 h after transfection and filtered through a 0.45- $\mu$ m membrane. The viral supernatant was added to medium in a ratio of 1:3 for viral infection. After 24 h, stably transfected cell lines were selected using 2  $\mu$ g/ml puromycin. All vectors, mimics and inhibitors were purchased from GenePharma (Shanghai, China). The scramble shRNA and empty pcDNA4 vector were used as negative controls, respectively. Sequences for sh-DNMT1, sh-MEG3 and sh-OTUD4 were detailed in **Supplementary material 1**. According to the preliminary experiments, the shRNA with a better interference efficiency was selected and the results were presented in the Results section.

### Dual-luciferase reporter gene assay

The 3'-UTR of MEG3 or OTUD4 was ligated to psiCHECK2 vector that was fused with luciferase gene and had been digested with Xhol and NotI restriction enzymes. The QuikChange multi-site-directed Mutagenesis kit (Stratagene, LaJolla, CA) was used to mutate the targeted sites of miR-494-3p on 3'-UTR. Luciferase activities were determined by dual-luciferase assay (Promega), and Renilla luciferase activity was used for normalization of Firefly luciferase activity.

### RNA binding protein immunoprecipitation (RIP) assay

RIP was performed using the Magna RIP RNA-Binding Protein Immunoprecipitation kit (Millipore, Burlington, MA). MDA-MB-231 cells in each group were lysed, and then the cell extracts were cultured with protein magnetic beads and incubated with 2  $\mu$ g of Ago2 antibody (ab186733, 1:30, Abcam, UK) or control IgG antibody (ab205718, 1:50, Abcam, UK) overnight at 4 °C. The immunoprecipitated RNA was purified and the expression levels of MEG3, miR-494-3p and OTUD4 were detected by qRT-PCR.

### Chromatin immunoprecipitation (ChIP) assay

Enrichment of DNMT1 in MEG3 promoter region was analyzed using ChIP kit (Millipore, USA). When the MDA-MB-231 cells were grown to 70-80% in confluence, 1% formaldehyde was added to the cells and cells were fixed at room temperature for 10 min to make the DNA and proteins in the cells immobilized and cross-linked. Then, the cross-linked products were randomly fragmented into fragments of appropriate size by 10 s of ultrasonication for 15 cycles with an interval of 10 s. After centrifugation at 13,000 rpm at 4 °C, the collected supernatant was transferred into 3 tubes and cultured with positive control antibody RNA polymerase II, negative control antibody IgG of normal mice (ab6721, 1:30, Abcam, UK) and methylation transferase specific antibody DNMT1 (ab13537, 1:50, Abcam, UK) overnight at 4 °C,

respectively. Protein Agarose/Sepharose was used to precipitate endogenous DNA-protein complexes, and the supernatant was adsorbed after a short centrifugation. The non-specific complexes were washed and de-crosslinked overnight at 65 °C. The DNA fragments were extracted and purified by phenol/chloroform. qRT-PCR was used to test the combination of DNMT1 and MEG3 promoter region. Primer sequences were detailed in **Supplementary material 1**.

### Methylation-specific PCR (MSP)

Genomic DNA was treated with sodium bisulfite and DNA methylation was tested by MSP using EZ DNA Methylation-Direct kit (Zymo Research). Two primer groups were used to amplify the promoter region of MEG3 containing multiple CpG sites, and the primer sequences were shown in **Table 1**. PCR reaction conditions: pre-denaturation at 95 °C for 10 min, followed by 35 cycles of 95 °C for 45 s, 56 °C (methylation) / 45 °C (non-methylation) for 45 s and 72 °C for 45 s, and finally extension at 72 °C for 10 min. The reaction products were subjected to agarose gel electrophoresis and images were captured for further analysis.

### Western blot

RIPA lysis buffer (Takara Biotechnology, Dalian, China) was used to extract total proteins from cells. A total of 20 µg of proteins were isolated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Billerica, MA, USA). After blocked in TBS buffer containing 5% skim milk (50 mmol/L NaCl, 10 mmol/L Tris, pH7.4), the membranes were washed with TBST three times for 5 min each time and incubated with primary antibodies at 4 °C overnight. Primary antibodies were DNMT1 (ab188453, 1:1000, Abcam, UK), OTUD4 (ab106368, 1:500, Abcam, UK) and GAPDH (ab181602, 1:10,000, Abcam, UK). Then, the membranes were washed with TBST as the above procedures, and incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) at room temperature for 1 h. Finally, immunoreactive proteins were treated with enhanced chemiluminescence reagent (Amersham, Little Chalfont, UK) and protein bands were analyzed using Amersham Imager 600 system (GE Healthcare Life Sciences, Shanghai, China).

### qRT-PCR

TRIzol reagent (Invitrogen) was used to extract total RNA from cells, and the OD260/280 value of each RNA sample was determined by an UV spectrometer. RNA concentration was calculated and samples were stored at -80 °C for subsequent experiments. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencis, CA, USA). cDNA of mRNA was obtained by reverse transcription kit (RR047A, Takara, Japan), while cDNA of miRNA was obtained by miRNA First Strand cDNA Synthesis kit (B532451-0020, Shanghai Sangon Biotech, China). The samples were loaded using the SYBR® Premix Ex TaqTM II (Perfect Real Time) kit (DRR081, Takara, Japan) and subjected to qRT-PCR reaction on a real-time fluorescence quantitative PCR instrument (ABI 7500, ABI, Foster City, CA, USA). The PCR amplification procedure was set as below: pre-denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s

and 60 °C for 34 s. Each sample treatment was repeated in triplicate. Primers were synthesized by Shanghai Sangon Biotech Company (**Table 1**). Ct value of each well was recorded. Relative expression of target genes was calculated by  $2^{-\Delta\Delta Ct}$  method with GAPDH or U6 as internal reference.  $\Delta\Delta Ct = (\text{average Ct value of target gene in experimental group} - \text{average Ct value of housekeeping gene in experimental group}) - (\text{average Ct value of target gene in control group} - \text{average Ct value of housekeeping gene in control group})$ .

## CCK-8

Cell proliferation was assessed by cell counting kit-8 (CCK-8; Beyotime Biotechnology, China). A total of  $3 \times 10^3$  MDA-MB-231 cells were inoculated into 96-well plates. After transfection on day 1, day 2, day 3 and day 4, 10  $\mu$ l of CCK-8 reagent was added to each well. The plates were placed at 37 °C for 2 h, and the absorbance was read at 450 nm using a microplate reader (Bio-Rad, San Diego, CA, USA). The absorbance value was expressed as a percentage of the experimental group to the control group.

## Wound healing assay

Cell motility was assessed by a wound-healing assay, as described in a previous study[16]. In brief,  $2 \times 10^5$  MDA-MB-231 cells were inoculated on 6-well plates and incubated in appropriate complete medium at 37 °C for 16 h. The monolayer was scraped and cells were cultured in a fresh medium without FBS for 24 h. Finally, three different fields of each well were observed and photographed under an inverted microscope to measure the scratch width. Relative scratch width = 24 h scratch width / 0 h scratch width.

## Transwell invasion assay

As mentioned previously, changes in cell invasion were analyzed through Transwell assay[17]. In this assay, 8.0- $\mu$ m Millipore Transwell chambers containing Matrigel were used. Firstly,  $1 \times 10^5$  MDA-MB-231 cells were resuspended in 200  $\mu$ l medium without FBS and then inoculated into the upper chambers. Next, 500  $\mu$ l medium containing 10% FBS was added to the lower chambers. After 48 h of culture, un-invading cells were removed from the upper surface of the membranes with a cotton swab, and the invading cells were fixed and stained with crystal violet. Finally, 5 randomly selected fields were observed under an inverted microscope to calculate cell number.

## Nude mice experiment[18]

Ten 6-week-old BALB/c female nude mice were purchased from Beijing HFK bio-technology (Beijing, China). The mice were randomly divided into two groups with 5 in each group. Then,  $5 \times 10^6$  MDA-MB-231 cells with sh-NC or sh-DNMT1 were resuspended in 100  $\mu$ l PBS and sequentially injected into each mouse by tail vein injection. After the mice were fed for 5 days, tumor volume was measured by a caliper every 5 days and calculated as follow:  $V = D \times d^2 \times 0.5$  (D, longer diameter; d, shorter diameter). After 35 days, the mice were euthanized by CO<sub>2</sub> inhalation. This experiment was approved by the Animal Care and Use Committee of Jinhua Municipal Central Hospital.

## Statistical analysis

All data were processed by SPSS 21.0 statistical software (SPSS, Inc., Chicago, IL, USA), and the measurement data were expressed as mean  $\pm$  standard deviation. The comparison between two groups was analyzed by Student's *t*-test, and the comparison among multiple groups was analyzed by one-way ANOVA.  $P<0.05$  indicated the difference was statistically significant.

## Results

### Silencing DNMT1 inhibits the proliferation, migration and invasion of breast cancer cells by promoting the expression of MEG3 through demethylation

Current studies find that lncRNA MEG3 is regulated by DNMT1, and the promoter of MEG3 is methylated under the influence of DNMT1, while MEG3 appears to be hypermethylated and lowly expressed in tumors[9, 15, 19]. In view of this finding, we further examined the effect of the interaction of MEG3 with DNMT1 on the breast cancer cells. DNMT1 and MEG3 expression levels in human breast cancer cell lines MCF-7, MDA-MB-231, SKBR3 and human normal breast epithelial cell line MCF10A were tested by western blot (**Figure 1A**) and qRT-PCR (**Figure 1B**), respectively. The results showed that protein expression of DNMT1 was significantly higher in breast cancer cells, while MEG3 was significantly lowly expressed ( $p<0.05$ ). MDA-MB-231 cell line with the lowest MEG3 expression was chosen for subsequent experiments. ChIP assay was used to detect whether DNMT1 could bind to MEG3 promoter region (**Figure 1C**). Compared with the IgG control group, DNMT1 enrichment in MEG3 promoter region was significantly increased ( $p<0.05$ ). Besides, CpG islands were found in the MEG3 gene promoter region by analyzing 2100 bp nucleotide sequences near the MEG3 gene promoter region through the MethPrimer software, and the results indicated that MEG3 expression would be affected by promoter methylation (**Figure 1D**). Then, DNMT1 interference efficiency in MDA-MB-231 cells was examined by western blot (**Figure 1E**), and sh-DNMT1-1 was used in subsequent experiments for better interference efficiency. MEG3 methylation level detected by MSP (**Figure 1F**) presented that MEG3 methylation level was significantly decreased upon DNMT1 knockdown. While qRT-PCR results revealed that MEG3 gene expression was remarkably up-regulated when silencing DNMT1 (**Figure 1G**) ( $p<0.05$ ). It indicated that silencing DNMT1 could promote MEG3 expression by inhibiting MEG3 promoter methylation.

It is reported that DNMT1 can promote the malignant progression of breast cancer[8], while MEG3 can elicit a suppressive effect[12]. In this study, we proved that DNMT1 could potentiate MEG3 promoter methylation in turn inhibiting MEG3 expression. Based on the above results, we put forward a hypothesis that DNMT1 regulates the proliferation, migration and invasion of breast cancer cells by inhibiting MEG3 expression. To validate the speculation, the level of MEG3 expression was firstly interfered, and sh-MEG3-1 with a better interference efficiency as directed by qRT-PCR was selected for subsequent experiments (**Figure 1H**). Then, expression levels of DNMT1 and MEG3 in three groups (sh-NC, sh-DNMT1+sh-NC, sh-DNMT1+sh-MEG3) were detected by qRT-PCR (**Figure 1I**). As demonstrated, the expression of DNMT1 was conspicuously down-regulated while the expression of MEG3 was remarkably up-regulated in the sh-

DNMT1+sh-NC group relative to the sh-NC group. Besides, MEG3 expression was obviously down-regulated when DNMT1 and MEG3 were both silenced, and there was no significant diversity in DNMT1 expression, with a comparison of those in the sh-DNMT1+sh-NC group. Then, the results of CCK-8 (**Figure 1J**), wound healing (**Figure 1K**) and Transwell (**Figure 1L**) assays displayed that silencing DNMT1 decreased cell activity, migratory and invasive abilities while these abilities were increased when DNMT1 and MEG3 were silenced simultaneously. In conclusion, silencing DNMT1 inhibited the malignant progression of breast cancer via up-regulating MEG3.

### **miR-494-3p is a target of MEG3 in breast cancer cells**

Many studies have pointed out that MEG3 can play a regulatory role by acting as a ceRNA[20, 21]. Further prediction of the downstream regulatory miRNAs of MEG3 (**Figure 2A**) discovered that miR-494-3p might be a target of MEG3 and there were potential targeted binding sites linking these two genes (**Figure 2B**). Moreover, a study indicated that the expression level of miR-494-3p in tumors is significantly increased[22]. Hence, expression level of miR-494-3p in breast cancer cell lines *in vitro* was further examined and found to be significantly higher than that in control cell line (**Figure 2C**). Moreover, RIP was used to validate the binding relationship between MEG3 and miR-494-3p (**Figure 2D**). Compared with the IgG group, the quantity of MEG3 and miR-494-3p bound by Ago2 was significantly increased. Dual-luciferase reporter gene assay further verified the targeted binding sites of miR-494-3p on MEG3 3'-UTR (**Figure 2E**). The results showed that overexpression of miR-494-3p markedly reduced the luciferase activity of the MEG3-wt group but had no effect on that of the MEG3-mut group. Expression analysis was conducted by qRT-PCR for the levels of MEG3 and miR-494-3p in the oe-NC group and oe-MEG3 group (**Figure 2F**), demonstrating that the expression of miR-494-3p was remarkably down-regulated when MEG3 was overexpressed, indicating that MEG3 targeted and negatively regulated miR-494-3p.

Then, rescue experiments were conducted to study the regulation of the MEG3/miR-494-3p axis on breast cancer cells. Firstly, the expression levels of MEG3 and miR-494-3p in 3 groups (oe-NC+NC mimic group, oe-MEG3 +NC mimic group and oe-MEG3+ miR-494-3p mimic group) were detected by qRT-PCR (**Figure 2G**). Overexpression of MEG3 significantly down-regulated miR-494-3p expression, but when MEG3 and miR-494-3p were overexpressed simultaneously, miR-494-3p expression was elevated greatly. The results of CCK-8 (**Figure 2H**), wound healing (**Figure 2I**) and Transwell (**Figure 2J**) assays displayed that overexpression of MEG3 decreased cell activity, migratory and invasive abilities, while these abilities were recovered when MEG3 and miR-494-3p were overexpressed at the same time ( $p<0.05$ ).

### **Silencing miR-494-3p inhibits proliferation, migration and invasion of breast cancer cells by targeting and promoting OTUD4 expression**

Furthermore, DEGs in breast cancer dataset GSE70905 that was included in GEO database were analyzed (**Figure 3A**), and downstream target genes of miR-494-3p were predicted on bioinformatics databases. It was found that OTUD4 was with specific binding sites of miR-494-3p and it was lowly expressed in breast cancer (**Figure 3B-D**). Similarly, *in vitro* cell experiments also showed that the expression of OTUD4 mRNA in breast cancer cell lines was significantly lower than that in normal cell line (**Figure 3E**). To

confirm the relationship of miR-494-3p with OTUD4, firstly, RIP assay was performed and the results shown in **Figure 3F** revealed that compared with the IgG group, the quantity of miR-494-3p and OTUD4 bound by Ago2 was significantly increased. Dual-luciferase assay was conducted for further verification (**Figure 3G**). The result showed that overexpression of miR-494-3p significantly decreased the luciferase activity of the OTUD4-wt group ( $p<0.05$ ) but had no effect on that of the OTUD4-mut group ( $p>0.05$ ). OTUD4 expression in NC inhibitor and miR-494-3p inhibitor groups was tested by western blot (**Figure 3H**) and the results suggested that silencing miR-494-3p significantly up-regulated OTUD4 expression ( $p<0.05$ ).

Rescue experiments were also used to test the regulatory effect of miR-494-3p/OTUD4 on breast cancer development. Firstly, sh-OTUD4-1, which had a better interference efficiency as judged by western blot, was chosen for further tests (**Figure 3I**). The expression levels of miR-494-3p and OTUD4 in 3 groups (NC inhibitor+sh-NC group, miR-494-3p inhibitor+sh-NC group, and miR-494-3p inhibitor+sh-OTUD4 group) were detected by qRT-PCR (**Figure 3J**). As the results displayed, silencing miR-494-3p significantly up-regulated OTUD4 expression, but when OTUD4 and miR-494-3p were silenced simultaneously, OTUD4 expression was decreased greatly ( $p<0.05$ ). Finally, CCK-8 (**Figure 3K**), wound healing (**Figure 3L**) and Transwell (**Figure 3M**) assays were carried out, demonstrating that silencing miR-494-3p decreased cell viability, migratory and invasive abilities while the effect was reversed when miR-494-3p and OTUD4 were silenced at the same time ( $p<0.05$ ).

### **MEG3 negatively regulates miR-494-3p to promote OTUD4 expression and inhibits the proliferation, migration and invasion of breast cancer cells**

For deeply understanding the influence of MEG3/miR-494-3p/OTUD4 as the regulatory axis on breast cancer cells, the expression levels of MEG3, miR-494-3p and OTUD4 in 3 groups (oe-NC+sh-NC group, oe-MEG3+sh-NC group and oe-MEG3+sh-OTUD4 group) were tested by qRT-PCR (**Figure 4A**), and the protein level of OTUD4 was detected by western blot (**Figure 4B**). Overexpression of MEG3 significantly increased OTUD4 protein and mRNA expression levels, but reduced miR-494-3p expression. In comparison with the oe-MEG3+sh-NC group, the mRNA and protein expression levels of OTUD4 were greatly down-regulated in the oe-MEG3+sh-OTUD4 group, while the expression levels of MEG3 and miR-494-3p had no significant difference ( $p>0.05$ ). The results of CCK-8 (**Figure 4C**), wound healing (**Figure 4D**) and Transwell (**Figure 4E**) assays indicated that overexpression of MEG3 decreased cell viability, migratory and invasive abilities while silencing OTUD4 in MEG3-overexpressed cells could partially alleviate the inhibition.

### **Overexpression of MEG3 inhibits the tumorigenic ability of breast cancer *in vivo***

Finally, we overexpressed MEG3 in nude mice to observe the effect of MEG3 on the tumorigenicity of breast cancer. The tumor weight and volume of each group were detected as shown in **Figure 5A-C**. Overexpression of MEG3 reduced tumor volume and weight. The expression levels of DNMT1, MEG3, miR-494-3p and OTUD4 upon MEG3 overexpression were detected by qRT-PCR (**Figure 5D**), while the protein expression levels of DNMT1 and OTUD4 were detected by western blot (**Figure 5E**). The results exhibited that miR-494-3p was significantly down-regulated when MEG3 was overexpressed, while MEG3

and OTUD4 were remarkably up-regulated ( $p<0.05$ ). Besides, overexpression of MEG3 had no significant effect on DNMT1 expression ( $p>0.05$ ).

## Discussion

Aberrant DNA methylation plays an important role in gene expression[23]. This study explored the regulation of MEG3/miR-494-3p/OTUD4 axis in breast cancer from the perspective of DNMT1 promoting MEG3 hypermethylation, to clarify its potential effect on the biological behaviors of breast cancer cells. Previous studies demonstrated that DNMT1 (DNA methylase) can promote the methylation of lncRNA MEG3, and methylation of MEG3 promoter along with changes in gene region is the main reason for abnormal expression of MEG3 in tumors[24]. Another study showed that DNA methylation inhibitor (5'-Aza-2'-deoxycytidine) plays an important regulatory role in MEG3 expression in glioma cells[25]. Additionally, aberrant methylation promoted by DNMT1 can increase the resistance of breast cancer cells to anticancer drugs, and DNMT1 also can promote the development of breast cancer by reducing the expression of MEG3[9]. Here, this study verified the role of DNMT1 in regulating MEG3, and the results were consistent with previous reports. The rescue experiments further verified that knockdown of DNMT1 could demethylate MEG3 and promote its expression, thus inhibiting the progression of breast cancer cells.

It is known that MEG3 can function as a ceRNA in tumors[15]. For example, MEG3 may regulate the progression of gastric cancer as a ceRNA binding to miR-181a[26], and regulate ischemic neuronal death by targeting miR-21/PDCD4 signaling pathway[27]. MEG3 modulates EMT of cells by targeting miR-421 in breast cancer[15]. This study further explored the downstream targets of MEG3, and found that MEG3 could target and bind to miR-494-3p. To verify this relationship, RIP and dual-luciferase assays were used and the results implied that MEG3 could regulate the expression of miR-494-3p as a ceRNA. Zhou *et al.* reported that miR-494-3p could promote cell proliferation and tumor growth in breast cancer through targeting and inhibiting TRIM21 expression[28], and miR-494-3p was also found to inhibit self-renewal of breast cancer stem/progenitor cells[29], which both indicate the important regulatory role of miR-494-3p in breast cancer. In the study, we overexpressed MEG3 expression in cancer cells and found suppressed cell activity, invasive and migratory abilities. In rescue experiments, overexpression of miR-494-3p reversed the inhibitory effect which functioned by MEG3 overexpression on the development of breast cancer cells. The results showed that high level of MEG3 inhibited the proliferation, invasion and migration of breast cancer cells by targeting and inhibiting miR-494-3p expression.

In order to further understand the underlying regulatory mechanism, target genes of miR-494-3p were predicted, and the DEMRNAs in breast cancer were screened using GEO database. The results suggested that OTUD4 was poorly expressed in breast cancer tissue samples, and the 3'-UTR of OTUD4 had potential binding sites of miR-494-3p. Furthermore, RIP and dual-luciferase assays were used to verify the binding relationship, and western blot assay results confirmed the down-regulation of OTUD4 expression in the case of miR-494-3p overexpression. This indicated that miR-494-3p targeted and down-regulated the expression of OTUD4 in breast cancer. OTUD4 has been found to be lowly expressed in non-small cell

lung cancer and is able to inhibit the proliferation of cancer cells[30]. In addition, alkylation damage which is critical for cancer chemotherapy can also be regulated by OTUD4[31]. However, the mechanism of OTUD4 in breast cancer has not been reported. Rescue experiments were performed in this study again to find that silencing OTUD4 reversed the negative influence of miR-494-3p inhibitor on breast cancer cells. Moreover, the downstream regulatory effects of DNMT1 and MEG3 were further investigated to determine their impact on breast cancer progression.

## Conclusions

We explored the role of DNMT1 in reducing MEG3 methylation, and in turn regulating the expression of genes in the MEG3/miR-494-3p/OTUD4 axis to influence progression of breast cancer cells (**Figure 6**). This study reveals the mutual interactions among MEG3, miR-494-3p and OTUD4, providing a new approach for targeted therapy of breast cancer.

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Availability of data and materials

The data and materials in the current study are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no potential conflicts of interest.

### Funding

Not applicable.

### Authors' contributions

Xiaotao Zhu contributed to the study design. Fan Wang conducted the literature search and performed data analysis. Lin LV acquired the data and wrote the article. Mingzheng Wang and Chen Fan drafted. Xiaofeng Lu, Miaomiao Jin and Shuguang Li revised the article and gave the final approval of the version to be submitted. All authors read and approved the final manuscript.

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Not applicable.

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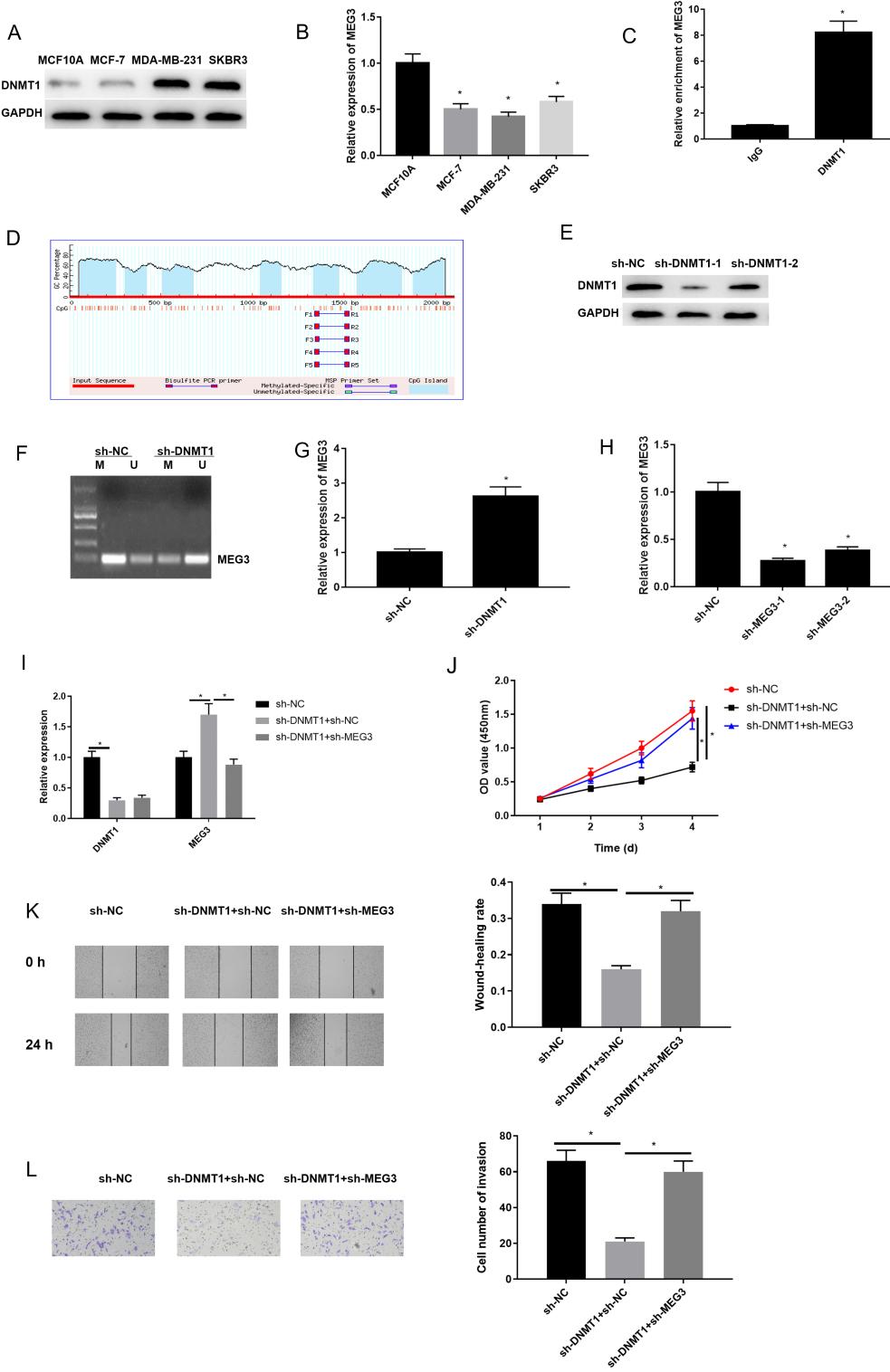
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## Tables

**Table 1 Primer sequences**

## Figures

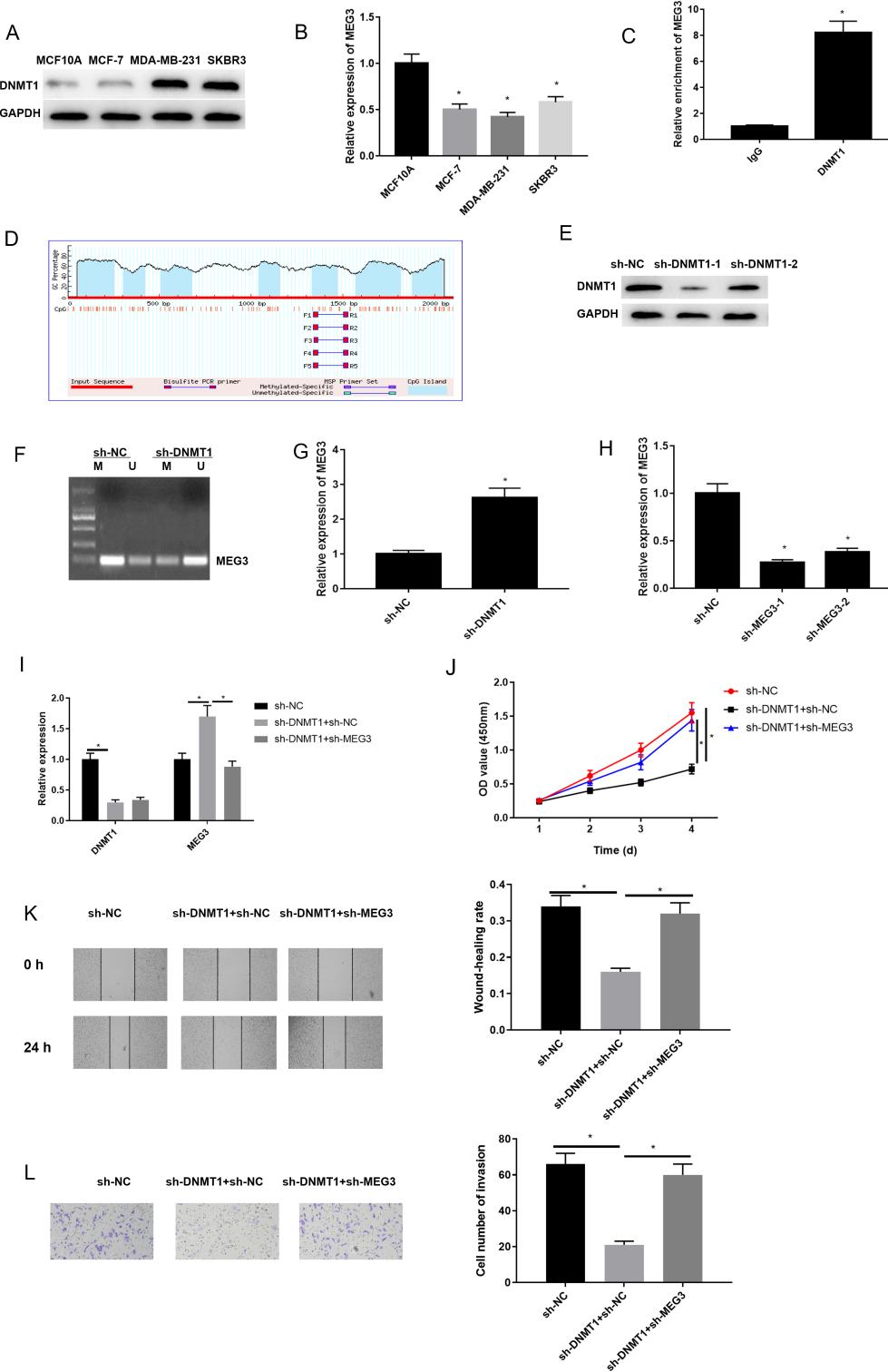
Genes	Primer sequences
miR-494-3p	F: 5'- GAAACATACACGGGAAAC C -3' R: 5'-GTGCAGGGTCCGAGG T-3'
U6	F:5'- CTCGCTTCG GCAGCACA-3' R:5'- AACGCTTCACGAATTGCT GT-3'
DNMT1	F: 5'- CGGCTTCAGCACCTCATTG-3' R: 5'- AGGTCGAGTCGGAATTGCTC-3'
MEG3	F: 5'- ATCATCCGTCCACCTCCTTGTCTTC-3' R: 5'- GTATGAGCATAGCAAAGGTAGGGC-3'
MSP-MEG3 (Methylation)	F: 5'- TATGAGTTGTAAGCGGTAGAGTTC-3' R: 5'- TACGAACCTAACGAAAAAAAATCAT-3'
MSP-MEG3 (Non-methylation)	F: 5'- GAATATGAGTTGTAAGTGGTAGAGTTT-3' R: 5'- TACAAACTAACAAAAAAAATCATACT-3'
OTUD4	F: 5'-TTCTGATGTGGATTACAGAGGGC-3' R: 5'- ACGCATGTTGTCTTACTCCTGA-3'
GAPDH	F: 5'-GAGTCAACGGATTGGTCGT-3' R: 5'- TTGATTTGGAGGGATCTCG-3'



**Figure 1**

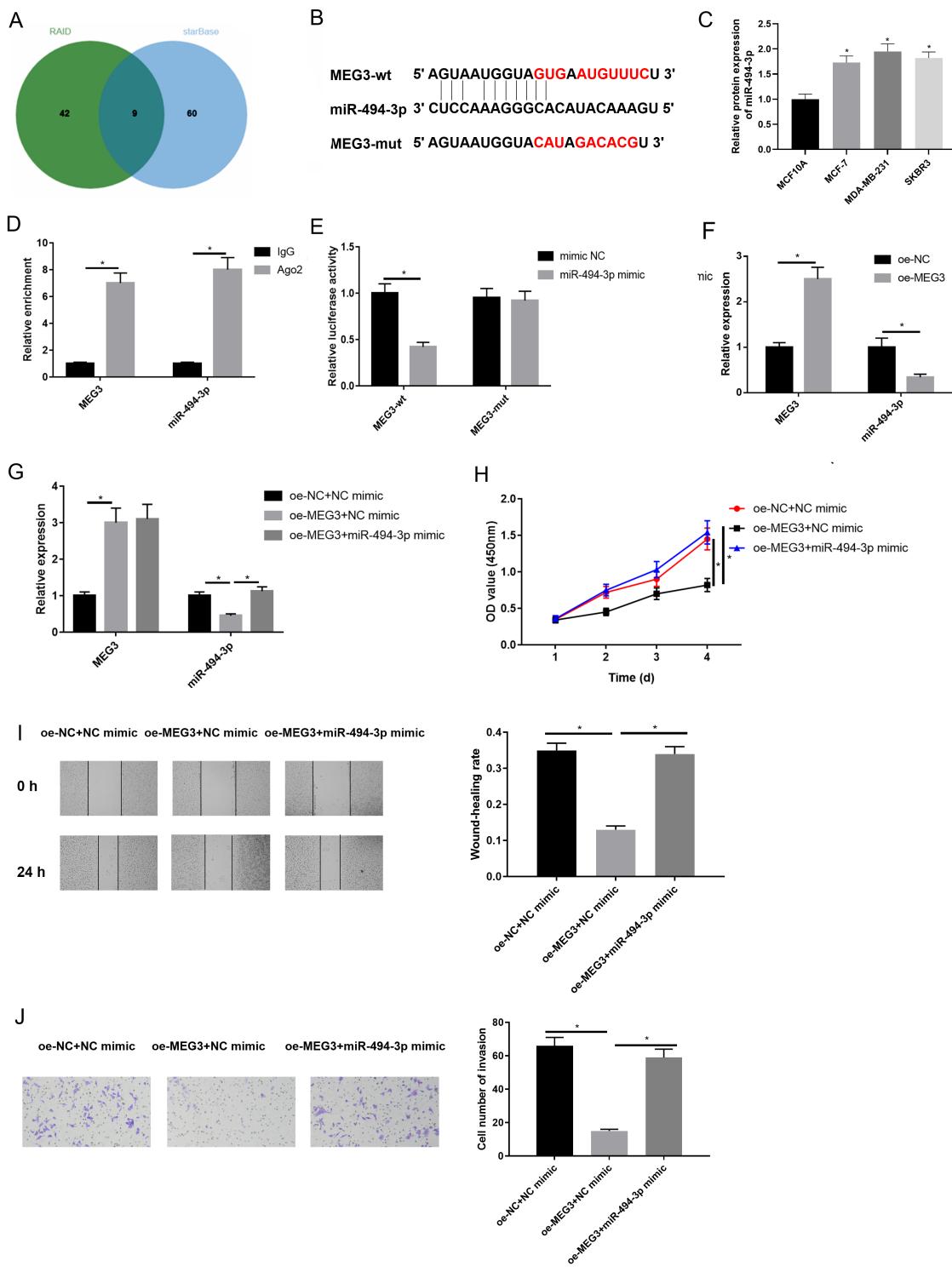
Silencing DNMT1 inhibits the proliferation, migration and invasion of breast cancer cells through promoting MEG3 expression. The expression levels of (A) DNMT1 and (B) MEG3 in human breast cancer cell lines (MCF-7, MDA-MB-231, SKBR3) and human normal breast epithelial cell line MCF10A were detected by western blot and qRT-PCR; (C) ChIP assay was used to determine whether DNMT1 could bind to the promoter region of MEG3; (D) MEG3 methylation was determined by MethPrimer software; (E)

Western blot was used to detect the interference efficiency of the shRNAs targeting DNMT1; (F) MEG3 methylation level was tested by MSP (U, unmethylated alleles; M, methylated alleles); (G) The expression of MEG3 was detected by qRT-PCR after DNMT1 was silenced; (H) Interference efficiency of the shRNAs targeting MEG3 was detected by qRT-PCR; (I) The expression levels of DNMT1 and MEG3 were detected by qRT-PCR in three groups (sh-NC, sh-DNMT1+sh-NC, sh-DNMT1+sh-MEG3); (J) Cell proliferation, (K) migration and (L) invasion were tested by CCK-8, wound healing and Transwell assays, respectively. Each experiment was carried out in triplicate; \* p<0.05.



## Figure 1

Silencing DNMT1 inhibits the proliferation, migration and invasion of breast cancer cells through promoting MEG3 expression. The expression levels of (A) DNMT1 and (B) MEG3 in human breast cancer cell lines (MCF-7, MDA-MB-231, SKBR3) and human normal breast epithelial cell line MCF10A were detected by western blot and qRT-PCR; (C) ChIP assay was used to determine whether DNMT1 could bind to the promoter region of MEG3; (D) MEG3 methylation was determined by MethPrimer software; (E) Western blot was used to detect the interference efficiency of the shRNAs targeting DNMT1; (F) MEG3 methylation level was tested by MSP (U, unmethylated alleles; M, methylated alleles); (G) The expression of MEG3 was detected by qRT-PCR after DNMT1 was silenced; (H) Interference efficiency of the shRNAs targeting MEG3 was detected by qRT-PCR; (I) The expression levels of DNMT1 and MEG3 were detected by qRT-PCR in three groups (sh-NC, sh-DNMT1+sh-NC, sh-DNMT1+sh-MEG3); (J) Cell proliferation, (K) migration and (L) invasion were tested by CCK-8, wound healing and Transwell assays, respectively. Each experiment was carried out in triplicate; \* p<0.05.



**Figure 2**

miR-494-3p is a target of MEG3 in breast cancer cells (A) Potential downstream miRNAs of MEG3 along with the (B) targeted binding sites of miR-494-3p on MEG3 were predicted by bioinformatics; (C) miR-494-3p expression levels in human breast cancer cell lines (MCF-7, MDA-MB-231, SKBR3) and human normal breast epithelial cell line MCF10A were detected by qRT-PCR; The binding relationship of MEG3 with miR-494-3p was verified by (D) RIP and (E) dual-luciferase assays; (F) The expression levels of MEG3 and

miR-494-3p in oe-NC group and oe-MEG3 group were detected by qRT-PCR; (G) The expression levels of MEG3 and miR-494-3p, (H) the cell proliferation, (I) migration and (J) invasion in oe-NC+NC mimic group, oe-MEG3+NC mimic group, and oe-MEG3+miR-494-3p mimic group were detected by qRT-PCR, CCK-8, wound healing and Transwell assays, respectively. Each experiment was carried out in triplicate; \* p<0.05.

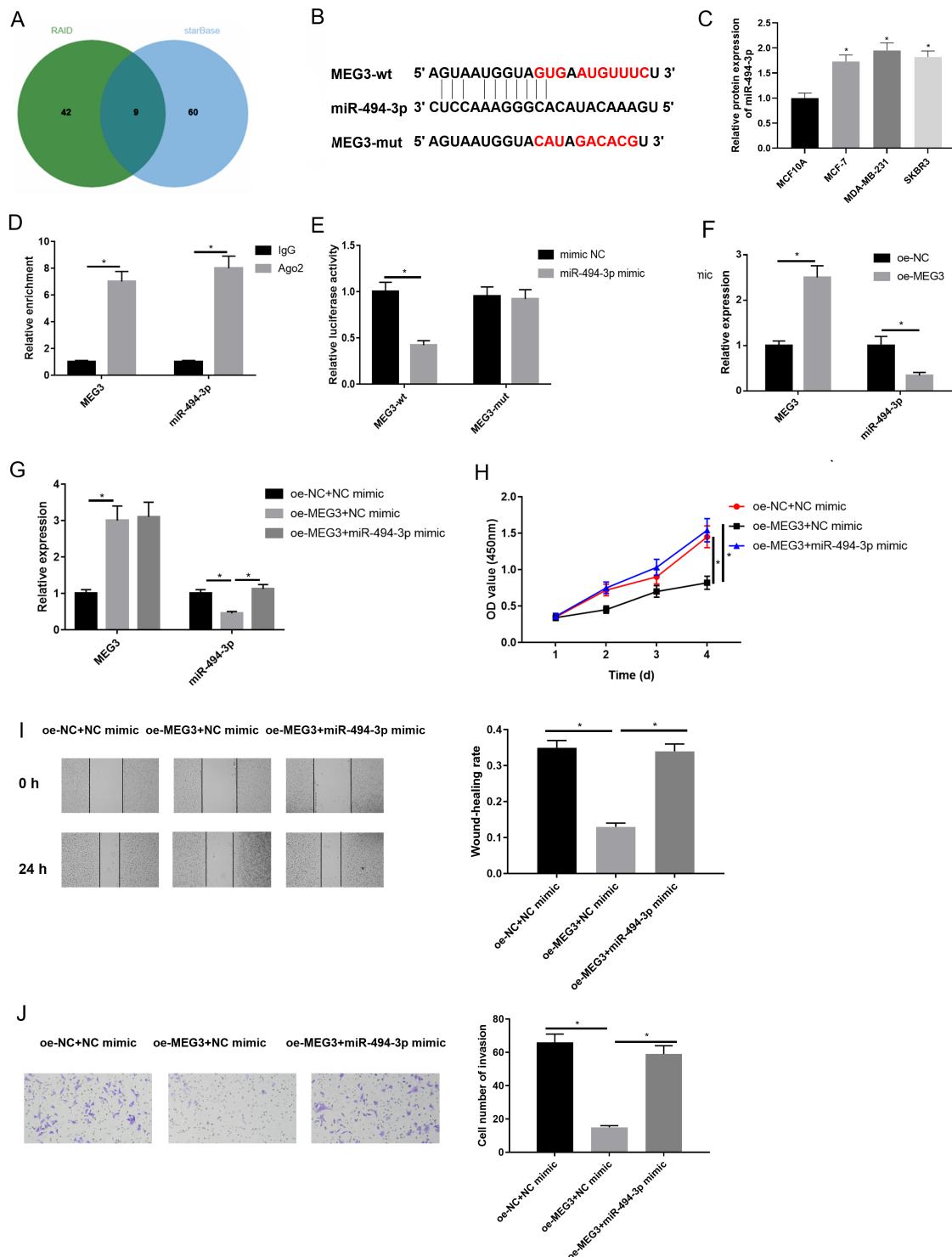
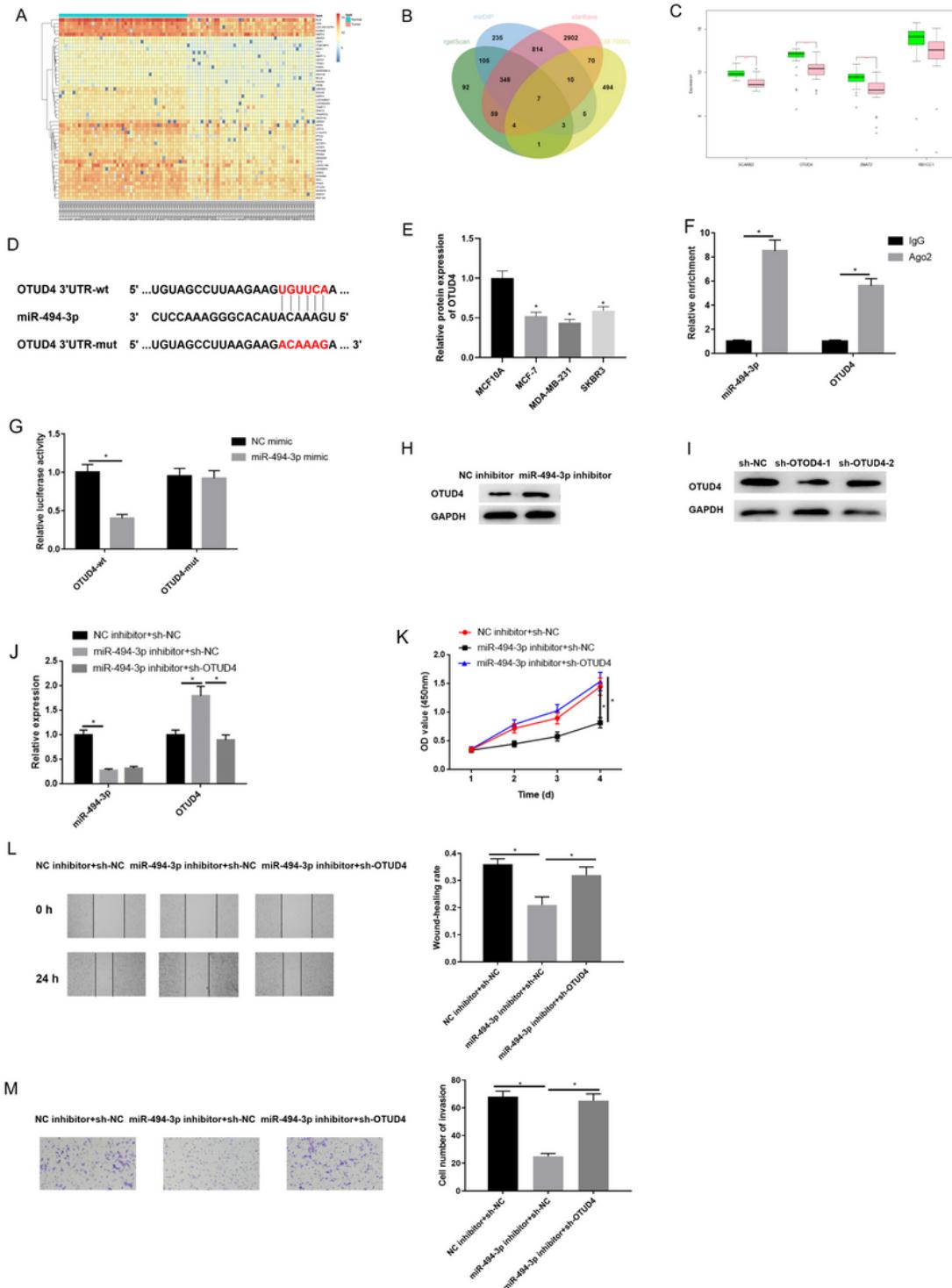


Figure 2

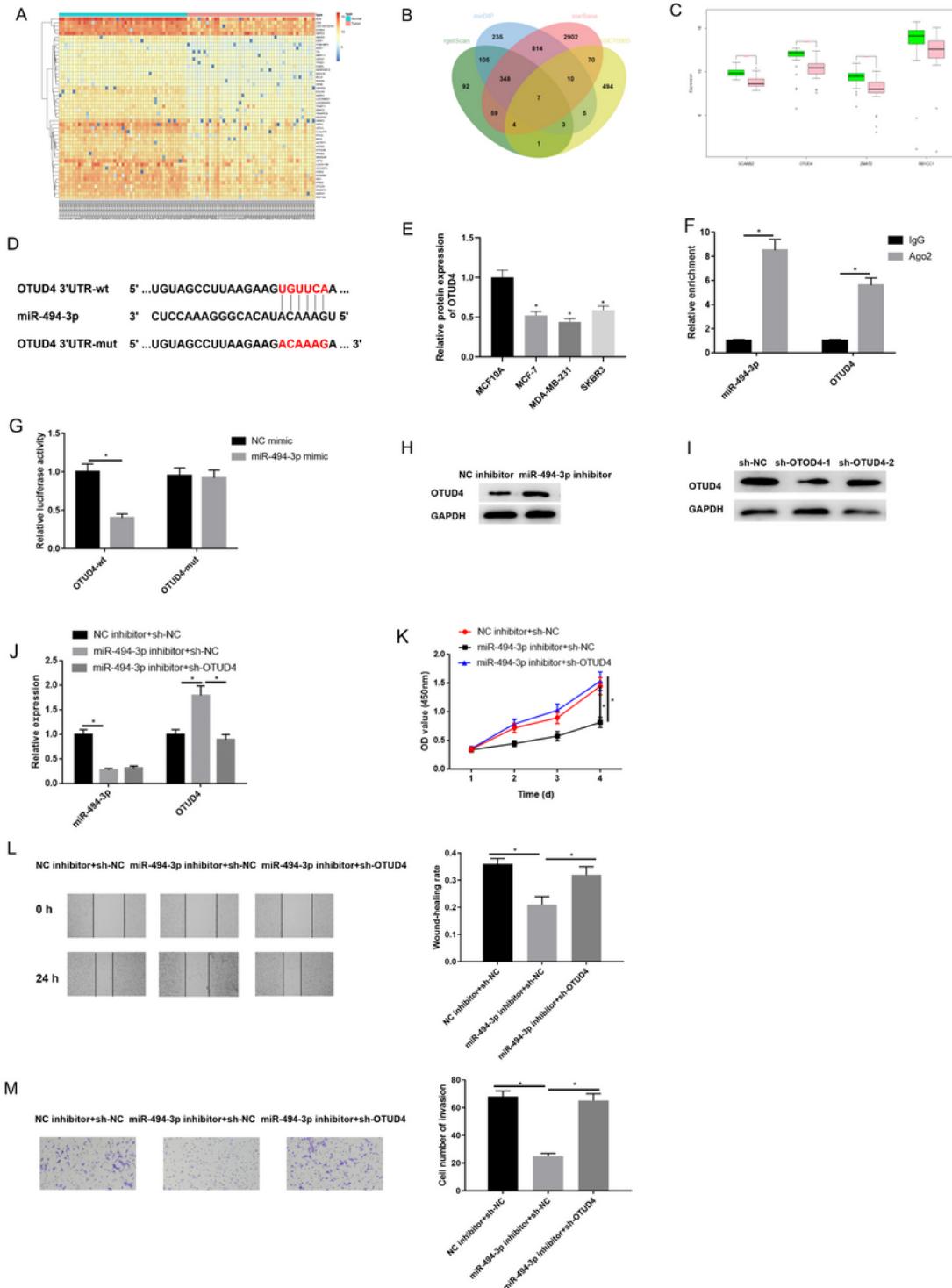
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**Figure 3**

Silencing miR-494-3p inhibits proliferation, migration and invasion of breast cancer cells by targeting and promoting OTUD4 expression (A) DEGs in GSE70905 dataset from GEO database were analyzed; (B) Venn diagram of DEGs and predicted target genes of miR-494-3p; (C) Differential expression of candidate genes in GSE70905 dataset; (D) The targeted binding sites of miR-494-3p on OTUD4 3'UTR were predicted by starBase database; (E) OTUD4 expression levels in human breast cancer cell lines (MCF-7, MDA-MB-

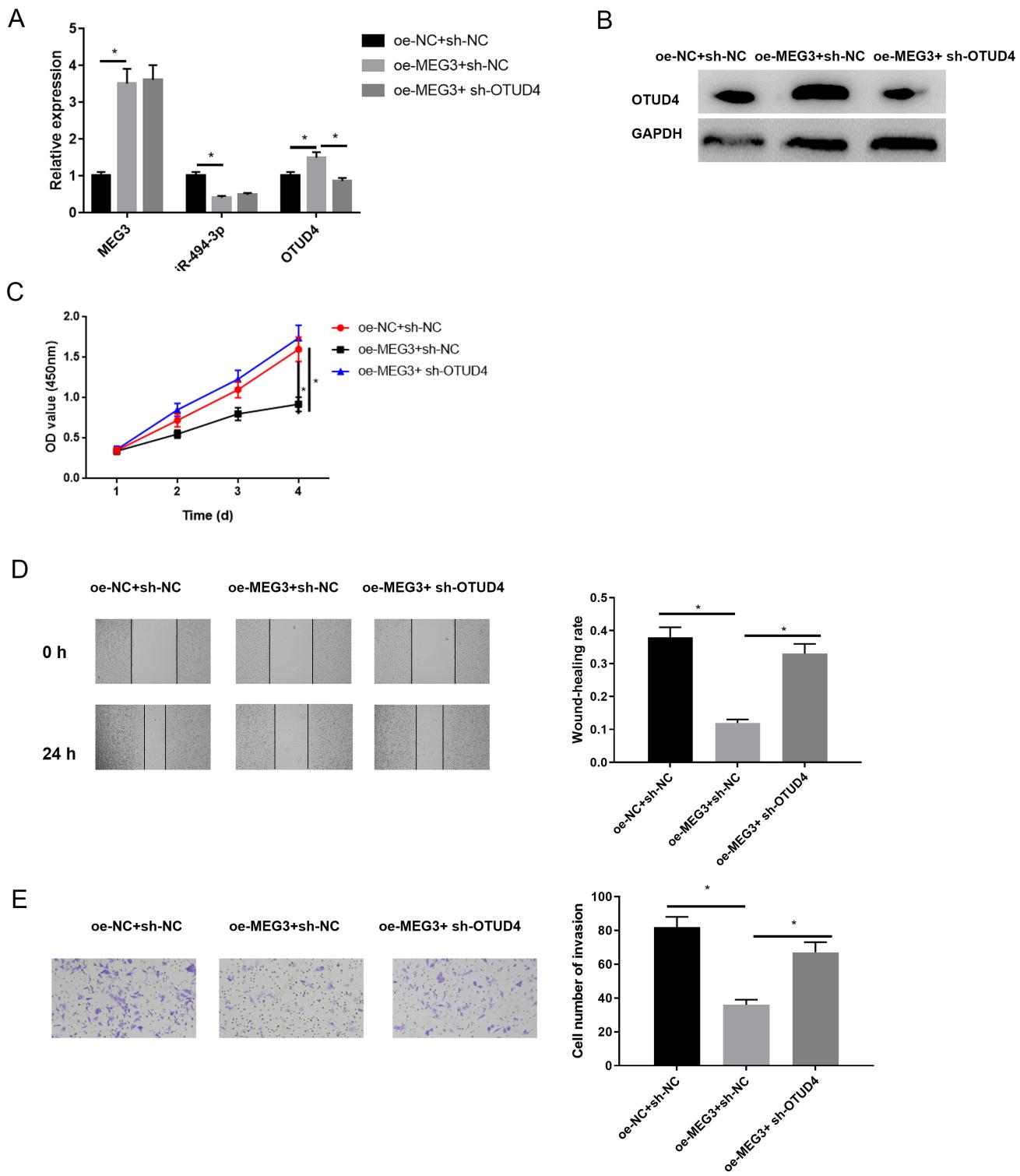
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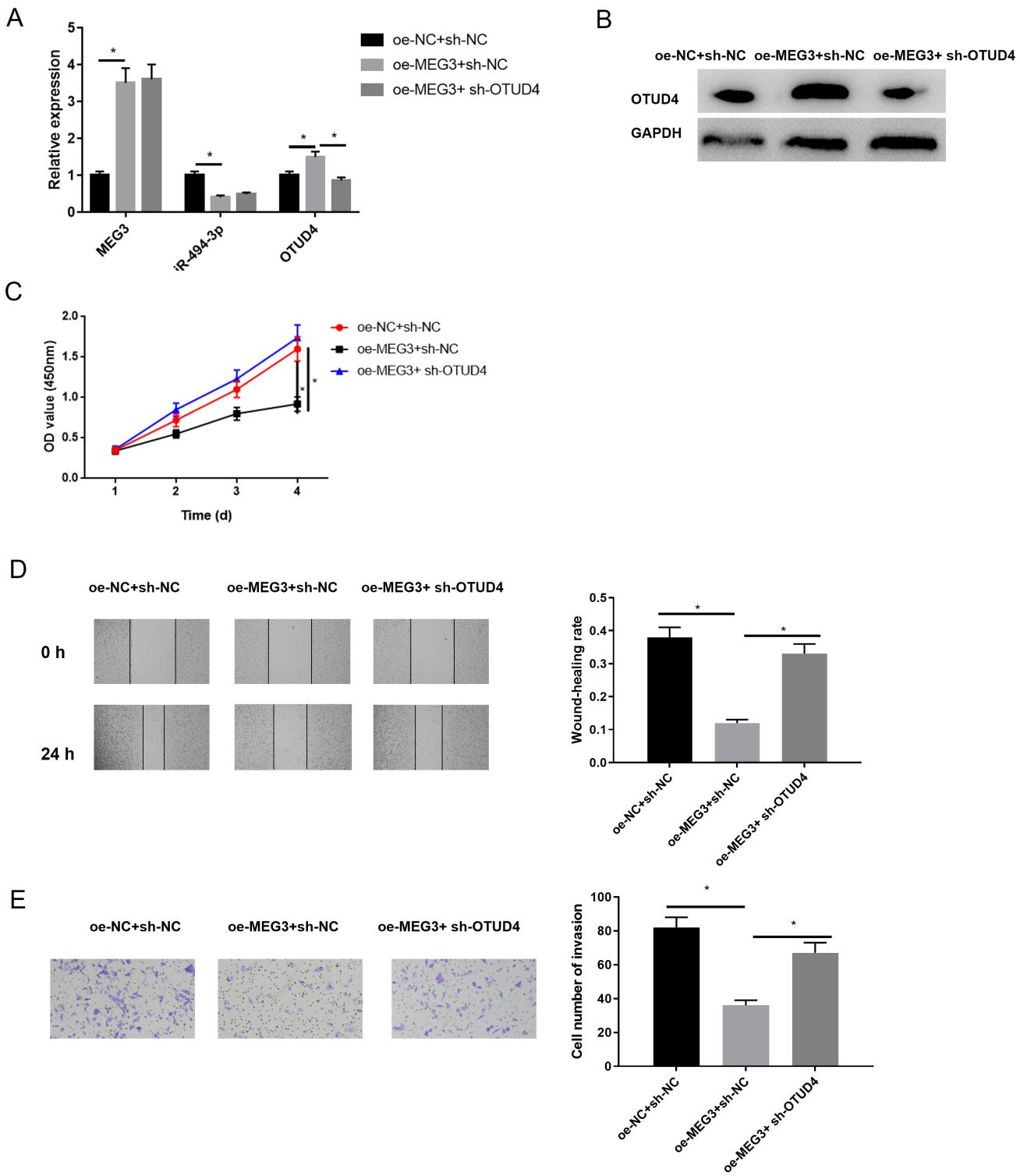
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**Figure 4**

MEG3 negatively regulates miR-494-3p to promote OTUD4 expression and inhibits the proliferation, migration and invasion of breast cancer cells (A) The expression levels of MEG3, miR-494-3p and OTUD4 in oe-NC+sh-NC, oe-MEG3+sh-NC and oe-MEG3+sh-OTUD4 groups were detected by qRT-PCR and (B) the protein expression of OTUD4 was tested by western blot; (C) Cell proliferation, (D) migration and (E)

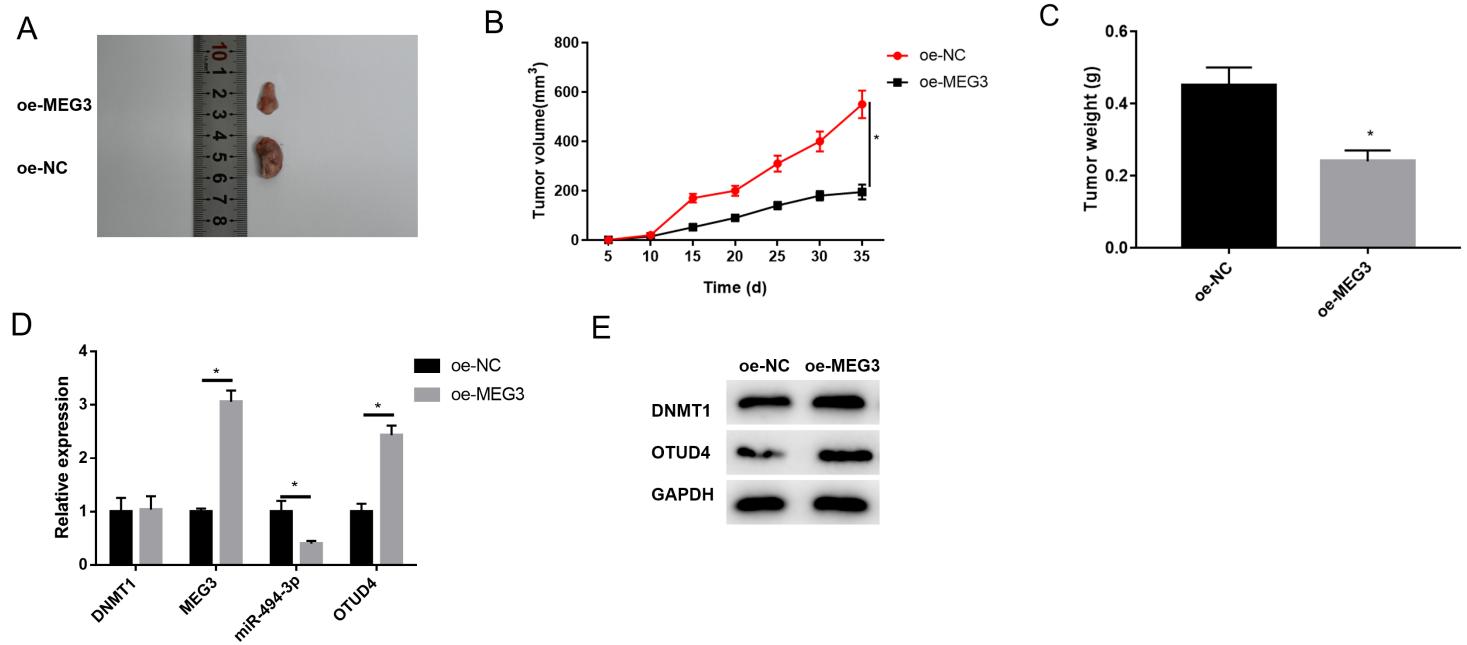
invasion were measured by CCK-8, wound healing and Transwell assays, respectively. Each experiment was carried out in triplicate; \* p<0.05.



**Figure 4**

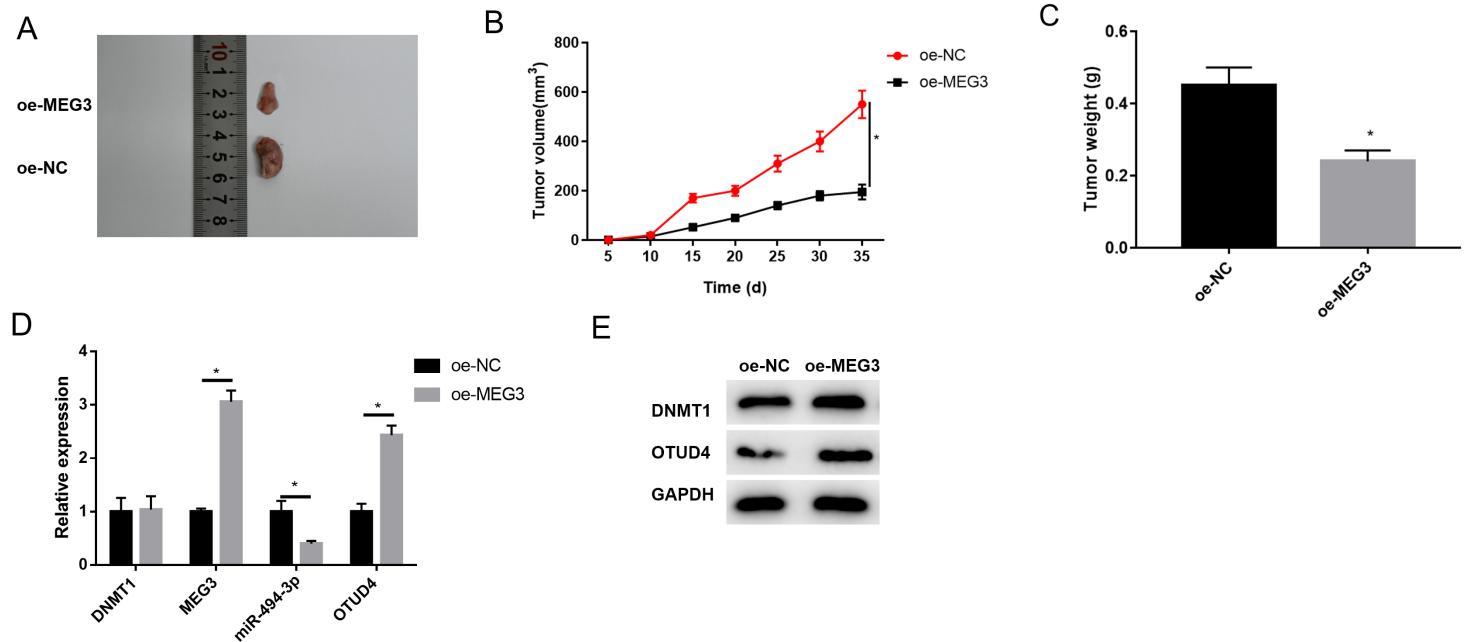
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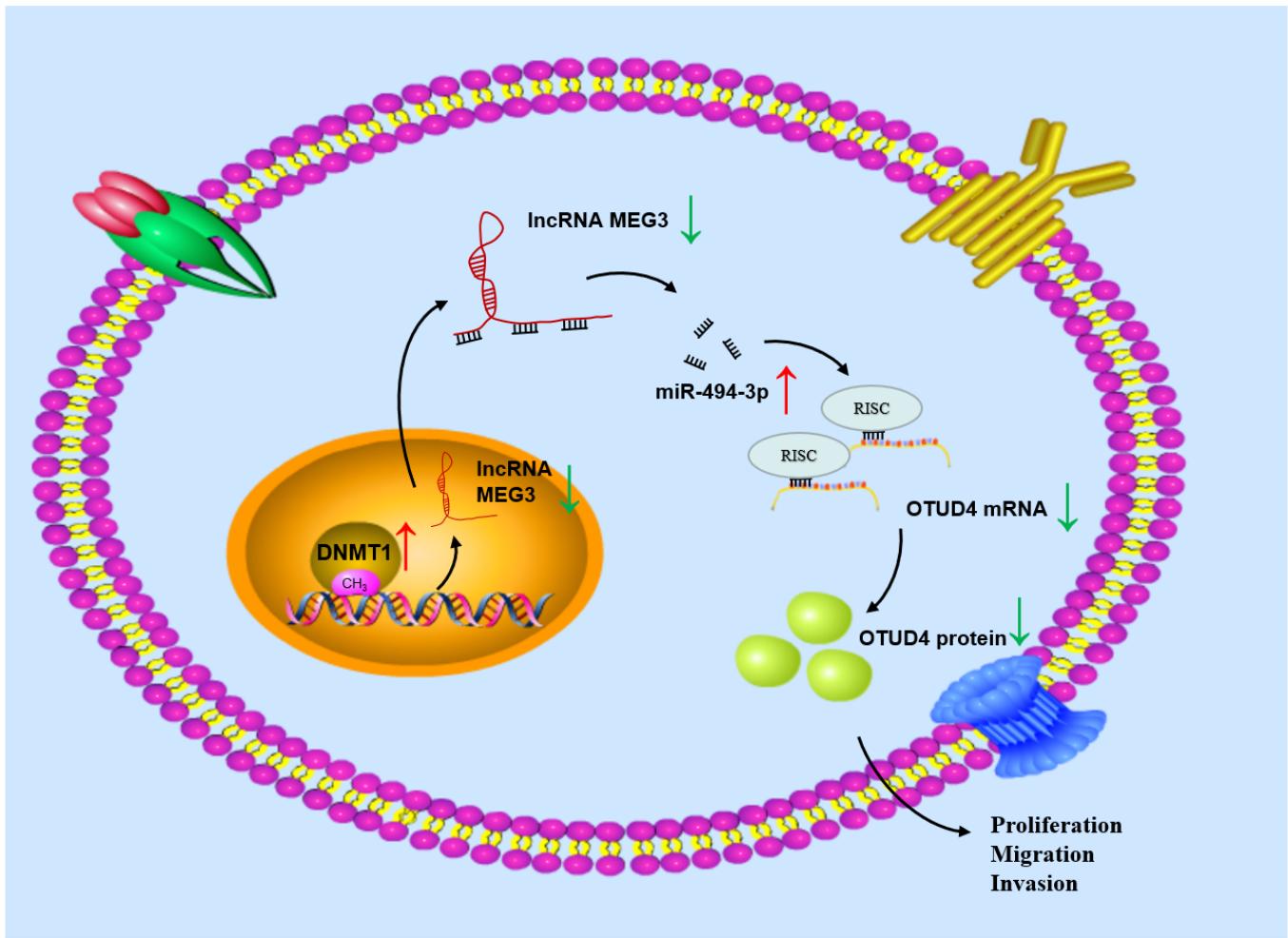
**Figure 5**

Silencing DNMT1 inhibits breast cancer cell tumorigenesis in vivo (A) Tumor stereogram, (B) tumor volume and (C) tumor weight of nude mice in each group were measured; (D) The expression levels of DNMT1, MEG3, miR-494-3p and OTUD4 upon MEG3 overexpression were detected by qRT-PCR and (E) the protein expression levels of DNMT1 and OTUD4 were tested by western blot. Each experiment was carried out in triplicate; \* p<0.05.



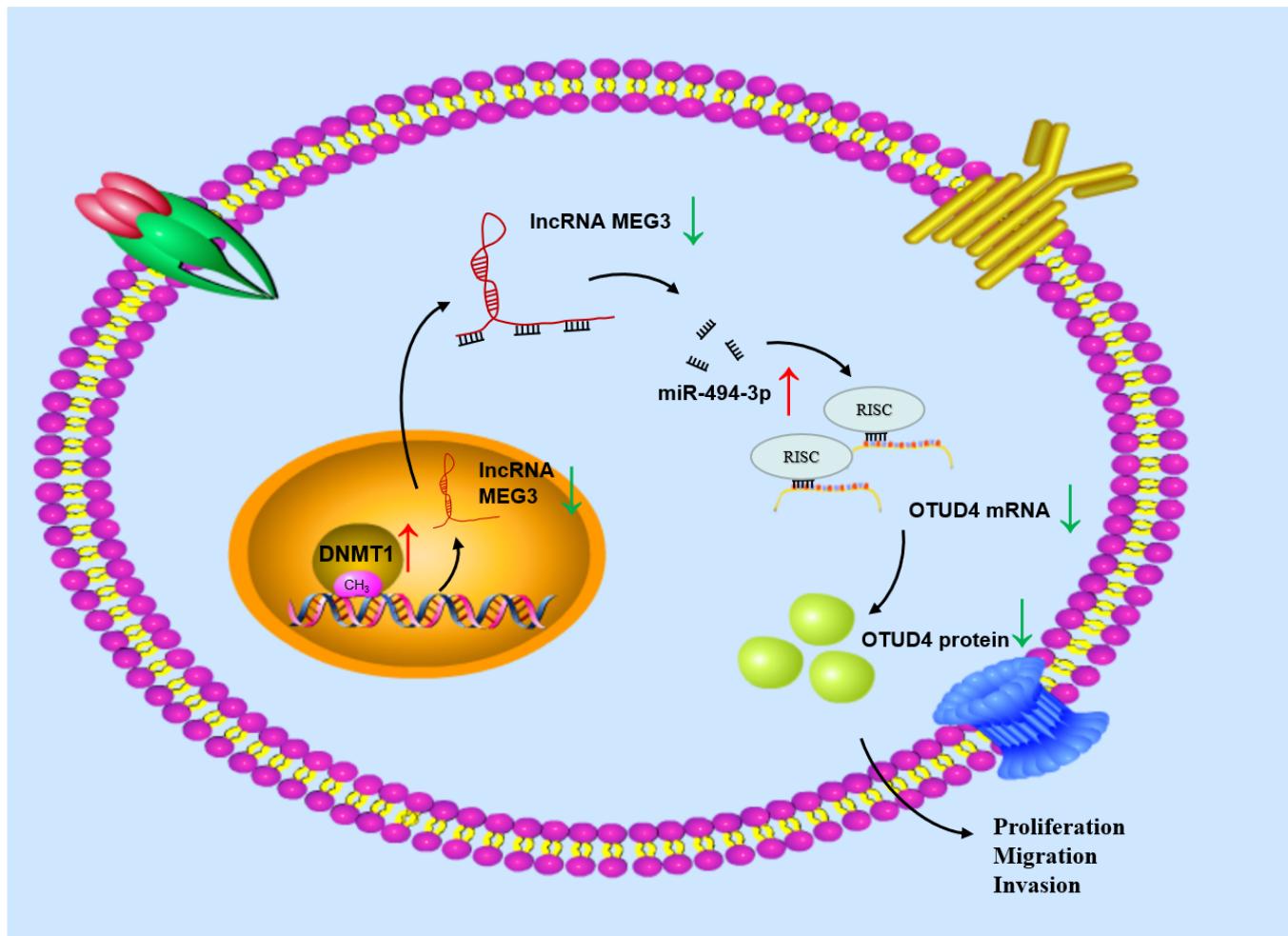
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**Figure 6**

The molecular mechanism of the DNMT1/MEG3/miR-494-3p/OTUD4 axis in breast cancer



**Figure 6**

The molecular mechanism of the DNMT1/MEG3/miR-494-3p/OTUD4 axis in breast cancer

## Supplementary Files

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