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Metastasis-associated protein 1, modulated by miR-30c, promotes endometrial cancer progression through AKT/mTOR/4E-BP1 pathway

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HIGHLIGHTS

- An overexpression of MTA1 was associated with poor prognosis in EC.
- MTA1 facilitates EC development both in vitro and vivo.
- MTA1 reverses the effect of its moderator, miR-30c, on EC cells.
- The AKT/mTOR/4E-BP1 pathway may be the key pathway regulated by MTA1 in EC.
- Silencing of MTA1 results in inhibition of EC.

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ABSTRACT

Objective. Though metastasis-associated protein 1 (MTA1) is widely overexpressed in human cancers and is associated with advanced clinicopathological characteristics and survival in related diseases, the association between MTA1 and endometrial cancer (EC) is little known and needs to be studied.

Methods. Western blot and immunohistochemistry were used to analyze protein expression level of cells and tissues, while real-time PCR was used for RNA detection. Bioinformatics tool analysis revealed the relationship between MTA1 and clinicopathological characteristics and survival. CCK-8 assay, colony-formation assay, cell scratch assay, and Transwell assay were performed to determine cell proliferation, migration and invasion abilities, respectively.

Results. The expression level of MTA1 was significantly higher in human EC tissues than in normal endometrium. MTA1 expression was correlated positively with lymph nodes metastasis and poor survival rate in EC. Experimentally overexpressed MTA1 could promote cell proliferation, migration and invasion abilities of EC cell lines Ishikawa, HEC-1B, and RL-952, while reduction of MTA1 inhibited these cell biological behaviors. Moreover, MTA1 could also reverse the negative effect of miR-30c, a direct modulator of MTA1, on EC cells. Our research also revealed that overexpression of MTA1 contributed to EC tumor growth, while knockdown of MTA1 resulted in tumor growth inhibition. Additionally, the phosphorylation levels of mTOR (S2448) and 4E-BP1 (T37/46) changed significantly along with AKT (T308) under regulation of MTA1, both in vivo and vitro.

Conclusion. Our results showed that MTA1, as a downstream target of miR-30c, might promote EC progression via AKT/mTOR/4E-BP1 pathway, which indicated the potential therapy target of MTA1 in EC.

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1. Introduction

Metastasis-associated protein 1 (MTA1) is a key component of the nucleosome remodeling and deacetylating (NuRD) complex and plays an important role in transcriptional regulation via histone deacetylation and chromatin remodeling [1]. It was first found that MTA1 can recruit HDAC2 and join together to the promoter of ER, which inhibits its transcriptional activity [2]. Later, MTA1 was found to be able to combine to the FosB promoter, together with Pol II and C-Jun, and stimulates FosB expression, which indicated that it is also a coactivator protein [3]. As these transcription factors regulate the expression of a large number of genes, MTA1 may thus exert a more wide range of regulatory functions [4].

MTA1 is overexpressed in a variety of human cancers, including breast, ovarian, pulmonary, gastrointestinal, and colorectal cancer [5,6]. Immunohistochemistry staining and clinicopathological analysis have revealed that overexpression of MTA1 is positively correlated with clinical stage, distant metastasis, and poor survival of patients in various cancers [7–9]. However, research on MTA1 expression in endometrial cancer (EC) tissue is very limited. Among patients undergoing surgery after neoadjuvant chemotherapy (NACT), MTA1 expression was significantly downregulated in EC tissues of patients receiving combined NACT of TAC (paclitaxel, adriamycin and carboplatin) regimen with medroxyprogesterone acetate, compared to those receiving the same NACT regimen alone [10]. Balasenthil et al. performed immunohistochemical staining microarray containing 70 endometrial endometrioid adenocarcinomas of various grades showed increased expression of MTA1 in 53 (75.7%) tumors and no significant association between MTA1 expression and EC grade was observed [11]. However, no other papers have looked at MTA1 and tumor grade. To our knowledge, little is known about the relationship between MTA1 expression and prognosis of EC.

In our previous study, we firstly reported that MTA1 is a direct target of miR-30c, which can be depressed by estrogen, and overexpression of miR-30c can negatively regulate EC cells [12,13]. However, we wonder whether the tumor suppressor effect of miR-30c resulted from its targeting of MTA1 and the exact mechanism remains to be fully elucidated. Thus, in the present study, we aimed to identify the MTA1 carcinogenic effect on EC and its role as a downstream regulator of miR-30c.

2. Materials and methods

2.1. Human tissue samples and patient characteristics

Paraffin-embedded tissue samples were obtained from 161 patients who were presented between January 2011 and May 2018 and pathologically diagnosed as EC at Nanjing Drum Tower Hospital (Nanjing, China). In addition, 52 cases of fresh EC tissues and 28 paracancerous tissues obtained from some of the same 52 patients were frozen and stored in liquid nitrogen for RNA extraction. For our 161 IHC samples, including 52 cases for tissue RNA extraction, there were 141 endometrioid adenocarcinoma samples, 16 serous adenocarcinoma samples, and 4 clear cell carcinoma samples. Informed consent and approval for research purpose according to the medical ethics committee of the Affiliated Drum Tower Hospital, Nanjing University Medical School. Experiments were undertaken with the understanding and written consent of each subject.

2.2. Immunohistochemistry (IHC) analysis

Paraffin tissue sections were deparaffinized and followed by rehydration steps through a graded ethanol series and distilled water. The slides were incubated in 3% H_2O_2 at room temperature for 10 min to block endogenous peroxidase activity. Antigen retrieval was performed by boiling in a pressure cooker at 120 °C in 0.01 M citrate buffer solution for 10 min. The samples were incubated with primary anti-rabbit antibodies at 4 °C overnight. After being washed with phosphate-buffered saline (PBS), the slides were incubated with the HRP-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA) at room temperature for 30 min. After that, the sections were stained with DAB according to the manufacturer's protocols and photographed using a microscope and analyzed expression level. MTA1, AKT (T308), mTOR (S2448), and 4E-BP1 (T37/46) protein expression levels in subcutaneou transplantation tumors were detected, while only MTA1 protein level in patients EC tissues was analyzed. We stained one section for each patient tissue sample and two sections for each mice tissue sample, which were then evaluated independently by two experienced pathologists who had no knowledge of the patients' identities or clinical status. It was scored according to the staining intensity and quantity of tumor cells. Only strong staining with denoting >50% of tumor cells was defined as high expression. Primary antibodies dilutions were performed as the following: MTA1 at 1:200 (#5646, Cell Signaling Technology), Phospho-4E-BP1 at 1: 200 (#2855, Cell Signaling Technology), Phospho-Thr308 at 1: 200 (#13038, Cell Signaling Technology), Phospho-mTOR at 1: 200 (#13038, Cell Signaling Technology).

2.3. Cell culture

Ishikawa cell was kindly provided by Professor L.H. Wei (Peking University People's Hospital, China), while HEC-1B and RL-952 cells were purchased from KeyGEN BioTECH (Jiangsu, China). All the cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and penicillin (100 U/ml) and streptomycin (100 mg/ml). All cells were kept in an incubator at 37° C with 5% CO_2.

2.4. Plasmid construction, lentivirus production and cell transfection

Human full-length MTA1 was generated by PCR amplification of MTA1 cDNA fragments. It was cloned into a pCDH vector (CD511B-1) and named pCDH-MTA1. The MTA1-shRNA-plasmid (pMagic-shMTA1) was purchased from Obio Technology (Shanghai, China). All cloned regions were verified by sequencing. miRNA-30c mimics, inhibitor and scrambled negative control RNAs were purchased from Ribobio Company (Guangzhou, China). For plasmid and RNAs transfection, cell transfection was performed with Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's instructions. For pMagic-shMTA1, recombinant lentivirus was generated from 293 T cells using calcium phosphate precipitation. Ishikawa cells were transfected with lentivirus using polybrene (8 μg/ml), followed by puromycin selection (3 μg/ml) for 10d to select stably-expressing cells. Lentivirus for overexpressing MTA1 was purchased from GeneChem Technology (Shanghai, China), and used following its instructions.

2.5. RNA extraction and quantitative RT-PCR

Total RNA was extracted from tissue or cells with isolater reagent (Vazyme, China). After measurement of the RNA concentration, cDNAs were generated from reverse transcription with the HiScrip II 1st Strand cDNA Synthesis Kit (Vazyme, China). The expression levels of mRNA and miR-30c were analyzed according to the instructions of the ChamQTM Universal SYBR qPCR Master Mix kit (Vazyme, China) using the ABI-7300 Real-Time PCR Detection System (Applied Biosystems, USA). The bulge-loop™ miRNA Primer Sets (one RT primer and a pair of qPCR primers) specific for miR-30c were purchased from RiboBio (Guangzhou, China). The levels of mRNA and miR-30c were normalized to GAPDH and U6, respectively. The relative primers for MTA1 were forward, 5′-AGCTACGAGCAGCACAACGGGGT-3′ and reverse, 5′-CACGCT TGGTTTCCGAGGAT-3′; for GAPDH forward, 5′-CGTGGGCCGCCCTAGG CACCA-3′ and reverse, 5′-TTGGCTTAGGGTTCAGGGGGG-3′; and for U6, forward, 5′-CTCGCTTCGGCAGCACA-3′ and reverse, 5′-AACGCTTCACG AATTTGCGT-3′. The SYBR-Green qPCR was performed using the ABI

7500 Fast Real-Time PCR system, and fold-changes were calculated using the $2^{-\Delta\Delta Ct}$ method. Each plate was run in triplicate.

2.6. Cell proliferation assay

Cellular proliferation was analyzed using a cell counting kit-8 (vazyme, China). 1×10^3 cells were seeded in 96-well plates and incubated for 1–5 days, respectively. Ten microliters of CCK-8 solution was added to each well and the cultures were incubated at 37 °C for 2 h. Absorbance at 450 nm was measured using a spectrometer reader.

2.7. Colony-formation assay

The cells were seeded at a density of 500 cells/ml (1000 cells per dish) into 3.5 cm-cell culture dishes and cultured for 10 days in 2 ml DMEM with 10% fetal bovine serum at 37 °C with 5% CO_2. Then, cell colonies were fixed with methanol and stained with Giemsa. Colonies were counted. Cloning efficiency (%) = (number of clones formed from untreated cells/number of untreated cells inoculated) × 100%.

2.8. Cell scratch assay

Cell migration ability was examined by cell scratch assay. Briefly, transfected cells were seeded on 6-well plates and incubated to almost full confluence. Scratching was performed with a 200 μl plastic pipette tip, and the cells were cultured in serum-free medium. The initial gap width (0 h) and the residual gap width at 24–48 h after scratching were observed and photographed under the inverted microscope.

2.9. Cell invasion assay

We conducted Transwell invasion assays using transwell chambers (24-well, 8 μm pore size). Matrigel-coated membranes were used for invasion assays. Briefly, 2×10^4 cells were seeded into the upper transwell chambers with culture medium without FBS, and lower chambers were filled with culture media containing 10% FBS as a chemoattractant. The chambers were incubated at 37 °C for 24 h. The invaded cells on the lower side were fixed with paraformaldehyde, stained with 1% crystal violet, and then counted in five random fields under a microscope.

2.10. Western blotting analysis

Western blotting was performed as previously described [12]. Primary antibodies dilutions were performed as the following: MTA1 at 1:1000 (#5646, Cell Signaling Technology), Phospho-4E-BP1 at 1: 1000 (#2855, Cell Signaling Technology), Phospho-Thr308 at 1: 1000 (#13038, Cell Signaling Technology), Phospho-mTOR at 1: 1000 (#13038, Cell Signaling Technology).

2.11. Immunofluorescence (IF) staining

Cells were grown on a glass petri dish and fixed with 4% formaldehyde for 30 min at room temperature. After three times of quick wash with PBS, cells were incubated with MTA1 antibody (Rabbit monoclonal, Cell Signaling Technology) and GFP antibody (Rabbit Polyclonal, Cell Signaling Technology) in culture medium at 1:200 for 1 h. Then, after three times of wash, cells were incubated with Alexa-conjugated secondary antibodies at 1:500, 1:300 DAPI and 1:10000 whole cell stain and incubated for 1 h in the dark followed by final wash steps. To permeabilize cells, 0.1% Triton X-100 was added to the culture medium during fixing, antibody incubation, and washing. Images were captured using a confocal laser scanning microscope.

2.12. Tumor xenografts in nude mice

Tumorigenesis experiments in nude mice were performed in strict accordance with protocols approved by the Ethics Committee of Drum Tower Hospital Affiliated to Nanjing University Medical School. Female nude mice (four- to six-week-old) were purchased from the Laboratory Animal Center of the Drum Tower Hospital Affiliated to Nanjing University Medical School (Nanjing, China). Stably expressed Ishikawa cells (1×10^6 cells/100ul), which were cultured in DMEM without FBS and mixed with Matrigel at a ratio of 4:1, were subcutaneously injected to the flank of female nude mice ($n = 5$ mice per group). The tumors of nude mice were observed every 3 days and tumor volume was measured using caliper with the traditional formula: $V = 1/2 \times \text{length (mm)} \times \text{width}^2\text{ (mm)}$. At 3 weeks (for the Control and MTA1 groups) and 4 weeks (for the pControl and shMTA1groups) thereafter, the nude mice were euthanized by CO_2 asphyxiation and tumor tissues were stored for further experiments.

2.13. Bioinformatic analysis

RNA-Seq and miRNA-seq data for the TCGA-UCEC Project were downloaded from TCGA official website (https://cancergenome.nih.gov). The corresponding clinical information was downloaded from http://www.cbioportal.org. For the association between MTA1 expression and clinicopathology, we performed analyses using UALCAN (http://ualcan.path.uab.edu) based on TCGA level 3 RNA-seq expression and clinical data [14]. We also performed in-depth analyses of miR-30c expression and survival based on the R language.

2.14. Statistical analysis

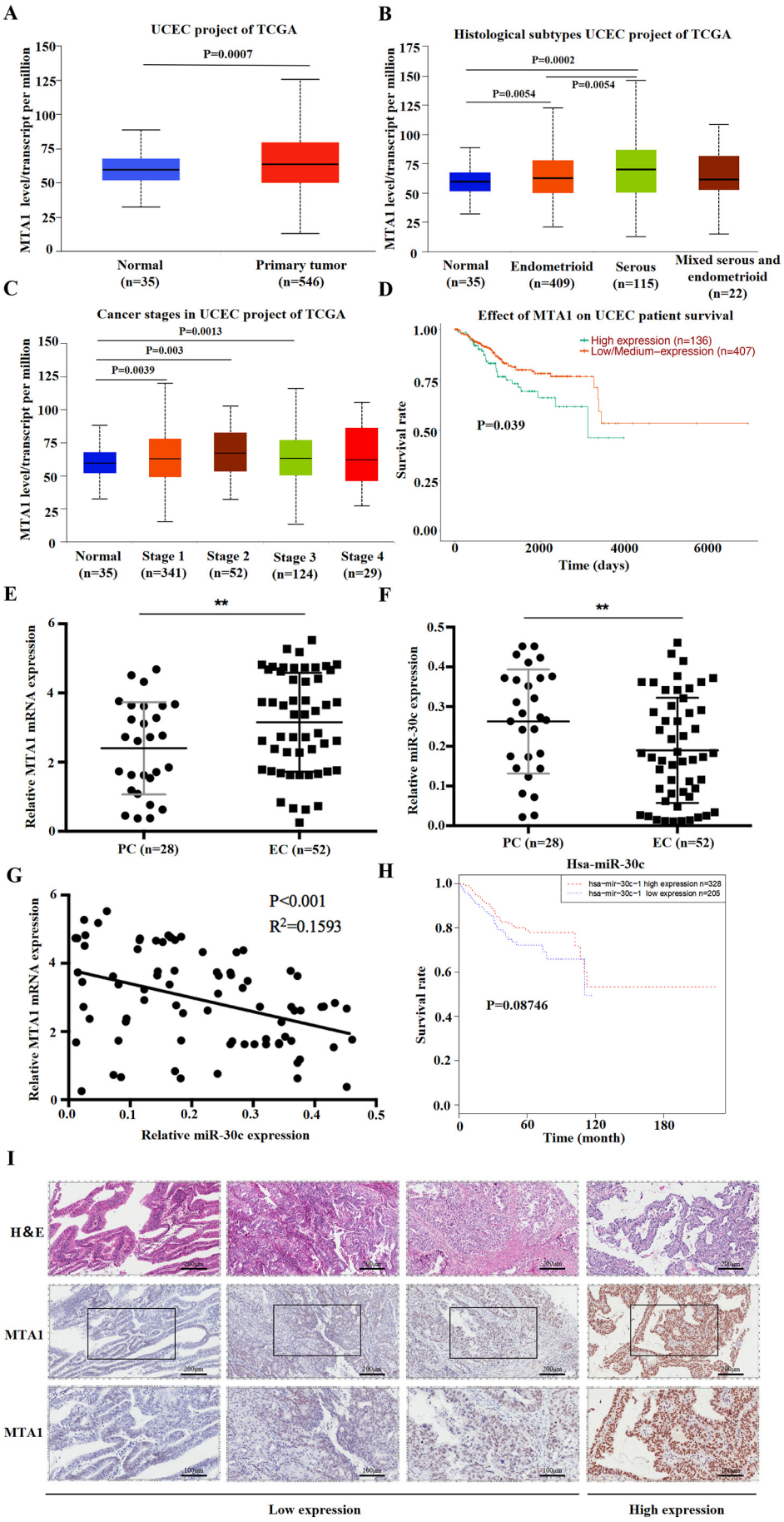
Univariate survival analysis was plotted using the Kaplan–Meier method and analyzed with the log-rank test. A $\chi 2$ test was used to evaluate the associations between expression lever with related clinicopathological factors. The Student's *t*-test analysis of variances was used to evaluate statistical differences between experimental groups. Pearson correlation analysis was used to analyze the relationship of associated factors. Statistical analysis was performed using SPSS version 17.0 and $P < 0.05$ was considered to indicate a statistically significant difference. Statistical significances are presented as * according to the following scheme: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. All assays were independently performed for 3 times.

3. Results

3.1. Overexpressed MTA1, opposite of miR-30c, correlated with poorer prognosis in EC

Data for MTA1 expression and clinical information in TCGA were extracted from the UALCAN. Scrutiny of the database determined the higher MTA1 mRNA expression in uterine corpus EC tissue compared with normal endometrial tissue ($P < 0.01$, Fig. 1A). In addition, cancer of individual stages showed higher MTA1 expression compared with normal tissue ($P < 0.01$, Fig. 1C), except stage 4. For histological subtypes, the MTA1 expression of serous EC was higher than endometrioid EC ($P < 0.01$, Fig. 1B). Moreover, Kaplan-Meier survival curves indicated that MTA1 high expression patients ($n = 136$) correlated with poorer survival probability than low expression patients ($n = 407$, $P = 0.039$, Fig. 1D).

We then measured the MTA1 mRNA expression in our EC samples compared with paracancerous control (PC) samples. Based on our previous proof that MTA1 was a direct target of miR-30c [12], we also conducted miR-30c measurement on these same clinical samples. Interestingly, while MTA1 showed a significant upregulation, miR-30c was downregulated in EC tissues compared to normal PC tissues ($P < 0.01$, Fig. 1E and F). An inverse linear correlation between


A
UCEC project of TCGA
P=0.0007
MTA1 level/transcript per million
Normal (n=35)
Primary tumor (n=546)
B
Histological subtypes UCEC project of TCGA
P=0.0002
P=0.0054
P=0.0054
MTA1 level/transcript per million
Normal (n=35)
Endometrioid (n=409)
Serous (n=115)
Mixed serous and endometrioid (n=22)
C
Cancer stages in UCEC project of TCGA
P=0.0013
P=0.003
P=0.0039
MTA1 level/transcript per million
Normal (n=35)
Stage 1 (n=341)
Stage 2 (n=52)
Stage 3 (n=124)
Stage 4 (n=29)
D
Effect of MTA1 on UCEC patient survival
High expression (n=136)
Low/Medium-expression (n=407)
P=0.039
Survival rate
Time (days)
E
**
Relative MTA1 mRNA expression
PC (n=28)
EC (n=52)
F
**
Relative miR-30c expression
PC (n=28)
EC (n=52)
G
P<0.001
R2=0.1593
Relative MTA1 mRNA expression
Relative miR-30c expression
H
Hsa-miR-30c
hsa-mir-30c-1 high expression n=328
hsa-mir-30c-1 low expression n=205
P=0.08746
Survival rate
Time (month)
I
H&E
MTA1
MTA1
Low expression
High expression

them appeared under Pearson analysis with $R^2 = 0.1593$ and $P < 0.001$ (Fig. 1G). Although no significant difference was seen, the Kaplan–Meier analyses based on TCGA-UCEC database showed a tendency that low expression of miR-30c related to poorer survival with a P value of 0.08746 (Fig. 1H).

Further, MTA1 protein expression level in 161 paraffin-embedded specimens of EC sample tissues were also evaluated under IHC (Fig. 1I). Patient clinicopathological characteristics and its association with MTA1 expression were shown in Table 1, which indicated that the expression level of MTA1 was significantly related to lymph nodes metastasis ($P = 0.007$).

3.2. MTA1 promoted proliferation, migration and invasion of EC cells in vitro

In an attempt to investigate the biological functions of MTA1 in EC cells, Ishikawa, HEC-1B, and RL-952 cells were transduced by pCDH-MTA1, pMagic-shMTA, and their controls, respectively. The MTA1 protein levers were detected by Western blot (Fig. 2A and B). The cell immunofluorescence targeting MTA1 (red) in Ishikawa cell was mainly expressed in the nucleus (Fig. 2C). These results indicated that the expression of MTA1 was significantly changed by our interference. Ishikawa cell proliferation was measured by CCK8 (Fig. 2D), overexpression of MTA1 promoted cell proliferation and silencing of MTA1 inhibited cell proliferation significantly in Ishikawa cell line. The colony formation assay showed that MTA1 knockdown suppressed Ishikawa cell colony-forming ability (Cloning efficiency: 11.5% ± 2.83% vs 17.3% ± 3.97%, $P = 0.0072$) and inversely, overexpression of MTA1 enhanced their colony-forming ability (Cloning efficiency: 74.2% ± 19.90% vs 18.3% ± 6.53%, $P = 0.0018$, Fig. 2E and F). A scratch wound assay was applied to measure the effect of MTA1 on the migration of EC cells. The result showed that overexpression and knockdown treatment markedly enhanced or inhibited the motility of Ishikawa cells, respectively, as determined by the migration area (Fig. 2G and H). To investigate whether overexpression of MTA1 induces the invasive capacity of EC cells, Transwell assay was carried out to determine the ability of the Ishikawa cells to invade through biological matrices in vitro. The invasive cells with MTA1 overexpression were more than that of the control cells (123 ± 27.5 vs 49 ± 18.8, $P = 0.0031$), whereas the number of MTA1-knockdown cells significantly reduced (17 ± 8.5 vs 68 ± 17.6, $P = 0.0013$, Fig. 2I and J).

Further, we performed these biology experiments with the other two EC cell lines HEC-1B and RL-952. Interestingly, upregulation of MTA1 also enhanced the proliferation, migration, and invasion abilities of both the two cell lines, while downregulation of MTA1 could inhibit their biological abilities, respectively (Figs. S1 and S2). Taken together, these results indicated that MTA1 could promote proliferation, migration, and invasion in both ER positive and negative EC cell lines in vitro.

3.3. MTA1 reversed the effect of miR-30c on EC cell

As mentioned in our previous study, overexpression of miR-30c could inhibit the proliferative, migratory and invasive abilities of EC cells [12]. We wonder whether it affects cell behavior through MTA1, which was a direct target of miR-30c. Firstly, we detected the protein level of MTA1 after co-transfecting MTA1-associated plasmid and miR-30c mimics/inhibitor based on western blotting (Fig. 3A). Interestingly, transiently co-transfection of MTA1 plasmid and miR-30c mimics into Ishikawa cells could reverse cell proliferation and colony-forming abilities, which were weakened by overexpression of miR-30c, based on CCK8 (Fig. 3B) and colony formation assays (Cloning efficiency, Control: 23.9% ± 4.72% vs Mimics: 9.4% ± 4.22%, $P = 0.0022$; Mimics: 9.4% ± 4.22% vs Mimics+MTA1: 53.1% ± 8.74%, $P = 0.0012$; Fig. 3C). Furthermore, overexpression of MTA1 could reverse migration ability of Ishikawa cells, which was inhibited by miR-30c, through the scratch wound assay (Fig. 3D). The same effect was also seen in Transwell invasion assay (Invasive cell number, Control: 22 ± 5.9 vs Mimics: 13 ± 3.9, $P = 0.0013$; Mimics: 13 ± 3.9 vs Mimics+MTA1: 32 ± 6.8, $P = 0.0010$; Fig. 3E).

Contrarily, reduction of miR-30c enhanced cell proliferation, while knockdown of MTA1 could reverse it (Fig. 3B). Downregulation of miR-30c enhanced cell colony-forming ability, while knockdown of MTA1 could reverse the effect (Cloning efficiency, Control: 14.8% ± 4.11% vs Inhibitor: 41.5% ± 5.72%, $P = 0.0084$; Inhibitor: 41.5% ± 5.72% vs Inhibitor+shMTA1: 7.8% ± 3.28%, $P = 0.0014$; Fig. 3C). The scratch wound assay revealed that silencing MTA1 resulted in reversed inhibition of cell migration ability strengthened by miR-30c inhibitor (Fig. 3D). Similar phenomena was also seen in Transwell invasion assay (Invasive cell number, Control: 21 ± 2.1 vs Inhibitor: 29 ± 3.0, $P = 0.0089$; Inhibitor: 29 ± 3.0 vs Inhibitor+shMTA1: 12 ± 4.7, $P = 0.0010$; Fig. 3E).

These results indicated that miR-30c regulated biological behavior of Ishikawa cell through targeting MTA1, at least partially.

Table 1
Association of MTA1 expression with clinicopathological parameters from EC patients.

Clinicopathologic variable	N	MTA1 IHC expression		χ^2	P
		Low	High		
Age					
≤58	87	36	51	0.658	0.417
>58	74	26	48		
Histology					
EA	141	58	83	1.914	0.166
Non-EA	20	5	15		
Serous	16	4	12		
Clear cell	4	1	3		
Grade					
G1	28	10	18	0.665	0.717
G2	83	35	48		
G3	50	18	32		
Stage					
I–II	88	25	63	0.073	0.787
III–IV	39	12	27		
Myometrial invasion					
≤50%	87	19	68	0.514	0.473
>50%	26	4	22		
Lymph nodes					
Positive	15	9	6	7.199	0.007*
Negative	118	31	87		

EA, endometrioid adenocarcinoma; Non-EA, non-endometrioid adenocarcinoma.
* $P < 0.05$ was considered to indicate a statistically significant difference.

Fig. 1. Overexpressed MTA1, opposite of miR-30c, correlated with poorer prognosis in EC. A-D: UALCAN (http://ualcan.path.uab.edu) scrutiny of the TCGA-UCEC database. (A) MTA1 mRNA expression level was significantly higher in uterine corpus EC tissue compared with normal endometrial tissue. $P = 0.0007$. (B) MTA1 expression of serous EC was higher than endometrioid EC in TCGA-UCEC Project. $P = 0.0054$. (C) Cancer of individual stages showed higher MTA1 expression compared with normal tissue. $P = 0.0039$ (Stage 1 vs. Normal), $P = 0.003$ (Stage 2 vs. Normal), $P = 0.0013$ (Stage 3 vs. Normal). (D) Kaplan–Meier survival curves indicated that MTA1 high expression patients (green, n = 136) correlated with poorer survival probability than low expression patients (red, n = 407), $P = 0.039$. (E) Real-time PCR revealed that MTA1 mRNA expression was much higher in clinical EC samples (n = 52) than in normal PC endometrium (n = 28). **$P < 0.01$. (F) Hsa-miR-30c, a direct modulator of MTA1, was downregulated in EC samples (n = 52) compared to normal PC endometrium(n = 28). **$P < 0.01$. (G) An inverse linear correlation between MTA1 and miR-30c expressions appeared under Pearson analysis with $R^2 = 0.1593$. ***$P < 0.001$. (H) Kaplan–Meier analysis based on TCGA-UCEC database showed a tendency that low expression (blue) of miR-30c related to poorer survival. $P = 0.08746$. (I) Immunohistochemistry (IHC) following HE staining demonstrated MTA1 protein expression level in 161 paraffin-embedded specimens of EC sample tissues (×100, ×200). Only strong staining with denoting >50% of tumor cells was defined as high expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

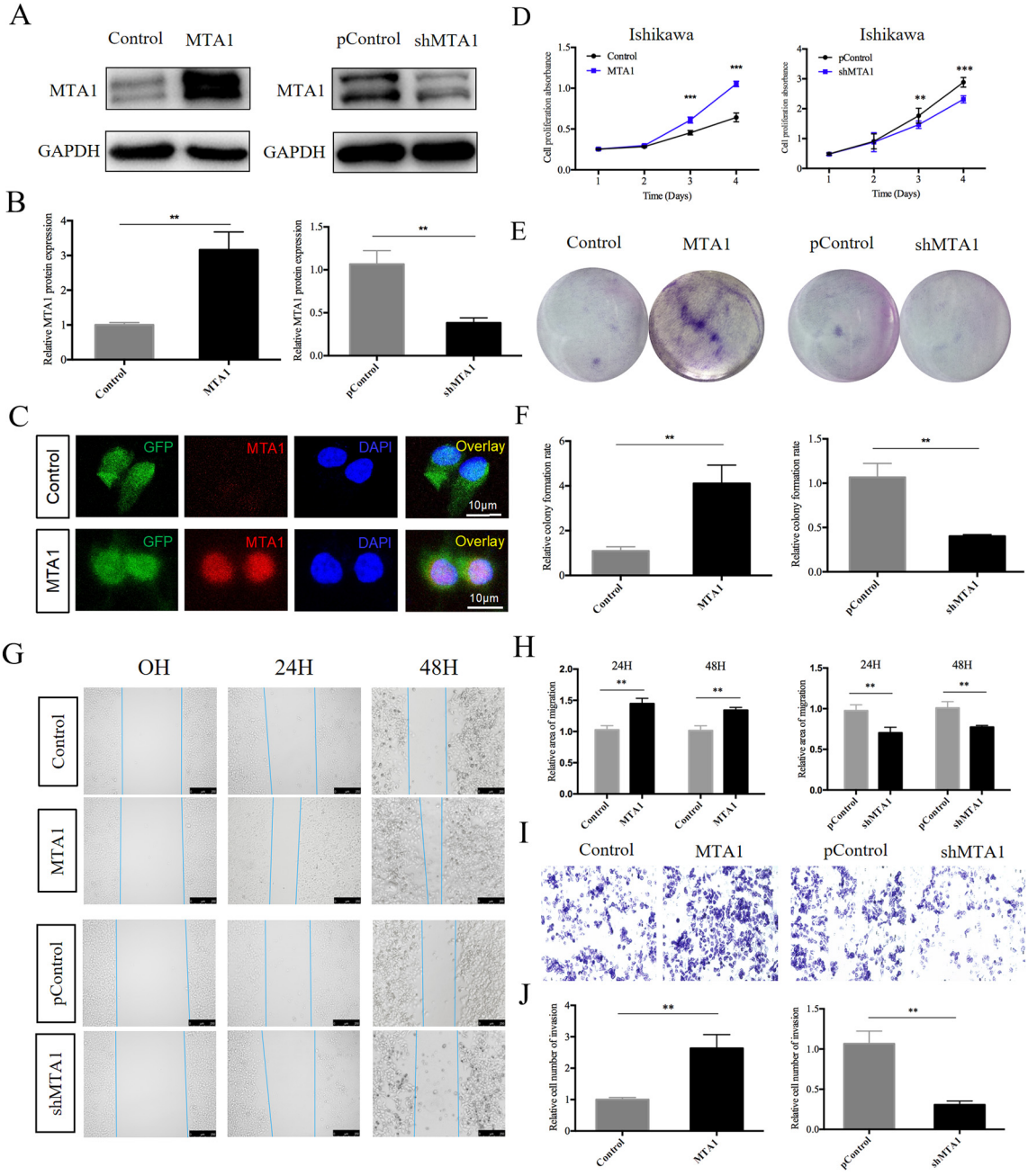


Fig. 2. MTA1 promoted proliferation, migration, and invasion of EC Ishikawa cells in vitro. (A and B) Western blot showed the MTA1 protein level after transfection with generated lentivirus in Ishikawa cells. **P < 0.01. (C) Cell immunofluorescence revealed the protein expression level and location, GFP (green), MTA1 (red) and DAPI (blue), in Ishikawa cells. (D) CCK-8 cell proliferation assay after overexpression and knockdown of MTA1 in Ishikawa cells. **P < 0.01, ***P < 0.001. (E and F) Colony-formation assay after overexpression and knockdown of MTA1 in Ishikawa cells. Error bars represent the relative rate of colon numbers compared to control from three independent experiments. **P < 0.01. (G and H) The effects of the indicated treatments on cell migration using a scratch wound-healing assay are shown. Error bars represent the migration area from three independent experiments. **P < 0.01. (I and J) Transwell invasion assay after overexpression and knockdown of MTA1 in Ishikawa cells. Error bars represent the relative rate of invasive cell numbers compared to control from three independent experiments (×100). **P < 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. MTA1 promoted endometrial tumor growth via AKT/mTOR/4E-BP1 pathway

Since AKT/mTOR/4E-BP1 signaling pathway has been demonstrated to play an important role in the development of EC [15,16], we wonder whether MTA1 effects EC through this way and first detected the expression of AKT in Ishikawa cells. As shown in Fig. 4A, overexpression of MTA1 upregulated the expression level of phosphorylated AKT (T308), not total AKT, while knockdown of MTA1 resulted in a reduction of pAKT (T308). We next detected the possible downstream proteins, phosphorylated mTOR (S2448) and 4E-BP1 (T37/46). The results showed that the phosphorylation levels of mTOR and 4E-BP1 changed significantly along with pAKT (T308) under the regulation of MTA1 (Fig. 4A–B).

To further explore the role of MTA1 in EC in vivo, we injected stably-expressing Ishikawa cells into nude mice. In line with in vitro analysis, MTA1 overexpression significantly increased the tumor volume as compared with control group (0.9080 ± 0.02703 vs 0.3353 ± 0.08261 cm^3, P = 0.0002), while MTA1 knockdown resulted in decreased tumor volume compared to control group (0.1137 ± 0.04238 vs 0.4254 ±

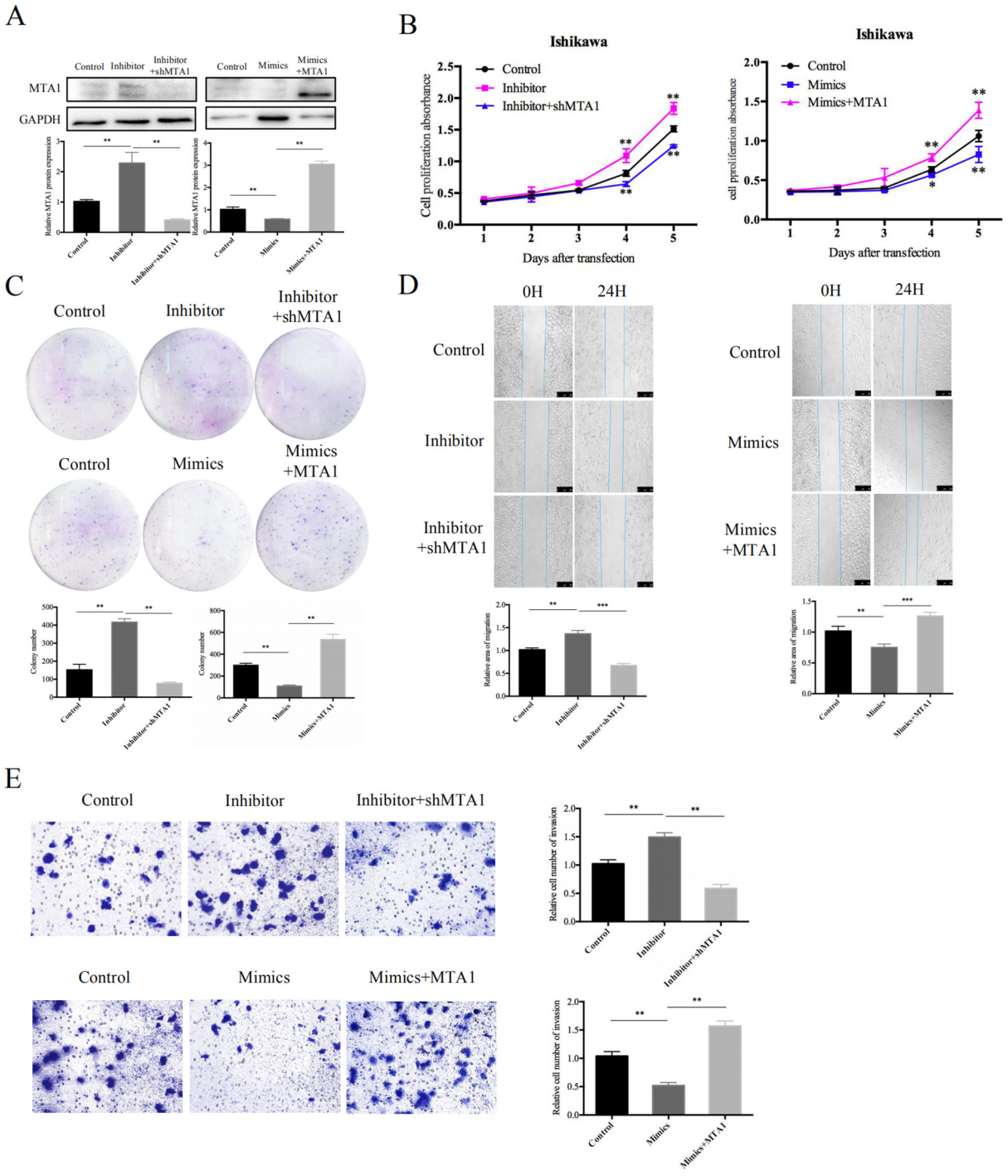


Fig. 3. MTA1 reversed the effect of miR-30c on EC cell. (A) Western blot analysis for MTA1 protein after treatments on Ishikawa cells with miR-30c mimics/inhibitor, constructed MTA1 plasmid and their controls. (B) CCK-8 cell proliferation assay after treatments on Ishikawa cells. *P < 0.05, **P < 0.01, ***P < 0.001. (C) Colony-formation assay after treatment on Ishikawa cells. Error bars represent the colon numbers from three independent experiments. **P < 0.01. (D) The effects of the treatments on cell migration using a scratch wound-healing assay. Error bars represent the migration area from three independent experiments. **P < 0.01, ***P < 0.001. (E) Transwell invasion assay after treatment on Ishikawa cells. Error bars represent the relative rate of invasive cell numbers compared to control from three independent experiments (×200). **P < 0.01.

0.01461 cm^3, P = 0.0001, Fig. 5A–C). We further examined all the important organs of these mice, but found no metastasis. We further extracted protein from these xenografted tumors and performed IHC and Western blotting to confirm the signaling pathway in vivo. As a result, the phosphorylated level of AKT(T308), mTOR and 4E-BP1 altered accompanied by the changes of MTA1 (Fig. 5D–E). These results indicated that MTA1 might promote endometrial tumor growth via AKT/mTOR/4E-BP1 pathway.

4. Discussion

With the first identification of MTA1 as a metastasis-relevant oncogene in 1994 [17], many studies focusing on the relationship between MTA1 and human cancer have been reported. Patients with high expression of MTA1 had significantly worse OS and recurrence-free survival (RFS) than those with low expression of MTA1 among lung adenocarcinoma of stage I-III [18]. Immunohistochemical staining

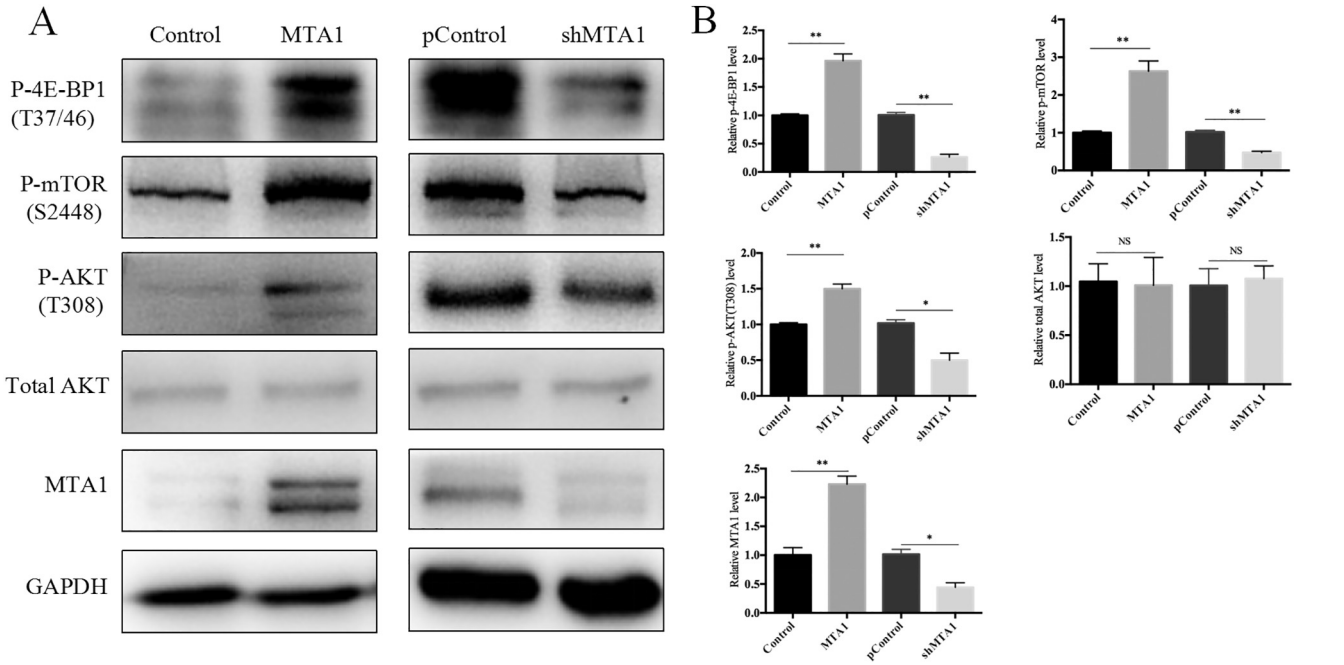
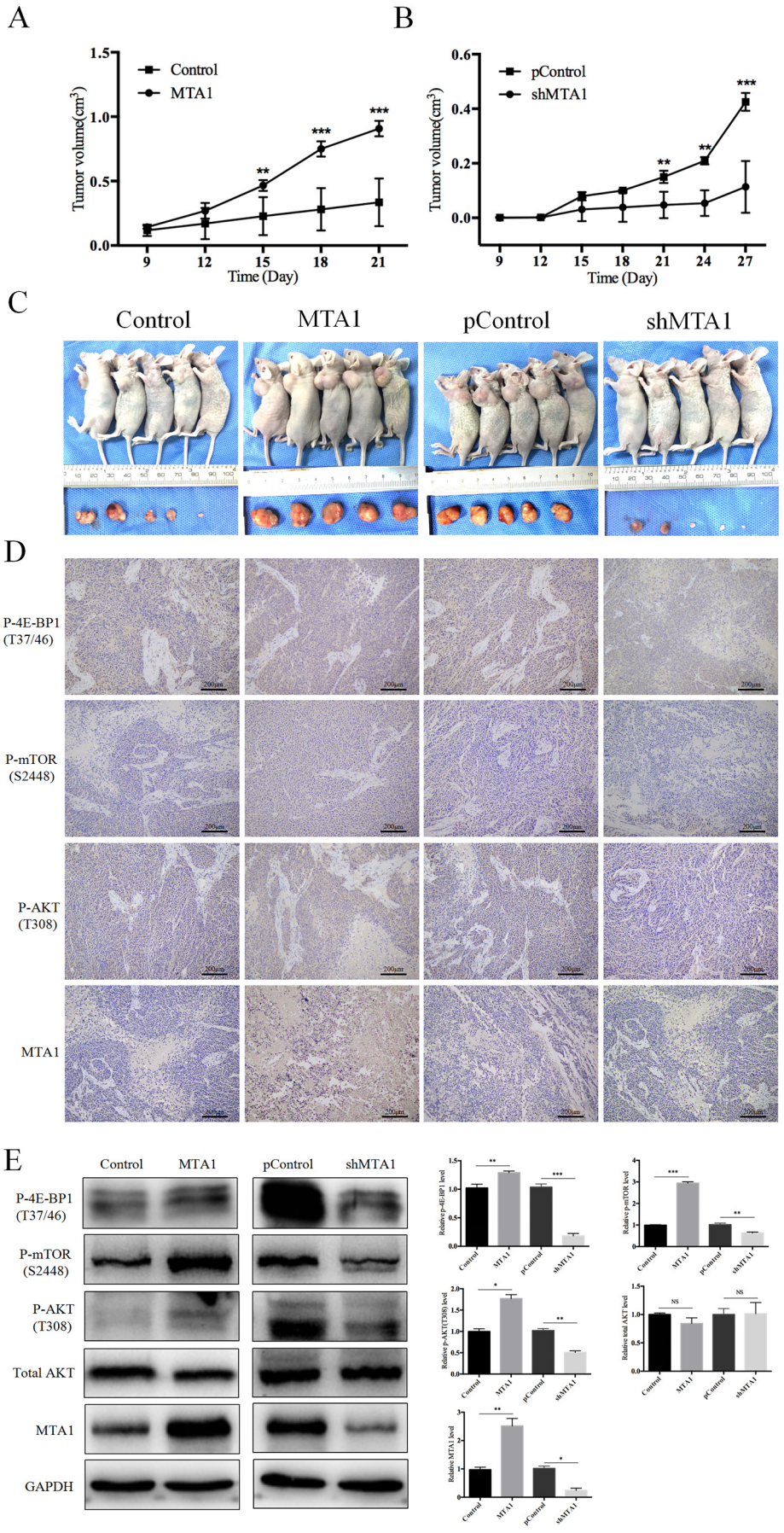


Fig. 4. MTA1 facilitates EC cell progression via AKT/mTOR/4E-BP1 pathway. (A) Representative immunoblot of multiple proteins after overexpression and knockdown of MTA1 in Ishikawa cells. (B) Quantitative graphs of the proteins level. Data were from three independent experiments and were expressed as means ± SEM. *P < 0.05, **P < 0.01.

revealed that MTA1 was overexpressed in gastric cancer tissues compared to matched normal tissues and patients with high MTA1 expression had a significantly lower five-year survival rate compared with patients with low MTA1 expression in gastric cancer [19]. Similarly, high levels of MTA1 were present and associated with poorer survival in nasopharyngeal cancer, hepatocellular cancer, ovarian cancer [7,8,20]. However, to our knowledge, the expression level of MTA1 and its role in EC have not been studied thoroughly. Based on the bioinformatic and experimental technology, we for the first time revealed that MTA1 was overexpressed both at transcriptome and protein levels in EC compared to normal samples and its high level was also associated with an advanced clinicopathology and poor prognosis. For our human studies looking at MTA1 expression, there seems to be a discrepancy that increase in mRNA expression of MTA1 was associated with histology and poor survival while increased IHC protein expression was only associated with lymph node metastases. As we known, RNA-to-protein (RTP) ratio varies significantly with a few hundred copies per mRNA molecule for some genes to several hundred thousands of protein copies per mRNA molecule for others. The transcript and protein levels do not correlate well unless a gene-specific RTP conversion factor independent of the tissue type is introduced, thus significantly enhancing the predictability of protein copy numbers from RNA levels [21]. On the other hand, too few non-endometrioid cancer samples for IHC detection might affected our statistical results for the relationship between MTA1 protein expression and cancer histology. Furthermore, considering that lymph node metastasis is always associated with advanced clinical cancer stages and poor prognosis, overexpression of MTA1 protein might be associated with a poorer survival, though MTA1 protein-associated survival analysis was not performed. Thus, it was reasonable between what is found when looking at protein expression by IHC versus mRNA expression.

It was interesting that MTA3, also a member of MTA family, was significantly reduced in poor differentiated endometrioid adenocarcinomas compared to those well differentiated tumors [22], which seemed to be opposite of MTA1. Similar phenomena were also observed in gastrointestinal cancer [23], ovarian cancer [24] and breast cancer [25]. For advanced breast and ovarian cancer were cancer types whose first progression were related to steroid hormone receptor expression and activation, a contrived explanation for different expressions of the two MTA members in a same cancer was that the expression level of MTA3 itself was shown to be hormone-dependent [26]. Further studies on its mechanism were needed.

As reported for the first time in our previous study, MTA1 was a target of miR-30c [12]. Since miR-30c suppressed EC not only on cell invasion but also on cell proliferation, we performed both cell invasion and cellular growth to assess the function of MTA1. It was surprising that MTA1 had both carcinogenic functions and could reverse the carcinostatic effects of miR-30c on EC cells. Thus, we demonstrated that the negative regulatory effect of miR-30c on EC cells resulted from targeting MTA1, at least in part. MTA1 was first identified to be regulated by microRNA (miR-661) and thereby showed tumor-promoting functions in breast cancer cell [27]. Several other different miRNAs including miR125a, miR-543, miR-421, and miR-30e were subsequently reported to target MTA1 in an anticancer way like miR-30c [28–31]. Based on our results, miR-30c/MTA1 might play an important role in the progression of EC. Since the PI3K/AKT/mTOR pathway has been implicated in the pathogenesis of endometrial cancer and activated AKT initiates a cascade of downstream signaling events, including cellular metabolism, proliferation, migration, apoptosis, and angiogenesis [16], we chose this signal pathway preferentially to be investigated. Fortunately with a surprise, the selected phosphorylated level of AKT (T308) and mTOR altered accompanied by the changes of MTA1, which indicated a downstream signaling pathway of MTA1. The 4E-BP1 protein could be phosphorylated directly by mTOR on T37/T46, and exerted significant control over cap-dependent translation, cell growth, cancer initiation, and progression [32], so we also detected the 4E-BP1 protein level and resulted in a similar effect subsequently. Given the importance of PI3K/AKT/mTOR signaling in the development of human cancers, the promoting function of MTA1 towards cell proliferation, migration and invasion may be attributed to its activation of AKT/mTOR signaling. MTA-associated epithelial-mesenchymal transition (EMT) was known as an important process during cancer metastasis [33]. Overexpressed MTA1, accompanied by reduced MTA3, resulted in aberrant expression of the transcriptional repressor Snail and down-regulation of E-cadherin, which favors EMT and progression of cancer [24,25]. These data led us to speculate that MTA1 might also promote


A
Control
MTA1
Tumor volume(cm3)
Time (Day)
B
pControl
shMTA1
Tumor volume(cm3)
Time (Day)
C
Control
MTA1
pControl
shMTA1
D
P-4E-BP1 (T37/46)
P-mTOR (S2448)
P-AKT (T308)
MTA1
E
Control
MTA1
pControl
shMTA1
P-4E-BP1 (T37/46)
P-mTOR (S2448)
P-AKT (T308)
Total AKT
MTA1
GAPDH

cell migration and invasion via Snail-mediated EMT in EC, though there was no basic experimental support.

Amount of clinicopathological data that MTA1 overexpression was positively linked to the involvement of advanced cancer stages and poor prognosis promoted researchers to investigate the molecular mechanism directly relied on MTA1. Through recruitment of HDACs to target gene promoters, MTA1 was implicated in the transcriptional repression of gene expression via a NuRD-dependent way [34]. It could recruit HDAC4 along with the transcription factor Yin-Yang 1 (YY1) onto the PTEN promoter and transcriptionally represses its expression [35]. By combining to two distinct regions of the Six3 regulatory chromatin with HDAC complexes, MTA1 transcriptionally repressed the expression of Six3 [36]. MTA1 also acted as a transcriptional activator for many target genes through the nucleosome remodeling factor (NURF) coactivator complex [37,38]. MTA1 could promote transcription of ErbB2 by binding of MTA1/HACD2 complex to the promoter region of ErbB2 in hepatocellular carcinoma [28]. MTA1 also could stimulate transcription of STAT3 through action on the MTA1/STAT3/Pol II coactivator complex in breast cancer [39]. The underlying mechanism for MTA1-mediated switch between gene activation and repression events might lie in dynamic change from methylation to demethylation [37]. However, the direct downstream effectors of MTA1 were not present in our study and the exact mechanism in EC needed further study.

Given its functional importance in tumor growth and metastasis, MTA1 has emerged a potential target for developing anticancer strategies. While resveratrol could reactivate PTEN through inhibiting MTA1/HDAC complex and recalling its acetylation status and resulted in inhibition of prostate cancer progression [40], the experimental downregulation of MTA1 promoted cancer cells to resveratrol-induced apoptosis in prostate cancer [9]. Chen et al. showed that beta-elemene as a noncellular antineoplastic agent, could downregulate MTA1 expression and inhibit bladder cancer [41]. MTA1 overexpression induced cisplatin resistance, while depletion of MTA1 increased cisplatin susceptibility in nasopharyngeal cancer [42]. Pterostilbene could attenuate prostate cancer progression by targeting MTA1/HIF-1α signaling in combination with histone deacetylase inhibitor [43]. Our experimental data showed that silencing of MTA1 could inhibit EC both in vitro and vivo, which indicated the potential therapy target of MTA1 in EC.

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Author contribution

X. X., J. L. and H. Z. designed the experiments and prepared the manuscript; X. X., X. K., T. L., L. Z., Y. W., M. Z., L. D., S. Y., J. W., Q. Y. and J. F. performed the experiments; J. L., R. L., X. Z. X. T., Y. Z.,Y. D. and H. Z. collected clinical specimens; all authors analyzed the data; H. Z. conceived and supervised the project.

Declaration of interest

The authors report no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygyno.2019.04.005.

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Fig. 5. MTA1 promoted endometrial tumor growth via AKT/mTOR/4E-BP1 pathway. (A and B) Tumor volumes were evaluated every 3 days, and tumors were harvested and tumor growth curves were made after 21 days (overexpression group, n = 5) and 28 days (knockdown group, n = 5). (C) Representative photos of tumorigenesis of mice treated with MTA1-overexpressed and MTA1-silenced Ishikawa cells. (D) Representative immunohistochemistry (IHC) of the level of MTA1, p-AKT(T308), p-mTOR(S2448) and p-4E-BP1(T37/46) in the tumors (×200). (E) Western blot analysis for MTA1, p-AKT(T308), p-mTOR(S2448) and p-4E-BP1(T37/46) in the tumors. *P < 0.05, **P < 0.01, ***P < 0.001.

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