DNMT1-mediated demethylation of IncRNA MEG3 promoter suppressed breast cancer progression by repressing Notch1 signaling pathway

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ABSTRACT
Breast carcinoma is one of the common causes of cancer-related mortality in women. Maternally expressed gene 3 (MEG3), a IncRNA located at 14q32, can be involved in carcinogenesis. In this study, we discovered that MEG3 was downregulated by CpG hypermethylation within its gene promoter. Functionally, treatment of breast cancer cells with the DNA methylation inhibitor 5-AzadC as well as silencing of DNA methyltransferase-1 (DNMT1) could decrease the abnormal hypermethylation of the MEG3 promoter, reverse MEG3 expression, inhibit cell proliferation and promote cell apoptosis. In addition, we found that MEG3 expression was negatively correlated with DNMT1. Mechanistically, MEG3 knockdown combined with 5-AzadC or sh-DNMT1 treatment restored the expression of Notch1 receptor, leading to the Notch1 pathway activation, and promoted the progression of epithelial mesenchymal transformation (EMT). Finally, the mice tumor model experiments showed that DNMT1 knockdown can increase MEG3 expression and inhibit tumor growth. Collectively, our findings uncovered that DNMT1-mediated MEG3 demethylation leads to MEG3 upregulation, which in turn inhibits the Notch1 pathway and EMT process in breast cancer.

1. Introduction
Breast cancer remains a major health problem and is the most frequently diagnosed cancer in women [1]. It is also one of the most common malignant tumor in China [2]. Advances in early diagnosis and therapies have resulted in a 38% decrease in mortality of breast cancer patients, but almost all the patients who develop organ metastatic disease and high postoperative recurrence will succumb to it [3–5]. Therefore, there is a pivotal need for novel strategies, especially molecular targets, to improve the treatment of breast cancer.

Long non-coding RNAs (lncRNAs) are a class of transcripts more than 200 nucleotides in length with limited or no protein-coding ability [6]. Increasing evidence indicates that significant numbers of lncRNAs are involved in a variety of tumor biological processes such as proliferation, apoptosis, invasion and metastasis, etc. [7,8]. Aberrant expressions of lncRNAs including GAS5, NEAT1, ROPM, and MEG3 have been reported in breast cancer [9–12]. Among them, lncRNA MEG3 is an imprinted gene belonging to the imprinted DLK1-MEG3 locus located at chromosome 14q32.3 in humans [13]. The loss of MEG3 expression has been found in many human tumors [12,14]. Notably, it was reported that MEG3 is downregulated in breast cancer and plays a negative role in tumor cell biological processes [15,16]. Therefore, it has been hypothesized that MEG3 is a tumor suppressor gene. However, the specific mechanisms of unusually low expression of MEG3 in
breast cancer are still unclear. Previous studies indicated that MEG3 is downregulated by aberrant CpG methylation at its promoters in a variety of tumors such as esophageal cancer, hepatocellular carcinoma, etc., which is associated with the expression of DNA methyltransferases (DNMTs) [17,18]. It also suggested that DNMT1 overexpression may be responsible for the aberrant DNA methylation of tumor-related genes in gliomas [19]. Meanwhile, MEG3 overexpression inhibited breast cancer cell proliferation and invasion, suggesting that MEG3 expression may also be regulated by aberrant CpG methylation [15]. More importantly, treatment with a DNA methylation inhibitor, 5-aza-2-deoxycytidine (5-AzadC), resulted in re-expression of MEG3 in multiple human breast cancer tumor cell lines [20]. Collectively, DNA methylation-mediated epigenetic control of MEG3 may play an important role in the process of breast cancer. Therefore, in this study, we sought to investigate the expression and promoter methylation status of MEG3 as well as its function and molecular mechanisms in breast cancer.

2. Materials and methods

2.1. Patients and specimens

A total of 28 paired cancerous and matched para-cancerous tissues that were at least 2 cm away from the edge of the tumors were collected from patients by radical breast cancer resection in the First Affiliated Hospital of the University of Science and Technology (USTC) of China. The diagnosis of all the tumor tissues was confirmed by pathologists based on World Health Organization (WHO) classification. The data, including age, primary tumor size, lymph nodes metastasis, histological grade, estrogen receptor (ER) status, progesterone receptor (PR) status, HER-2 status, and subtype, were obtained from clinical and pathological records. The clinicopathological characteristics of all the breast cancer patients are outlined in Table 1. Fresh tumor samples were immediately frozen in liquid nitrogen, perfectly preserved at −80°C and stored until use. The present research was approved by the Ethics Committee of the USTC of China. Informed consent was signed by all of the enrolled patients.

2.2. Cell culture and transfection

The human breast tumor cell lines MCF-7 and MDA-MB-231 were a kind gift from Pro. Tao Zhu (USTC of China). The normal breast mammary epithelial cell line MCF-10A was obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). All cells are cultured in DMEM containing 10% fetal bovine serum (FBS, Gibco, USA), 100 U/mL penicillin and 50 μg/mL streptomycin, and maintained at 37°C incubator with 5% CO2. Small-interfering RNA oligonucleotides against DNMT1 gene (si-DNMT1), scrambled sequence (scnRNA), shRNA-DNMT1 (sh-DNMT1), shRNA-MEG3 (shMEG3) or control (shRNA-NC) were synthesized by GenePharma (Generay, Shanghai, China). Furthermore, MEG3 overexpression plasmid pcDNA3.1-MEG3 (pcDNA-MEG3) or control (pcDNA3.1 vector) was also synthesized by GenePharma. All transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer’s protocol. Thus, the cells were harvested for further evaluation at 48 h after transfection.

Table 1. Clinical characteristics of breast cancer patients.

| Clinicopathological Data | Number (n = 28) | Percentage (%) |
|--------------------------|-----------------|----------------|
| Age (ys)                 |                 |                |
| <50                      | 12              | 42.9           |
| ≥50                      | 16              | 57.1           |
| Primary tumor size (cm)  |                 |                |
| <3                       | 21              | 75             |
| ≥3                       | 7               | 25             |
| Lymph node metastasis    |                 |                |
| Present                  | 13              | 46.4           |
| Absent                   | 15              | 53.6           |
| Histopathological grade  |                 |                |
| II–III                   | 14              | 50             |
| III                      | 14              | 50             |
| ER status                |                 |                |
| Positive                 | 24              | 85.7           |
| Negative                 | 4               | 14.3           |
| PR status                |                 |                |
| Positive                 | 24              | 85.7           |
| Negative                 | 4               | 14.3           |
| HER-2 status             |                 |                |
| Positive                 | 10              | 35.7           |
| Negative                 | 18              | 64.3           |
2.3. Methylation analysis of MEG3 CpG islands by methylation-specific polymerase chain reaction (MSP) and bisulfite genomic sequencing (BGS)

Genomic DNA was extracted using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany). The MSP and BGS were performed as previously described [21]. The primers of the MEG3 gene for MSP that included one specific for the methylated sequence (MEG3-M, forward, 5’-TTCTGTATTTCGATGGATGTTC-3’; reverse, 5’-ATATATAAAAATCGCCCGAT-3’) and the other for the unmethylated sequence (MEG3-U, forward, 5’-TTGTATTTTTGATGATGTTTC-3’; reverse, 5’-CAATATATAAAAAATCGCCCGAT-3’). The primers of the MEG3 gene for BGS were used: forward, 5’-TTTAGTGGAGAGGAAGGGGTATAG-3’; reverse, 5’-CCACAAAAATAAATTCAAA CACAAT-3’.

2.4. Demethylation experiments using 5-aza-2’-deoxycytidine

Demethylation treatment with 5-AzadC (Sigma-Aldrich, St Louis, USA) was performed as previously reported [22].

2.5. Quantitative real-Time PCR

Total RNA was extracted from tissues and cells by using TRIzol reagent (Invitrogen, Carlsbad, USA). Reverse transcription was performed using a first-strand cDNA synthesis kit (Fermentas, USA) according to the manufacturer’s instructions. Quantitative real-time PCR analysis was conducted by using SYBR Green PCR master mix (QIAGEN, Germany) in an Applied Biosystem 7500 instrument (ABI, USA). The relative gene expression was calculated by using the 2^(-ΔΔCt) method and expressed as fold change relative to the respective control. All amplifications were normalized by GAPDH. Primers are listed in Supplementary Table 1.

2.6. Western blot analysis

Cells were lysed and harvested in RIPA lysis buffer (Beyotime, China). Total protein concentrations were quantified with a Bicin Choninic Acid (BCA) protein assay kit (Boster, Wuhan, China). Fifty micrograms of protein was separated by denaturing 8% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to PVDF membranes (Millipore Corporation, Billerica, USA). After blocking with 5% milk, membranes were incubated with the following antibodies overnight at 4°C: anti-DNMT-1, anti-Notch1, anti-HES1, anti-N-cadherin, anti-E-cadherin, anti-cyclinD1, anti-Vimentin and anti-Ki-67 (Cell Signaling Technology Inc., USA), anti-Bax (Zen BioScience, Chengdu, China), anti-Bcl-2 (Boster Biological Technology, Wuhan, China), anti-GAPDH (Santa Cruz Biotechnology Inc., Santa Cruz, CA) as a control. Anti-mouse IgG and anti-rabbit IgG horseradish peroxidase-conjugated antibodies were used as secondary antibodies correspondingly. The proteins were detected by chemiluminescence reagents (Model No. ChemiQ 4600, Bioshine).

2.7. Cell proliferation assay and colony formation assay

The cell proliferation capacity was assessed by using Cell Counting Kit-8 (CCK-8) and colony formation assay. At 0, 24, 48 and 72 h after siRNA transfection or 5-AzadC treatment, the CCK-8 solution was added to each well and incubated for 4 h. The absorbance was measured at 450 nm using a microplate reader SpectraMax i3x microplate (Molecular Devices, USA).

With respect to colony formation assay, cells were seeded in six-well plates after 72 h of siRNA transfection or 10 μM 5-AzadC treatment, and then cultured for 14 d in culture medium with 10% FBS as previously performed [9].

2.8. Apoptosis analysis

Seventy-two hours after the transfection or treatment as described above, quantification of apoptotic cells was measured by Annexin-V-FITC Apoptosis Detection Kit (Bestbio, China) according to the protocols. Analyses were conducted using a CytoFLEX flow cytometer (Beckman Coulter).
2.9. In vivo tumor model

BALB/c nude mice (aged 4–6 wk, weighing 18–22 g) were obtained from Slake Jingda Laboratory Animal Company (Hunan, China) and housed under pathogen-free conditions. The mice were randomly divided into two groups (n = 6/group). MDA-MB-231 cells with shRNA-DNMT1 or shRNA-NC were subcutaneously injected into the left armpits (200 µl) of nude mice, respectively. Tumor growth was measured every 5 d, and tumor volume was calculated with the equation: volume = 0.5 × width² × length. After 25 d, all mice were sacrificed and tumors were separated and photographed.

2.10. Statistical analysis

Data were expressed as the mean ± standard deviation and analyzed using SPSS 19.0 statistics software (SPSS Inc., USA). Significant differences were analyzed between two groups using Mann–Whitney U test or Student’s t test, and among several groups using one-way analysis of variance (ANOVA). The statistical significance was defined as *P < 0.05 or **P < 0.01. All experiments were performed in triplicate.

3. Results

3.1. LncRNA MEG3 was hypermethylated and downregulated in human breast cancer tissues and cells lines

We first detected the expression status of MEG3 in tumor tissues (n = 28) and adjacent tissues (n = 11) of breast cancer by qRT-PCR. The results showed that the expression of MEG3 was downregulated in tumor tissues than in normal tissues. Consistently, MEG3 was highly expressed in MCF10A cells, but greatly decreased in MCF-7 and MDA-MB-231 cell lines (Figure 1(a,b)). A typical CpG island (CGI) was identified in the MEG3 gene near exon 1 using the CGI Searcher (http://ccnt.hsc.usc.edu/cpgislands2) according to three parameters: Island size >200 bp, GC content >50%, and Obs CpG/Exp CpG >0.60 (Figure 1(c)), suggesting MEG3 was sensitive to methylation-mediated silencing. To explore whether the MEG3 silencing in breast cancer is related to promoter hypermethylation, we analyzed the methylation status of MEG3 CGI with MSP in tumor tissues. As expected, complete methylated status was demonstrated in tumor tissues. It showed that the methylation rate of the promoter region of MEG3 was 71.42% (20/28) in breast cancer tissues, and 18.18% (2/11) in 11 normal tissues (Figure 1(d)). The MSP results were further confirmed by BGS analysis of the methylation status of MEG3 in breast cancer and adjacent tissues (Figure 1(e,f)). The above results suggested that the promoter of MEG3 represented hypermethylation status in breast cancer.

In order to further ascertain downregulation of MEG3 is related to methylation, the breast cancer cells were treated with an inhibitor of DNA methylation 5-AzadC. As shown in Figure 2(a,b), after being treated with 10 µM 5-AzadC, the MEG3 expression was obviously upregulated, whereas DNMT1 expression was markedly downregulated, but there was no significance when treated with 2.5 µM or 5 µM 5-AzadC. Additionally, the promoter methylation of MEG3 in MCF-7 (Figure 2(c)) or MDA-MB-231 (Figure 2(d)) cells was reduced. These results revealed that the promoter methylation was responsible for the MEG3 downregulation in breast cancer cells.

3.2. Demethylation of MEG3 by 5-AzadC inhibits cell proliferation and induces apoptosis in breast cancer cells

To uncover the function of demethylation of MEG3 by 5-AzadC in breast cancer, the CCK8, colony formation, and apoptosis assays were performed. As described in Figure 3(a,b), the CCK8 assay indicated that the cell viability of MCF-7 and MDA-MB-231 cells was remarkably suppressed after treated with 10 µM 5-AzadC for 48 h and 72 h. Consistently, treatment with 10 µM 5-AzadC strikingly decreased colony formation ability of MCF-7 and MDA-MB-231 cells compared to the corresponding controls (Figure 3(c)). Furthermore, flow cytometry analysis observed that 10 µM 5-AzadC significantly induced MCF-7 and MDA-MB-231 cells apoptosis compared with the control group (Figure 3(d)). Overall, all of the data concluded that demethylation of the MEG3
promoter inhibited the proliferation and induced apoptosis of breast cancer cell lines.

3.3. **DNMT1 mediates hypermethylation of MEG3 in breast cancer cells**

To further investigate the possible effect of DNMT1 on the methylation of the MEG3 gene promoter, we evaluated the expression and function of DNMT1 in breast cancer. The results in Figure 4(a) demonstrated that the expression of DNMT1 was strongly increased in breast cancer tumor tissues (\( n = 28 \)) compared with that in adjacent tissues (\( n = 11 \)). Furthermore, the mRNA expression level of DNMT1 was negatively correlated with MEG3 level in breast cancer tissues (Figure 4(b)). Then, DNMT1 expression was downregulated in breast cancer cells by si-DNMT1 compared with their counterparts (Figure 4(c)). Further, the MSP assay showed that lowering of DNMT1 in MCF-7 and MDA-MB-231 cells reversed the MEG3 methylation and restored the MEG3 expression (Figure 4(d,e)).

Moreover, the data from CCK8 assay indicated that transfection with si-DNMT1 for 48 h and 72 h could reduce the cell viability of MCF-7 and MDA-MB-231 cells compared to its control sncRNA (Figure 5(a,b)). Subsequently, the colony...
formation assay observed that transfection with si-DNMT1 dramatically inhibited the colony formation ability in MCF-7 and MDA-MB-231 cells compared to scrRNA (Figure 5(c)). As described in Figure 5(d), it suggested that DNMT1 knockdown substantially increased the MCF-7 cells apoptosis by about 25% and MDA-MB-231 cells apoptosis by about 40% compared to their negative controls. Taken together, these data demonstrated that DNMT1 mediated hypermethylation of MEG3 expression levels, which positively correlated with breast cancer proliferation potentialities, suggesting that MEG3 was implicated in breast cancer progression.

3.4. Demethylation of MEG3 inhibits EMT and Notch1 signaling pathway in breast cancer cells

Next, we attempted to explore the underlying mechanisms of MEG3 methylation. As shown in Figure 6, compared with the controls, the mRNA expression levels of DNMT1, Notch1, HES1, N-cadherin, Vimentin, Cyclin D1 were significantly decreased, whereas E-cadherin was increased by treated with 10 µM 5-AzaC in MCF-7 and MDA-MB-231 cells (Figure 6(a)). In accordance with the aforementioned results, the protein expression levels of DNMT1, Notch1, HES1, N-cadherin, Vimentin, Cyclin D1 were decreased and E-cadherin was increased (Figure 6(c)). Meanwhile, as illustrated in Figure 6(b), DNMT1 silencing by transfected with si-DNMT1 notably declined the mRNA and protein expression levels of DNMT1, Notch1, HES1, N-cadherin, Vimentin, Cyclin D1, while enhanced the levels of E-cadherin in breast cancer cells compared to negative control (Figure 6(b–d))

Further, the expression level of MEG3 was overexpressed in the pcDNA-MEG3, sh-DNMT1 or 5-AzaC group relative to corresponding controls in the MDA-MB-231 cells (Figure 6(e,f)). More interesting, when MDA-MB-231 cells transfected with sh-MEG3 and then co-treated with 5-AzaC, we found that MEG3 expression level was obviously decreased (Figure 6(e)). Similar results were obtained after being co-treated with sh-DNMT1 (figure 6(f)). As described in Figure 6(g,h), the data revealed that compared to relevant controls, the protein expression levels of DNMT1, Notch1, HES1, N-cadherin, Vimentin, and Cyclin D1 were
Figure 3. 5-AzadC treatment inhibits cell proliferation and induces cells apoptosis in breast cancer cells. (a and b) Cells were treated with different concentrations of 5-AzadC (2.5, 5 or 10 μM). The cell viability was measured by CCK8 in MCF-7 and MDA-MB-231 cells. (c) Cells were treated with 10 μM 5-AzadC. The colony forming ability was detected by colony forming assay. (d) Cells were treated with 10 μM 5-AzadC. The cell apoptosis was detected by flow cytometry. Data are shown as the mean ± SD (n = 3). *P < 0.05, **P < 0.01 vs. respective control.

significantly decreased whereas E-cadherin distinctly increased in MDA-MB-231 cells transfected with pcDNA-MEG3, sh-DNMT1 or 5-AzadC. Moreover, the results were consistent with the previous findings after being treated with sh-MEG3 + 5-AzadC or sh-MEG3+ sh-DNMT1. The protein expression levels of DNMT1, Notch1, HES1, N-cadherin, E-cadherin, Vimentin, and Cyclin D1 were apparently reversed (Figure 6(g,h)). Remarkably, we found evidence that DMNT1-mediated epigenetic silencing of MEG3 led to activation of the Notch1 signaling pathway and promoted the occurrence of EMT in breast cancer cells.
3.5 Downregulation of DNMT1 inhibits tumor growth in nude mice

Nude mice xenografts model was applied to further assess the role of DNMT1 in tumorigenicity of breast cancer cells. As shown in Figure 7(a, b), the average volume and weight of the tumors were significantly lower in the sh-DNMT1 group than that in shRNA-NC. Consistent with previous results, we confirmed that the expression of MEG3 was upregulated in the sh-DNMT1 group compared with shRNA-NC (Figure 7(c)). In addition, downregulated DNMT1 suppressed the expression levels of Ki-67, Bcl-2 and increased the expression level of Bax (Figure 7(d)). Collectively, these data together demonstrated that downregulation of DNMT1 decreased MEG3 level, induced apoptosis and inhibited tumor growth in vivo.

4. Discussion

Despite the clinical development of screening and treatment, metastatic breast cancer is one of the leading causes of cancer-related death among women [23–25]. According to the cancer statistics estimates, about 266,120 new breast cancer cases and 40,920 breast cancer deaths occurred in 2018 [26]. Accumulating data support a key role for the targeted therapies in treatment of malignant tumor cells by interfering with the transduction of key signaling pathways or inhibiting the function of tumor-specific molecules, thus improving the survival of cancer patients significantly [27].

Recently, the explosion of clinical and experimental data on tumor-related lncRNAs have ushered new promises. Dysregulation of lncRNAs have been reported to play key roles in proliferation, invasion and metastasis of tumor cells [28–30]. LncRNA maternally expressed gene 3 (MEG3) was downregulated in a variety of human tumors, such as breast cancer, cervical cancer, gastric cancer, and colorectal cancer [31–34]. In addition, Zhang et al. [35] showed that upregulation of MEG3 inhibited cell proliferation, colony formation and promoted cell apoptosis in MDA-MB-231
Figure 5. DNMT1 knockdown inhibits cell proliferation and induced cells apoptosis in breast cancer cells. Cells were transfected with si-DNMT1 or snRNA. (a and b) The cell viability was measured by CCK8 in MCF-7 and MDA-MB-231 cells. (c) The colony forming ability was detected by colony forming assay. (d) The cell apoptosis was detected by flow cytometry. Data are shown as the mean ± SD (n = 3). *P < 0.05, **P < 0.01 vs. respective control.
cells. Consistently, we showed the similar result that MEG3 was excellently decreased in breast cancer tissues and breast cancer cell lines. Overexpression of MEG3 inhibits breast cancer cell proliferation and promotes cell apoptosis in vitro. All these data indicated that MEG3 serves as a tumor suppressor in breast cancer.

Epigenetic gene silencing of tumor suppressor genes is associated with promoter CpG island (CGI) hypermethylation, which is considered to be an early event during tumor development [36–38]. Previous studies have shown that aberrant DNA methylation resulted in the proliferation of malignant tumors mainly by silencing of tumor
suppressor genes and influencing their associated transcriptional pathways [39]. Mounting evidences reported that MEG3 promoter hypermethylation can lead to MEG3 expression loss in pituitary adenomas, gliomas, cervical cancer and hepatoma [12,20,40,41]. Based on these, we proposed a hypothesis that MEG3 expression loss may be connected with the methylation of promoter region in breast cancer. In this study, we showed that MEG3 was epigenetically methylated in primary breast tissues and cells, and remained unmethylated in normal breast tissues and cells. The reduced MEG3 expression was closely associated with promoter methylation. As we know, treatment with 5-AzadC can lead to genome-wide hypomethylation and thus inhibit cancer cell growth [42], which was expected to be a promising new-generation treatment regime for multiple types of cancer [43]. Additionally, a recent study by Liu et al. [44] showed that 5-AzadC increased MEG3 expression by inhibiting DNA hypermethylation, which suppressed the proliferation, migration, invasion and induced apoptosis in human hepatoma cells. We also affirmed that 5-AzadC incubation can diminish aberrant hypermethylation of MEG3 and inhibit
the proliferation and promote apoptosis. These results strongly indicated that hyper-methylation of the MEG3 promoter contributed to the down-regulation of MEG3 and supported an epigenetic mechanism of regulation of MEG3 expression in breast cancer.

In mammals, DNA methyltransferases (DNMTs) are enzymes that catalyze the methylation of mammalian genomic [45], including three major DNMTs: DNMT1, DNMT3a and DNMT3b [46]. In epigenetic events, DNMTs are responsible for the transfer of methyl groups from the universal methyl donor s-adenosine-lmethionine to the 5-position of DNA cytosine residues [47]. The increasing data supported that DNMT1 was frequently up-regulated in various human cancers [12,48,49]. For instance, long non-coding RNA-HAGLR suppressed tumor growth of lung adenocarcinoma through recruiting DNMT1 on E2F1 promoter to increase local DNA methylation [50]. Moreover, loss of DNMT1 inhibited the proliferation, migration and invasion of esophageal squamous cell carcinoma cells by suppressing the methylation of tumor suppressor gene promoter RASSF1A and death-related protein kinase [51]. Simultaneously, Kruer et al. [52] revealed that activation of pRb decreased expression of DNMT1, thus increasing MEG3 expression in lung A549 and SK-MES-1 cancer cells. In this study, we confirmed that DNMT1 was up-regulated in breast cancer tissues, and DNMT1 expression was negatively correlated with the MEG3 expression. Knockdown of DNMT1 strongly reversed the methylation of MEG3 promoter and subsequently promoted MEG3 expression, accompanied by inhibiting cells proliferation and accelerating the induction of cells apoptosis. Therefore, our result implied that inhibition of DNMT1 promoted the expression of tumor suppressor MEG3 and influenced the occurrence and development of breast cancer.

Notch signaling is a highly conservative signal regulated many physiological events, including cell fate determination, tissue maturity, tumor progression, and EMT [53]. Recently, Suman et al. [54] found that Notch1 activation or silence was critical for the proliferation, migration, and invasion of breast cancer cells. Moreover, Rui et al. [55] showed that both proliferation and invasion of breast cancer cells can be significantly weakened by reducing the expression of Notch1. Interesting, activation of

Figure 7. Downregulated DNMT1 inhibits tumor growth in vivo. MDA-MB-231 cells infected sh-DNMT1 or shRNA-NC were subcutaneously injected into the nude mice. (a) The tumor volume was measured every 5 d for 25 d. (b) The representative photographs and average weights of dissected tumors. (c) The expression of MEG3 in resected tumor masses was detected by qRT-PCR. (d) The protein expressions of Ki67, Bcl-2 and Bax in resected tumor masses were detected by western blot analysis. Data are shown as the mean ± SD (n = 3). *P < 0.05, **P < 0.01 vs. respective control.
Notch1 also promoted breast cancer progression by activating the EMT [56]. A research conducted by Zhang et al. [57] suggested that MEG3 inhibits EMT process through sponging miR-421 in breast cancer. In this study, we found that treatment with DNA methylation inhibitor 5-AzadC or sh-DNMT1 alone inhibited the expression of HES1, cyclin D1, N-cadherin, Vimentin and Notch1, whereas increased expression of E-cadherin in breast cancer cells. Of note, shMEG3 combined with 5-AzadC or sh-DNMT1 treatment was able to reverse these proteins levels. It provided sufficient evidence that epigenetic silencing of MEG3 mediated by DNMT1 led to the activation of Notch1 signaling pathway and subsequently regulated the growth of breast cancer cells. Likewise, our study provided noteworthy evidence that the EMT process was closely related to Notch1 signaling in breast cancer cells. Moreover, the in vivo results strongly proved that DNMT1-mediated demethylation of MEG3 inhibited breast cancer tumorigenesis.

In conclusion, our observations imply that MEG3 exerts its tumor suppressor activity by inhibiting cell proliferation and colony formation, inducing apoptosis, and suppressing EMT; this may be partly through DNMT1, which controls demethylation of MEG3 promoter and inhibits Notch1 signaling pathway. Notably, the expression of MEG3 can be restored by epigenetic modification, which implicates that MEG3 is a candidate tumor suppressor gene related to the pathogenesis and progression of human breast cancer.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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