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UXT, a novel DNMT3b-binding protein, promotes breast cancer progression via negatively modulating lncRNA MEG3/p53 axis

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Overexpressed ubiquitously expressed transcript (UXT) in breast tumors and derived cell lines modulated the transcriptional activity of estrogen receptor alpha. However, how UXT exerts its biological functions in the tumorigenicity of breast cancer remains largely unknown. Expressions of UXT and maternally expressed gene 3 (MEG3) were examined by qRT-PCR and Western blot. The capacity of cell proliferation, apoptosis, migration, and invasion was assessed using CCK-8, flow cytometry, and transwell assays. Methylation-specific PCR (MS-PCR) was employed to evaluate the methylation of the MEG3 imprinting control region. Co-immunoprecipitation was performed to verify the UXT/DNMT3b interaction. RNA immunoprecipitation (RIP) was subjected to assess the regulation of MEG3 on p53 activity. A xenograft tumor model was further conducted to certify the molecular mechanism. UXT was upregulated, while MEG3 was downregulated in breast cancer tissues and cell lines. UXT knockdown or MEG3 overexpression inhibited cell proliferation, promoted apoptosis, and weakened cell migration and invasion. Hypermethylation of the MEG3 imprinting control region was modulated by highly expressed DNMT3b. UXT inhibited MEG3 expression via recruiting DNMT3b to its imprinting control region. MEG3 positively regulated p53 activity. UXT negatively regulated the MEG3/p53 axis in a DNMT3b-dependent manner to promote tumor growth. UXT, a novel DNMT3b-binding protein, aggravates the progression of breast cancer through MEG3/p53 axis.

INTRODUCTION

In recent decades, cancers have been regarded as the leading cause of human death, which were traditionally recognized as genetic disorders. Although it is increasingly apparent that epigenetic alterations, such as microRNA dysregulation, DNA methylation, and histone modifications, have been found to play crucial roles in the development and progression of cancers, we are still unable to exactly explain the pathogenesis of cancers. Emerging evidence showed that several epigenetic modifier genes exert crucial functions participating in malignant tumor transformation and progression.[1] Therefore, it is urgent to obtain a better understanding of genetic and epigenetic alterations in the development of cancers for identifying new therapeutic targets in cancer treatment.

Ubiquitously expressed transcript (UXT), a putative member of an α-class prefoldin protein family, was first discovered by Andreas Schroer in 1998.[2,3] Located in Xp11.23-p11.22, the UXT gene is composed of seven exons and encodes a protein of 157 amino acids.[3] UXT is ubiquitously expressed and predominantly localizes in the nucleus.[4] It has been reported that UXT is markedly elevated in some human tumor tissues including bladder, breast, ovary, and thyroid, but not in the matching normal.[3] Abrogation of UXT protein expression by small interfering RNA leads to human osteoblast sarcoma U2 (U2OS) cell death.[5] Besides, UXT also suppresses cell transformation via interacting with survival stimulatory factors, such as Evi1,[6] and acts as an oncogene of sarcoma, promoting cell proliferation *in vitro* and tumor progression *in vivo*.[5] All these findings indicated that UXT might be a novel target against tumors. Nevertheless, further research remains to be carried out to explore the underlying mechanisms and regulatory networks of UXT.

Long noncoding RNAs (lncRNAs) are a heterogeneous group of noncoding transcripts longer than 200 nucleotides.[7] Abnormal expression of lncRNAs has been demonstrated to be involved in cancer tumorigenesis, development, and progression.[8–10] Maternally expressed gene 3 (MEG3), located on human chromosome 14q32, constitutes the imprinted domain reciprocally imprinted with the paternally expressed gene DLK1.[11] It is a lncRNA, with a length of ~1.6- kb nucleotides, and expresses in many human normal tissues.[12] However, the expression of MEG3 is lost or decreased in various tumors, such as meningioma, colon cancer, nasopharyngeal carcinoma, and leukemia,[13,14] implying its anti-tumor functions.

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Table 1. Association between UXT and clinicopathological characteristics of breast cancer

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Variables	Cases (n = 56)	Low (n = 28)	High (n = 28)	p Value
Age (years)				**0.296**
<50	25	15 (48%)	10 (52%)	
≥50	31	14 (45.2%)	17 (54.8%)	
Menopause				**0.399**
No	38	18 (47.4%)	20 (52.6%)	
Yes	18	11 (50%)	7 (50%)	
Tumor size				**0.015***
≤2.0 cm	25	17 (68%)	8 (32%)	
> 2.0 cm	31	10 (32.3%)	21 (67.7%)	
Lymph node metastasis				**0.031***
No	26	17 (65.4%)	9 (34.6%)	
Yes	30	10 (33.3%)	20 (66.7%)	
TNM stage				**0.006***
I-II	21	15 (71.4%)	6 (328.6%)	
III-IV	35	11 (31.4%)	24 (68.6%)	

*$p < 0.05$, statistically significant.

p53, a star of tumor suppressor gene, responds to diverse stress stimulation to prevent tumor development and avoid unnecessary pathological consequences.[15,16] In tumors, p53 is frequently mutated or lost.[17] Our previous study demonstrated that UXT binds to MDMX and suppresses the basal activity of p53, thereby inducing glycolysis by activated NF-κB in sarcoma.[5] Zhu et al. also found that ectopic expression of MEG3 inhibits hepatoma cell proliferation and induces apoptosis by interacting with p53 protein.[18] Therefore, both UXT and MEG3 participated in the regulation of p53 activity. However, whether there is an interaction between UXT and MEG3 remains unknown.

In the present study, we investigated the role of UXT and its potential mechanisms in the tumorigenesis of breast cancer. We found that UXT was upregulated, whereas MEG3 was downregulated in both breast cancer tissues and cell lines. Further mechanistic experiments revealed that UXT modulated the expression of MEG3 through methylation of the MEG3 imprinting control region via directly binding to DNMT3b, in turn inhibiting apoptosis, enhancing migration and invasion, and accelerating tumor growth, which provides a basis for the development strategies against cancers based on UXT, MEG3, and p53.

RESULTS

Upregulation of UXT and downregulation of MEG3 were observed in breast cancer

To investigate the expression of UXT and MEG3, we collected breast tumor tissues and their adjacent tissues from 13 cases of patients and conducted the following experiments. The clinical characteristics of tissue samples are shown Table 1. As shown in Figure 1A, the positive expression of UXT in tumor tissues was significantly stronger than adjacent tissues. The data of qRT-PCR and Western blot analysis also displayed that UXT was highly expressed, while MEG3 was low expressed in breast tumor tissues compared with adjacent normal tissues (Figures 1B–1D). In addition, higher expression of UXT or lower expression of MEG3 further indicated the poor prognosis of breast cancer patients (Figure 1E). Moreover, there was a negative correlation between UXT and MEG3 expression (Figure 1F). In parallel, we also examined the levels of UXT and MEG3 in two breast cancer cell lines including ZR-75-1 and MCF7, as well as normal breast cell MCF10A. Neither ZR-75-1 nor MCF7 cells possess gene amplification/deletion in chromosome Xp11.23 and 14q32.2, where the UXT and MEG3 genes exist. As expected, a higher level of UXT and a lower level of MEG3 were also observed in breast cancer cell lines, compared with that of control (Figures 1G–1I).

Knockdown of UXT or overexpression of MEG3 inhibited proliferation, promoted apoptosis, and delayed migration and invasion of MCF7 and ZR-75-1 cells

To explore the biological functions of UXT and MEG3 in the tumorigenesis of breast cancer, we downregulated UXT expression by using shRNA and upregulated MEG3 expression by introducing pcDNA3.1-MEG3 into ZR-75-1 and MCF7 cells, following by measuring the alteration of proliferation, apoptosis, migration, and invasion. As shown in Figure 2A, the level of MEG3 both in ZR-75-1 and MCF7 cells was remarkably increased 48 h after the transfection with pcDNA 3.1-MEG3. As expected, the mRNA and protein levels of UXT were significantly decreased at 48 h post-transfection (Figures 2B and 2C). Along with the increasing level of MEG3 or decreasing level of UXT, CCK-8 assay presented the inhibited cell viability of ZR-75-1 and MCF7 cells (Figure 2D), and the increased apoptotic cells (Figure 2E). Furthermore, both migratory and invasive cells were remarkably reduced in the plasmid pcDNA3.1-MEG3 group (Figure 2G). Likewise, UXT knockdown also inhibited the capacity of migration and invasion of MCF7 and ZR-75-1 cells (Figure 2H). Therefore, these data indicated that there is a negative correlation between UXT and MEG3 in regulating the carcinogenesis of breast cancer.

UXT bound with DNMT3b to promote MEG3 methylation and in turn downregulate MEG3 expression

To assess the methylation of MEG3 imprinting control region, methylation-specific PCR (MS-PCR) was conducted, and we found that the MS-PCR amplified DNA from a positive control, MCF7, and ZR-75-1, whereas the un-MS-PCR only amplified DNA from a negative control (Figure 3A), suggesting that the hypermethylation of the MEG3 imprinting control region is in breast cancer cells. It has been demonstrated that the methylation of MEG3 is regulated by DNA (cytosine-5)-methyltransferase 3b (DNMT3b) (19). Thus, DNMT3b level in breast cancer was detected in clinical specimens (13 cases) and cell lines. The results showed that DNMT3b was highly expressed in breast tumor tissues and cell lines (Figures 3B–3D). Knockdown of DNMT3b increased the level of MEG3

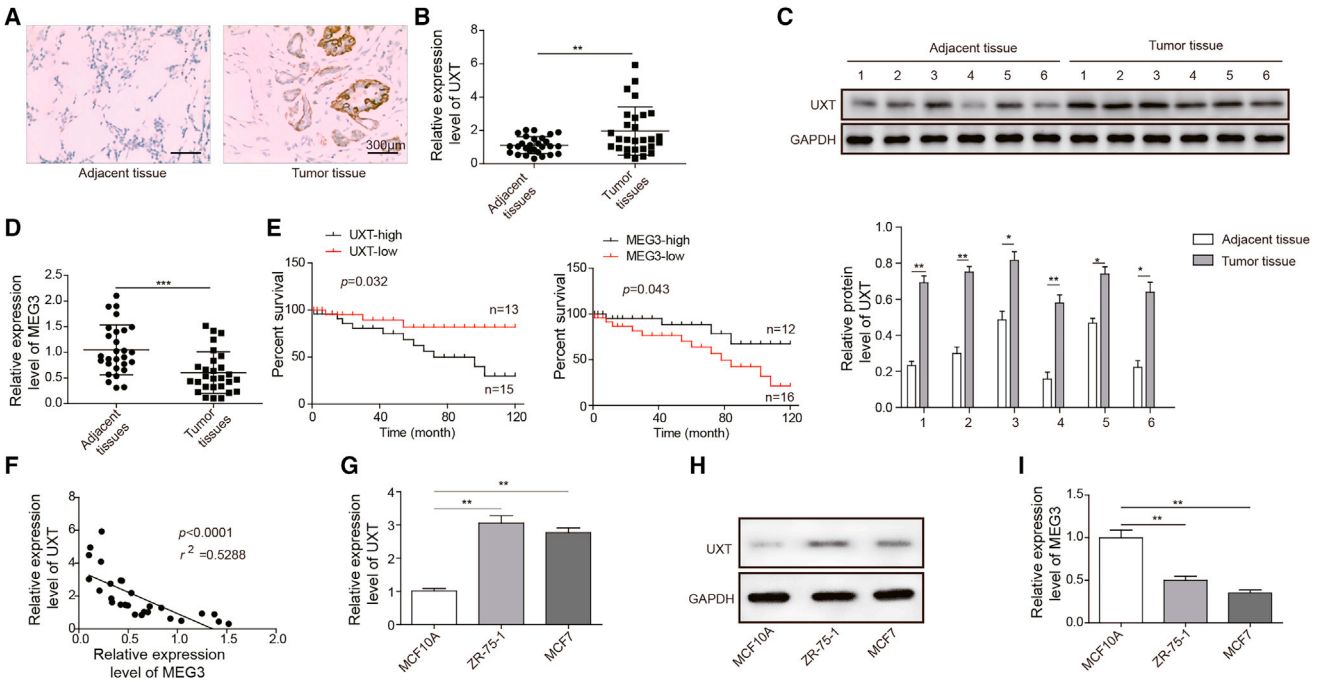


Figure 1. The expression patterns of UXT and MEG3 in breast cancer

(A) Immunohistochemistry analysis on UXT expression of breast cancer and adjacent nontumor tissue specimens from 28 cases of patients. Scale bar, 300 μm. (B) qRT-PCR analysis on UXT level in breast cancer and adjacent nontumor tissue specimens from 28 cases of patients using qRT-PCR assay. Each dot indicates the average of three experiments. (C) Western blot analysis of UXT in breast cancer and matching adjacent nontumor tissue specimens from 6 cases of patients. Each dot indicates the average of three experiments. (D) qRT-PCR analysis of MEG3 expression in breast cancer and adjacent nontumor tissue specimens from 28 cases of patients. (E) The Kaplan-Meier survival analysis was performed to assess the connection between levels of UXT or MEG3 and the prognostic performance. (F) The correlation between UXT and MEG3 in breast cancer was detected by the Spearman's correlation test. Each dot indicates the average of three experiments. (G) qRT-PCR analysis of UXT expression in breast cancer cells and normal breast cells. (H) Western blot analysis of UXT expression in breast cancer cells and normal breast cells. (I) qRT-PCR analysis of MEG3 expression in breast cancer cells and normal breast cells. **p < 0.01, ***p < 0.001.

(Figures 3E–3G). The data of co-immunoprecipitation identified the interaction between UXT and DNMT3b (Figure 3H). We further showed that knockdown of UXT reduced DNMT3b activity in breast cancer cells (Figure 3I). In addition, DNMT3b knockdown suppressed MEG3 gene methylation (Figure 3J). The silencing of UXT reduced the level of DNMT3b, while knockdown of DNMT3b did not alter the expression of UXT (Figure 3K), indicating that DNMT3b might be a downstream effector in UXT-mediated breast cancer tumorigenesis. Furthermore, the elevated level of MEG3 induced by UXT knockdown was dramatically reversed by DNMT3b overexpression (Figure 3L). To sum up, these data implied that the negative regulation of UXT on MEG3 expression was dependent on DNMT3b-mediated methylation modification.

MEG3 bound with p53 to regulate its transcriptional activity

To explore the downstream mechanism of MEG3, we detected the expression of p53 in normal and breast cancer cells and found that mRNA and protein levels of p53 were downregulated in breast cancer cells (Figures 4A and 4B). Both MCF-7 and ZR75-1 cells possess the wild-type p53 gene. As expected, overexpression of MEG3 increased the mRNA and protein levels of p53 (Figures 4C and 4D). Thus, we hypothesized whether MEG3 could bind to p53 and regulate its transcription. As MEG3 contains three conserved motifs, named as M1, M2, and M3, several relevant MEG3 deletion mutants were generated for further confirmation. Luciferase reporter assay was performed to test the mutant for the ability to activate p53-mediated transcription activity, and the results showed that each deletion mutants failed to stimulate the transcriptional activity of p53 (Figure 4E), indicating that full length of MEG3 is crucial for p53 transcription. Furthermore, RNA immunoprecipitation (RIP) assay suggested that MEG3 could directly bind to p53 (Figure 4F). Knockdown of p53 dramatically diminished the effects of MEG3 overexpression on cell apoptosis, migration, and invasion (Figures 4G and 4H), which was also further verified by the protein and mRNA expressions of apoptosis-related genes, including Bax, Bcl-2, p21, and p53 (Figures 4I and 4J). Collectively, it is suggested that p53 was involved in MEG3-mediated apoptosis, migration, and invasion of MCF7 and ZR-75-1 cells.

UXT negatively regulated the MEG3/p53 axis in a DNMT3b-dependent manner to promote cancer growth *in vivo*

To identify whether the regulation of UXT on the MEG3/p53 axis in a DNMT3b-dependent manner is fit *in vivo*, the stable cells of UXT knockdown combined with DNMT3b knockdown or overexpression were constructed and then subcutaneously injected into the right

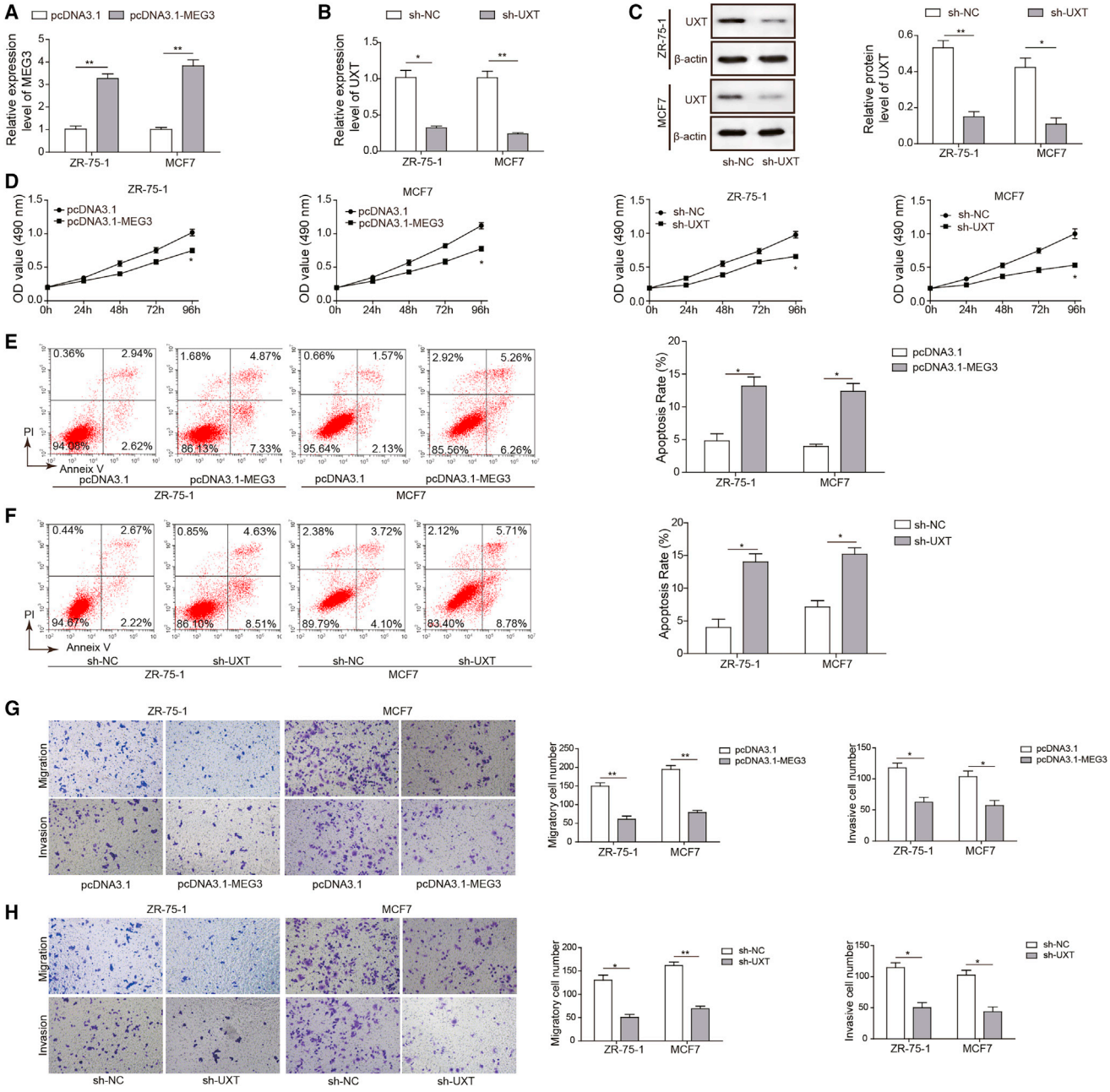


Figure 2. The effects of UXT and MEG3 on cell apoptosis, migration, and invasion in MCF7 and ZR-75-1 cells

MCF7 and ZR-75-1 cells were transfected by plasmid pcDNA3.1 MEG3 or shRNA UXT, and the proliferation, apoptosis, migration, and invasion were detected. Plasmid pcDNA3.1 and nonspecific control shRNA duplexes were, respectively, used as the matching control. (A) MEG3 expression level was determined using qRT-PCR assay in ZR-75-1 and MCF7 cells. (B and C) The mRNA and protein levels of UXT were examined using qRT-PCR and Western blot analysis in ZR-75-1 and MCF7 cells. (D) CCK-8 assay was performed to assess the cell viability within MEG3 overexpression or UXT knockdown. (E and F) The effects of MEG3 overexpression or UXT knockdown on cell apoptosis were detected using flow cytometry. (G and H) Transwell assay was employed to assess the cell migration and invasion in ZR-75-1 and MCF7 cells. *$p < 0.05$, **$p < 0.01$.

flank of nude mice to confirm the influence of UXT and DNMT3b on tumor growth. As shown in Figures 5A and 5B, the inhibition of UXT knockdown on tumor growth was further strengthened by DNMT3b knockdown but weakened by DNMT3b overexpression. Similarly, increasing levels of MEG3 and p53 were presented by the knockdown of both UXT and DNMT3b, while the upregulation of MEG3 and p53 induced by UXT knockdown was almost abolished by DNMT3b overexpression (Figures 5C and 5D). Whereas, the data of DNMT3b

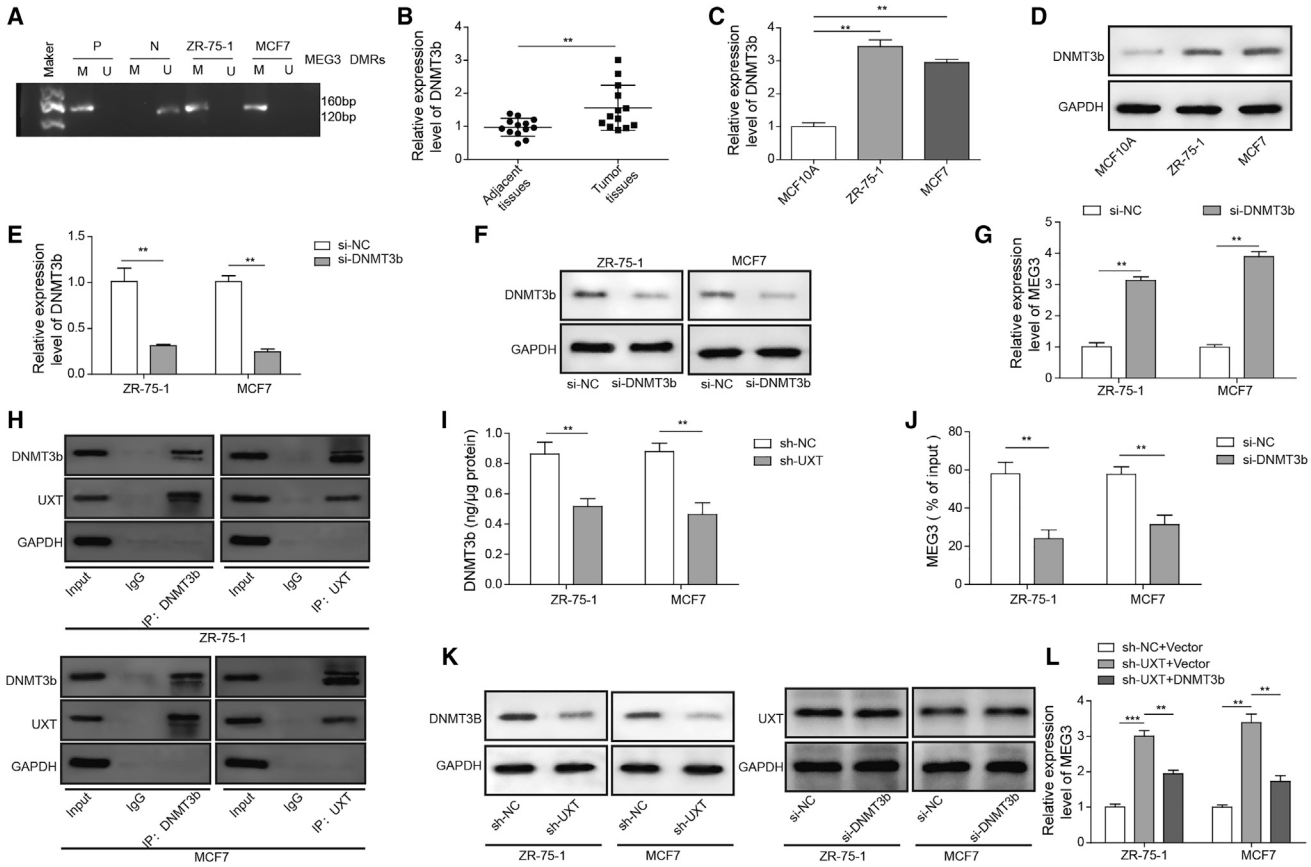


Figure 3. UXT regulated the methylation modification of MEG3 through binding to DNMT3b

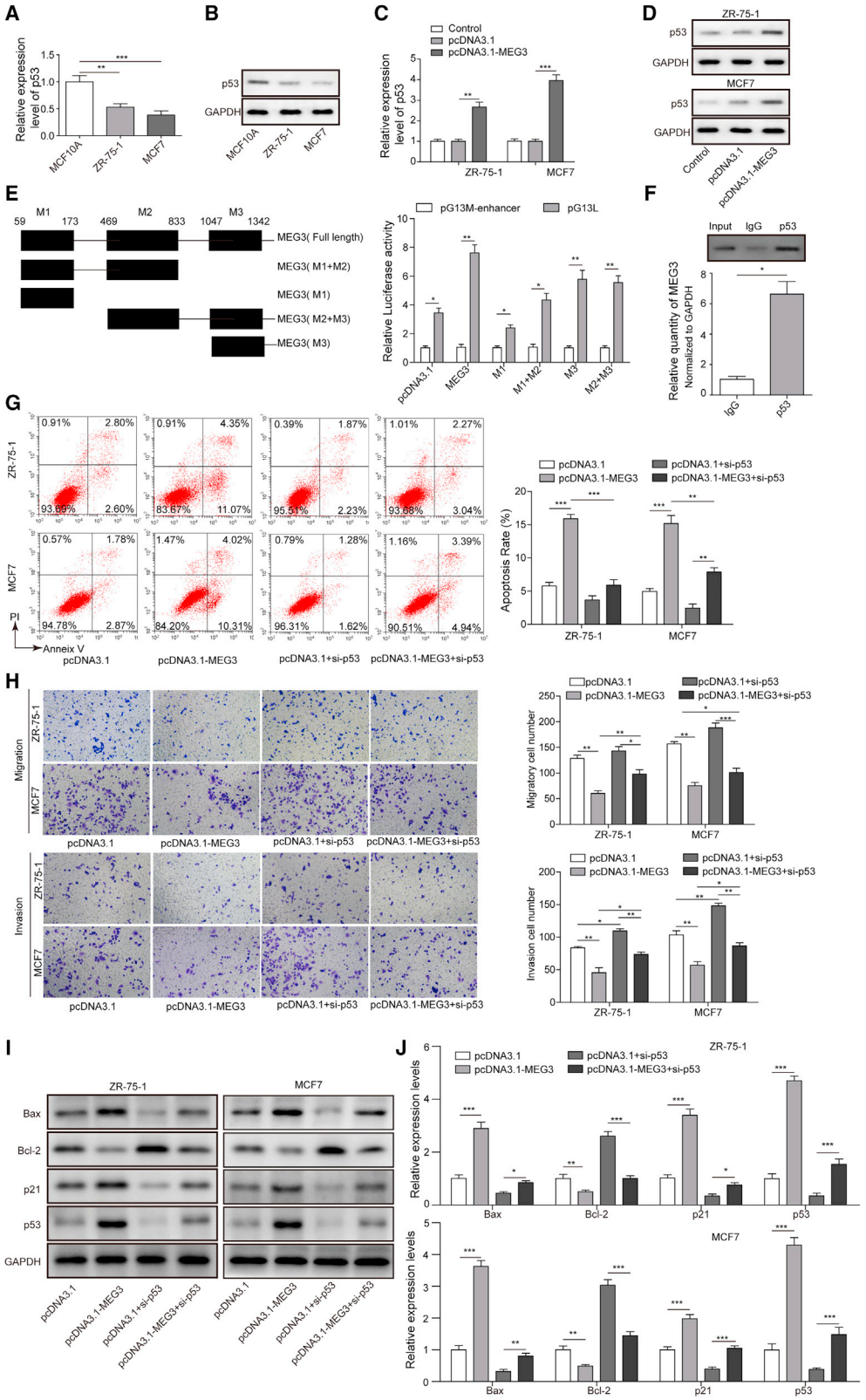
(A) MS-PCR assay was used to test the methylation of promoter DMR in MEG3. (B) qRT-PCR analysis of DNMT3b expression of breast cancer and adjacent nontumor tissue specimens from 13 cases of patients. (C) qRT-PCR analysis of DNMT3b expression in breast cancer cells and normal breast cells. (D) Western blot analysis of DNMT3b expression in breast cancer cells and normal breast cells. (E and F) DNMT3b mRNA and protein levels were determined using qRT-PCR and Western blot analysis. (G) The effects of DNMT3b knockdown on MEG3 expression. (H) Co-immunoprecipitation analysis on the interaction between DNMT3b and UXT. (I) The DNMT3b activity was measured in MCF7 and ZR-75-1 cells transfected with shRNA targeting UXT or control shRNA. (J) The MS-PCR was performed to determine the level of MEG3 in MCF7 and ZR-75-1 cells transfected with shRNA targeting DNMT3b or control shRNA. (K) Knockdown analysis on the interaction between DNMT3b and UXT. (L) The effects of UXT combined with DNMT3b on MEG3 levels in ZR-75-1 and MCF7 cells. **$p < 0.01$, ***$p < 0.001$.

exerted the opposite trend (Figure 5E). As expected, immunohistochemical staining of p53 and DNMT3b further validated the conclusion (Figure 5F). Thus, UXT negatively regulated the MEG3/p53 axis in a DNMT3b-dependent manner to promote the growth of nude mice xenograft.

DISCUSSION

Cancer is still the main threat to human health. The discoveries of new approaches and/or targets against cancer are always a hotspot in this field. It has been reported that UXT, a putative member of an α-class prefoldin protein family, is overexpressed in different types of human tumor tissues but not in the matching normal tissues.[3] Herein, we identified that UXT is also more abundant in breast cancer tissues or cells than normal tissues or cells through analyzing its expression in specimens from breast cancer patients and breast cancer cells, respectively. The reasons for UXT involvement in tumor progress were often attributed to enhancing NF-κB activities through forming an integral component of the NF-κB enhanceosome,[19] corrupting centrosome activity by forming a novel component of centrosomal processes associated with γ-tubulin,[5] or interacting with estrogen receptor or androgen receptor,[20,21] and so on. In the present study, we found that UXT negatively regulates the MEG3/p53 axis in a DNMT3b-dependent manner to promote apoptosis and inhibit migration and invasion of breast cancer cells, as well as the growth of nude mice xenograft.

Due to the important role in cell physiological activities and development of tumors, lncRNA has been emphasized in the therapy of tumors. MEG3, an imprinted gene, is highly expressed in normal human tissue but lost or downregulated in major human tumors.[13] Research on MEG3 suggests that MEG3 is a tumor suppressor.[12] In breast cancer, downregulated expression of MEG3 is associated



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with poor prognosis and serves as an unfavorable risk factor for the survival of patients.[22] Overexpression of MEG3 suppresses breast cancer cell proliferation, invasion, and angiogenesis.[23] Here, our results showed that MEG3 was significantly downregulated in breast cancer cells and tissues versus normal cells and tissues. Overexpression of MEG3 slowed down the proliferation, accelerated the apoptosis, and delayed the migration and invasion of MCF7 and ZR-75-1 cells.

Promoter hypermethylation is considered the major reason for low expression of MEG3.[24,25] Epigenetic changes are the vital factor for tumor occurrence and development and are also utilized as biomarkers for cancer early detection.[26] DNA methylation modifications occur the most frequently in epigenetic changes and function through silence gene expression via blocking the interaction between DNA and protein. DNA methylation is catalyzed by a family of DNA methyltransferase (DNMT) enzymes, namely, DNMT1, DNMT3a, and DNMT3b. DNMT1 is responsible for maintaining the methylation pattern of the genome in daughter cells during cell division, whereas DNMT3a and DNMT3b are essential for *de novo* methylation.[27] Zhou et al. suggested that elevated DNMT3b induced hypermethylation of MEG3 imprinting control region,[28] which is consistent with our results on MEG3 regulation. Intriguingly, we further identified that it is UXT that directly bound to DNMT3b to modulate its expression.

p53, a tumor suppressor gene, plays a critical role in tumor initiation, development, and progression.[29] Zhu et al. indicate that MEG3 functions in hepatoma cells through activation of p53 via interacting with p53 DNA binding domain.[18] Sun et al. suggested that overexpressed MEG3 in breast cancer inhibited the proliferation, colony formation, migration, and invasion capacities by enhancing p53's transcriptional activity on its target genes, including p21, Maspin, and KAI1.[30] Through investigating the interaction between MEG3 and p53 in breast cancer cells, we found that MEG3 did not only regulate the transcriptional activities of p53 but also bound to p53 protein. Furthermore, when siRNA p53 was transfected into cells pre-transfected with pcDNA3.1 MEG3, inhibition of p53 could abolish MEG3-mediated cell apoptosis, migration, and invasion.

In conclusion, UXT participated in tumor progress through downregulating the expression of MEG3 via methylation by binding to DNMT3b, in turn inhibiting the expression of p53 to influence the apoptosis, migration, invasion, and tumor growth of breast cancer cells. Those provide a basis for the development of drugs targeting UXT against cancer.

MATERIALS AND METHODS

Patient and tumor sample preparation

Twenty-eight pairs of breast cancer and adjacent nontumor tissue specimens were obtained from surgical specimens at The Third Xiangya Hospital of Central South University after informed consent. Breast cancer was diagnosed by a pathologist. Adjacent nontumor tissue specimens were taken from a standard distance (3 cm) from the margin of resected neoplastic tissues of patients with tumors who ensured surgical breast ablation. All these specimens were snap-frozen in liquid nitrogen after excision. This study was approved by the Medical Ethics Committee of The Third Xiangya Hospital of Central South University. Informed consent was obtained from each participant, and the procedures were carried out in accordance with the approved guidelines.

Immunohistochemistry

After paraffin section dewaxing and hydration, the sections of xenograft tumors were incubated with 3% methanol-hydrogen peroxide solution for 10 min at 37°C to inhibit endogenous peroxidase activity before blocking with 10% normal goat serum (MXB, Fuzhou, China) for 30 min. Subsequently, the sections were incubated overnight at 4°C with indicated antibodies (UXT, 1:50, #33510, Cell Signaling Technology, USA; DNMT3b, 1:100, #3598, Cell Signaling Technology, USA; p53, 1:100, #2527, Cell Signaling Technology, USA). Slides were washed with PBS three times and then incubated with the secondary antibody horseradish peroxidase-labeled IgG (1:200) for 2 h at room temperature. Finally, the sections were developed with diaminobenzidine (DAB), followed by hematoxylin counterstaining, dehydration, clearance, and neutral balsam sealing. Slides were imaged under a light microscope (Olympus, Japan).

Cell culture, plasmid construction, and transfection

The human breast cells MCF10A and breast cancer MCF7 and ZR-75-1 cells were obtained from American Tissue Culture Collection (ATCC, Manassas, VA, USA). These cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) and 1% antibiotics at 37°C with 5% CO_2. All cell transfection experiments were carried out by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

For gain-of-function of MEG3, full-length Homo sapiens MEG3 sequences (NR_002,766) were cloned into the pcDNA3.1 vector. The pcDNA3.1 vector was available commercially from OriGene. Then, MCF7 and ZR-75-1 cells were transfected with the vector pcDNA3.1-MEG3 to stably express MEG3 in two cell lines. The cell

Figure 4. The regulation of MEG3 on p53

(A and B) The mRNA and protein levels of p53 in normal and breast cells were detected by qRT-PCR and Western blot analysis. (C and D) The alteration of p53 after overexpression of MEG3 was tested using qRT-PCR and Western blot analysis. (E) Luciferase fluorescence intensity changed by being transfected with various components of MEG3 was measured via luciferase reporter assay. (F) RIP was used to test the binding relationship between p53 and MEG3. (G) Flow cytometry was performed to detect the apoptotic cell. (H) Transwell assay was subjected to assess cell migration and invasion of ZR-75-1 and MCF7 cells. (I) Western bolt analysis on the alterations of p53, p21, Bax, and Bcl-2. (J) qRT-PCR analysis on the alterations of p53, p21, Bax, and Bcl-2. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

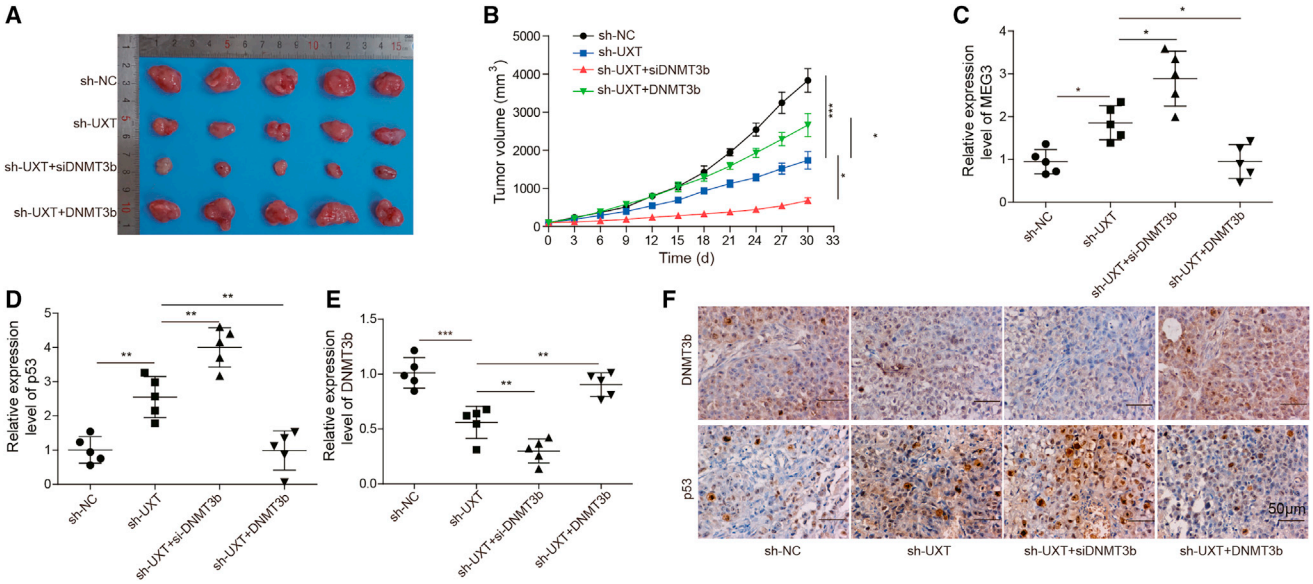

Figure 5. The effects of UXT and MEG3 on nude mice xenograft

After constructing the stable cells of UXT knockout combined with DNMT3b knockdown or overexpression by using matched plasmid, these cells were subcutaneously injected into the right frank of nude mice. (A and B) The image and curve of tumor volume varied with time. (C–E) qRT-PCR analysis of MEG3, p53, and DNMT3b expression levels was performed. (F) Immunohistochemistry analysis of DNMT3b and p53. *p < 0.05, **p < 0.01, ***p < 0.001.

line with integration of empty vector pcDNA3.1 served as control. The stably transfected cell lines were selected by puromycin and subsequent single cell cloning. Transection efficiency was evaluated at 48 h post-transfection by qRT-PCR assay.

For loss-of-function of UXT, target sequences for UXT (^{434}TAC AAG GCC TGC AGA ATT T^{452} and ^{362}GCA ACA GCC TCA CCA AGG A^{580}) were introduced into pGPU6/*Neo* (GenePharma). MCF7 and ZR-75-1 cells were transfected with the vector pGPU6/*Neo* or pGPU6/Neo-shUXT to stably knock down UXT. qRT-PCR and Western blot analysis were used to detect the knock-down efficiency of UXT. The nonspecific negative control sh-NC duplexes were used as the control.

Total RNA extraction and quantitative RT-PCR

Total RNA was isolated from breast cancer tissues or cells by using Trizol Reagent (Invitrogen), according to the manufacturer's instructions. Subsequently, 1 μg of total RNA was subjected to reverse transcription reaction for making cDNA (Takara), which served as the template of quantitative RT-PCR that was performed by SYBR Premix Ex TaqTM II (Takara) via ABI 7500 System (Applied Biosystems, CarIsbad, CA, USA). The n-fold change in gene relative expression was performed by the $2^{-\Delta\Delta Ct}$ method using the ABI software. The PCR cycling protocol was as follows: initiate incubation at 95°C for 15 s, followed by 40 amplification cycles of melting at 95°C for 5 s, annealing at 60°C for 31 s. The primer sequences designed by Primer Premier 5.0 were used as follows: MEG3: Forward 5′-AGACCCGC CCTCTGACTGAT-3′, Reverse 5′-AGGAGCCCACTTCCCACA-3′; UXT: Forward 5′-GACAAGCCGATTCCCAGCGTT-3′, Reverse 5′-TAGACCCTGGTGACACAGTTGCTT-3′; β-actin: Forward 5′-CTG TCCACCTTCCAGCAGATGT-3′, Reverse 5′-CGCAACTAAGTCA TAGTCCGCC-3′.

DNA extraction, bisulfite DNA modification, and MS-PCR

Genomic DNA from MCF7 and ZR75 cells was extracted using DNeasy Blood and Tissue Kit (Qiagen, Gaithersburg, MD, USA), according to the manufacturer's guidelines. Sodium bisulfite modification of DNA and subsequent purification was performed according to the manufacturer's guidelines for sodium bisulfite conversion of unmethylated cytosine in DNA using EpiTect Bisulfite kit (Qiagen). Bisulfite-treated genomic DNA was subjected to an optimized MS-PCR and was performed as Zhou et al. described.[28]

Total protein extraction and western blot

Cells were lysed in RIPA buffer supplemented with protease inhibitor cocktail (Roche) for 30 min on ice to extract total protein. Protein concentration was determined by BCA protein assay (ThermoScientific). The protein samples were boiled in 1 × sodium dodecyl sulfate buffer for 5 min. Protein in the same amount was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with TBST (100 mmol/L Tris-HCl pH7.4, 150 mmol/L NaCl, and 0.05% Tween 20) containing 5% nonfat milk for 1 h, followed by overnight incubation with indicated antibody at 4°C. After being washed three times, the membranes were probed by another matching second antibody. Eventually, it was visualized by enhanced chemiluminescence reagents super signal (ThermoScientific). Mouse monoclonal anti-p53 antibody (1:1,000), mouse monoclonal anti-DNMT3b antibody

(1:1,500), mouse monoclonal anti-UXT antibody (1:1,000), and mouse monoclonal anti-β-actin antibody (1:2,000) were purchased from Cell Signaling Technology. ImageJ was used for quantitative analysis of gray ribbon.

Co-immunoprecipitation

After quantitative analysis of extracted total protein by BCA, 200 μg of total cell lysate was incubated with a Flag antibody overnight at 4°C in a rocker platform. Protein A/G plus agarose beads (#sc-2003; Santa Cruz Biotechnology) were then added, in accompany with being incubated on a rocker platform for an additional 2 h at 4°C. After centrifugation and being washed three times in PBS, the beads were resuspended in SDS loading dye and subjected to SDS-PAGE. The following experimental procedure is the same that in Western blot described above.

Luciferase reporter assay

p53-TA-luc and internal control plasmid pRL-SV40 were co-transfected into HEK293T cells. The luciferase assay was performed by using a dual-luciferase reporter assay system (Promega). Luciferase activity was normalized against an internal control to correct for the variations in transfection efficiency.

Flow cytometry analysis of apoptosis

Cells transfected with pcDNA3.1 or pcDNA3.1-MEG3 were harvested by trypsinization. After double staining with FITC-Annexin V and Propidium isodide (PI), the cells were analyzed using flow cytometry (FACScan, BD, Biosciences). Cells were distributed into four parts including viable cells, dead cells, early apoptotic cells, and apoptotic cells. This experiment was independently performed at least three times.

Migration and invasion

Migration assays were performed in 24-well BD BiocoatTM MatrigelTM Invasion Chambers (8 μm pore size; BD Biosciences). After indicated treatment, 5×10^4 cells suspended in 300 μL medium with 2% FBS were seeded on the top chamber of the transwell, and medium containing 10% FBS was added to the bottom chamber. After 19 h of incubation, cells that migrated through the membrane were fixed using 70% methanol and stained with crystal violet. The average number of cells was calculated from sixteen fields of each insert. For invasion assay, the insert was covered by Matrigel diluted by serum-free medium with a ratio 1:7.

RIP

After indicated treatment, cells were washed twice in PBS with PMSF, lysed in RIPA buffer (ThermoScientific), and incubated at 4°C for 30 min with rotation. After being centrifugated at 13,000 RPM for 15 min at 4°C, the supernatant was divided into two parts, which were, respectively, used to test the expression of RNA-binding protein of interest by Western blotting and retrieve RNA for comparison in qRT-PCR. Samples were successively washed two times with low-salt wash buffer (RIPA buffer) and high-salt wash buffer (50 mMTris, pH 7.4, 1 M NaCl, 1 mM EDTA, 0.1% SDS, 1% NP–40, and 0.5% sodium deoxycholate). One hundred microliters each out of 1 mL of the beads suspension during the last wash was removed to test the efficiency of immunoprecipitation by Western blotting. The remaining beads and 1% input sample were treated with proteinase K buffer containing 117 μL of RIP wash buffer, 15 μL of 10% SDS, and 18 μL of 10 mg/mL proteinase K at 55°C for 30 s with shaking to digest the protein. The RNAs in the buffer were extracted by TriZol reagent. Quantitative PCR was performed to detect the MEG3 presented in the immune complex.

In vivo animal experiment

Twenty female immune-deficient mice (BALB/c nude mice, 19–22 g, 6 weeks old, Vital River Co.) were randomly divided into four groups (shNC, shUXT, shUXT + shDNMT3b, shUXT + DNMT3b) and housed in cages in pathogen-free conditions at 28°C with 50% humidity and then regularly observed. The indicated MCF-7 cells (1×10^7) suspended in 100 μL 0.9% sodium chloride (NaCl) solution were subcutaneously injected into the flanks. The volume of the xenograft tumor was calculated as $0.5 \times \text{length} \times \text{width}^2$. Tumor growth was observed every three days, and until 30 days, the mice were sacrificed and the tumors were harvested, weighed, and photographed. The experimental researches were approved by the Committee on the Ethics of Animal Experiments of The Third Xiangya Hospital of Central South University.

Statistical analysis

All experiments were independently performed at least three times. The values are presented as mean ± standard deviation (SD). The correlation between UXT and MEG3 expression was analyzed using Spearman's correlation test. Kaplan-Meier analysis was performed to determine the connection of UXT or MEG3 expression with the prognostic performance. Differences were assessed by two-tailed Student's *t* test or multi-groups one-way (ANOVA). Analysis was performed using GraphPad prism (prism 5 for windows). $P < 0.05$ was considered as statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omto.2021.12.008.

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AUTHOR CONTRIBUTIONS

Guarantor of integrity of the entire study: Z.-F.H.; study concepts: K.G.; study design: K.G.; definition of intellectual content: K.G.; literature research: Z.-F.H.; clinical studies: Z.-F.H., Y.-L.T., Z.-L.S., K.-Y.Y.; experimental studies: Z.-F.H., Y.-L.T., Z.-L.S., K.-Y.Y.; data acquisition, data analysis, and statistical analysis: Z.-F.H.; manuscript preparation: K.G.

DECLARATION OF INTERESTS

All authors declare that they have no conflict of interest.

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