Long Non-Coding RNA MEG3 Inhibits Cell Proliferation and Induces Apoptosis in Prostate Cancer

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Key Words
Long non-coding RNAs • MEG3 • Prostate cancer • Apoptosis

Abstract
Background/Aims: Long non-coding RNAs (IncRNAs) play important roles in diverse biological processes, such as cell growth, apoptosis and migration. Although downregulation of IncRNA maternally expressed gene 3 (MEG3) has been identified in several cancers, little is known about its role in prostate cancer progression. The aim of this study was to detect MEG3 expression in clinical prostate cancer tissues, investigate its biological functions in the development of prostate cancer and the underlying mechanism. Methods: MEG3 expression levels were detected by qRT-PCR in both tumor tissues and adjacent non-tumor tissues from 21 prostate cancer patients. The effects of MEG3 on PC3 and DU145 cells were assessed by MTT assay, colony formation assay, western blot and flow cytometry. Transfected PC3 cells were transplanted into nude mice, and the tumor growth curves were determined. Results: MEG3 decreased significantly in prostate cancer tissues relative to adjacent normal tissues. MEG3 inhibited intrinsic cell survival pathway in vitro and in vivo by reducing the protein expression of Bcl-2, enhancing Bax and activating caspase 3. We further demonstrated that MEG3 inhibited the expression of cell cycle regulatory protein Cyclin D1 and induced cell cycle arrest in G0/G1 phase. Conclusions: Our study presents an important role of MEG3 in the molecular etiology of prostate cancer and implicates the potential application of MEG3 in prostate cancer therapy.

G. Luo and M. Wang contributed equally to this article and should be considered co-first authors.
**Introduction**

Prostate cancer is one of the most commonly diagnosed cancer and the second most common cause of cancer death in men in the US [1-3]. About one million new cases are diagnosed with prostate cancer every year and it accounts for nearly 10% of all new cancer cases in men worldwide [4, 5]. In addition, about one-third of patients with organ-confined prostate cancer fail local therapy and progress to advanced-staged or metastatic disease in 10 years [6]. Metastatic prostate cancer may finally develop into androgen-independent prostate cancer and hormone refractory prostate cancer, which is the major cause of death in prostate cancer patients [7-9]. Thus, understanding carcinogenesis and development mechanism of prostate cancer is urgently needed for developing new therapies, especial for patients with androgen-independent prostate cancer and hormone refractory prostate cancer.

LncRNAs, which are currently defined as transcripts containing >200 nucleotides without evident protein coding function, were once considered to be transcriptional “noise”. But more and more studies have revealed that lncRNAs play significant roles in a large range of biological processes, including cell differentiation, proliferation and apoptosis [10-14]. Moreover, they are also important factors in pathophysiology including cancer. One such lncRNA gene is MEG3, located on chromosome 14q32. It is an imprinted gene expressed from the maternal allele with a length of about 1.6 kb nucleotides. Although MEG3 is expressed in many human normal tissues, the loss of MEG3 expression has been found in various types of human tumors, including non-small cell lung cancer, bladder cancer, prostate cancer and other cancers [15-20]. Furthermore, MEG3 can inhibit cancer cells proliferation or induce apoptosis in vitro and in vivo by stimulating p53-dependent transcription [21-23].

However, the biological role of MEG3 and its mechanism in prostate cancer remain largely unknown. In the present study, we characterized the expression of MEG3 in prostate cancer tissues and adjacent normal prostate tissues, and explored its effects on two androgen-independent prostate cancer cell lines (PC3 and DU145). To the best of our knowledge, these data establish for the first time that MEG3 plays a great role in the genesis and development of prostate cancer.

**Materials and Methods**

**Clinical samples and tissue processing**

Twenty-one paired prostate cancer tissues and adjacent normal prostate tissues were obtained from patients who underwent surgery at Wuhan Union Hospital between 2011 and 2014. All of the patients didn’t have any distant metastasis. No local or systemic treatment was conducted in these patients before surgery. All of the specimens were collected within 30 min after prostate resection and stored in liquid nitrogen immediately until RNA extraction. The research was approved by the Institutional Review Board of Tongji Medical College of Huazhong University of Science and Technology, and informed consent was obtained from each patient before surgery.

**Plasmids and small interfering RNA**

Full-length MEG3 sequence lacking a poly A tail was synthesized (based on the MEG3 sequence, NC_000014.9, in NCBI) and then subcloned into pCDNA3.1 vector (pCDNA-MEG3, Shanghai Genechem Co., Ltd., Shanghai, China). Empty vector (pCDNA) was used as a control. To ectopically express Cyclin D1, the synthetic Cyclin D1 sequence was subcloned into pCDNA3.1 vector (pCDNA-Cyclin D1, Shanghai Genechem Co., Ltd.). Three MEG3 siRNAs (si-MEG3-1, si-MEG3-2 and si-MEG3-3) and scrambled negative control siRNA (si-NC) were purchased from Invitrogen (Invitrogen, Carlsbad, CA, USA). The target sequences of these siRNAs were as follows: si-MEG3-1, 5′-GGG CTT CTG GAA TGA GCAT-3′; si-MEG3-2, 5′-CCT CTT ACC TAA AGA CTTA-3′; si-MEG3-3, 5′-GCT CAT ACT TTG ACT CTAT-3′.

**Cell culture and transfection**

Androgen-independent prostate cancer cell lines (PC3 and DU145) and hepatocellular cancer cell line (HepG2) were purchased from Chinese Academy of Sciences (Shanghai, China). Both were cultured in
RPMI-1640 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco). The cells were incubated at 37°C in a humid atmosphere with 5% CO₂. Cells were transfected with si-MEG3, si-NC, pCDNA-MEG3 or pCDNA using Lipofectamine2000 Transfection Reagent (Invitrogen) according to the manufacturer’s instructions. Cells were harvested after 48 hours of transfection for RNA and protein extraction. G418 (Gibco, final concentration: 500 μg/mL) was used 24 hours after transfection for 2 weeks to select the pCDNA-MEG3 and pCDNA stably transfected cells. The stably transfected cells were prepared for colony formation assay and tumor formation assay in nude mouse model.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from tissues or cultured cells with Trizol reagent (Invitrogen) according to the manufacturer’s protocol. The isolated RNA was reverse transcribed into cDNA using a reverse transcription kit (Takara Biotechnology, Dalian, China). The expression was quantified by qRT-PCR using a standard protocol from the SYBR Green PCR kit (Toyobo, Osaka, Japan) on the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The PCR primers were presented in Table 1. The PCR reaction was conducted at 95°C for 30 s followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Each sample was analyzed in triplicate and the relative expression was calculated using the 2^ΔΔCt method relative to GAPDH.

Cell viability assay

Prostate cancer cells were seeded at a density of 2,500 cells per well in 96-well plates and transfected with si-NC, si-MEG3, pCDNA or pCDNA-MEG3. At the indicated time-points, 100 μl MTT (0.5 mg/ml, Sigma, Saint Louis, MO, USA) was added to each well and cells were incubated for 4 hours at 37°C in a humidified chamber. Then the solution was discarded and 200 μl DMSO (Sigma) was added into the well to dissolve formazan crystals. Finally, plates were shaken for 15 min for complete dissolution. The optical density (OD) at 570 nm was measured by Windows Revelation QuickLink software, on Opsys MR spectrophotometer (DYNEX Technologies, Denkendorf, Germany). All the experiments were performed in triplicate.

Colony formation assay

Cells stably transfected with pCDNA-MEG3 or empty vector were seeded into 6-well plates at a density of 1,500 cells per well and cultured for 14 days. Cells were subsequently fixed with methanol for 15 min and stained with 0.1% crystal violet for 30 min. The number of colonies containing more than 50 cells was counted using an inverted microscope.

Flow cytometry analysis of apoptosis

PC3 and DU145 cells were cultured in six-well plates and harvested 48 hours after transfection. Apoptosis was measured using an Annexin V-PE/7-AAD kit accordance with the manufacturer’s protocols (BD Pharmingen, San Diego, CA, USA). Briefly, cells were resuspended in the binding buffer provided and reacted with 5 μl Annexin V-PE and 5 μl 7-AAD for 15 min at room temperature in the dark. 400 μl binding buffer was added and then stained cells were analyzed by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA, USA).

Table 1. Primers for qRT-PCR

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>MEG3 forward</td>
<td>5’-CTGCCCATCTAGCTACCTAGG-3’</td>
</tr>
<tr>
<td>MEG3 reverse</td>
<td>5’-CTCTCCGGCCCTGCTGCTAGGGC-3’</td>
</tr>
<tr>
<td>GAPDH forward</td>
<td>5’-GAAGTGAAGTGGCGAGTC-3’</td>
</tr>
<tr>
<td>GAPDH reverse</td>
<td>5’-GAAGATGGTGATGGCCT-3’</td>
</tr>
<tr>
<td>Cyclin D1 forward</td>
<td>5’-GCTGCGAAGTGAAAAACATC-3’</td>
</tr>
<tr>
<td>Cyclin D1 reverse</td>
<td>5’-ACCTCTCTCAGCACATTTGAA-3’</td>
</tr>
<tr>
<td>Bax forward</td>
<td>5’-CCCGAGAGGTTCTTTTCCG-3’</td>
</tr>
<tr>
<td>Bax reverse</td>
<td>5’-CCAGCCCATAGGTGTCAT-3’</td>
</tr>
<tr>
<td>Bcl-2 forward</td>
<td>5’-GGTGGGCTATGTGCATTGG-3’</td>
</tr>
<tr>
<td>Bcl-2 reverse</td>
<td>5’-CCGTYYAGGTGATCTAGTCC-3’</td>
</tr>
</tbody>
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Flow cytometry analysis of cell cycle

Transfected PC3 and DU145 cells were cultured in six-well plates. For cell cycle assay, the cells were harvested and fixed in 70% ethanol at -20°C overnight. Fixed cells were washed with PBS and incubated in 400 μl PBS, 50 μl RNase (1 mg/ml) and 10 μl propidium iodide (PI) (2 mg/ml, Keygen biotech, Nanjing, China) for 30 min at room temperature, followed by flow cytometry analysis (FACScan). The experiment was performed independently three times for each cell line.

Western blot assay

Cells were collected after 48 hours of transfection and resuspended in RIPA lysis buffer (Thermo Scientific, Rockford, IL, USA). 20 μg of total protein was separated on 10% SDS-polyacrylamide gels (SDS-PAGE) and transferred onto PVDF membranes. The membranes were blocked with 5% nonfat milk and incubated with specific antibodies overnight at 4°C. The specific antibodies for GAPDH, Cyclin A, Cyclin D1, Cyclin E, P21, Bax, Bcl-2 and Cleaved caspase 3 proteins were purchased from Cell Signaling Technology (Cell Signaling Technology, Danvers, MA, USA). The membranes were incubated with the specific HRP-conjugated secondary antibodies (Santa Cruz Biotech) at room temperature for 1 hour. ECL chromogenic substrate (Beyotime, China) was used to visualize the bands. GAPDH was used as a control.

Tumor formation assay in a nude mouse model

Animal care and protocols were approved by the Institutional Animal Care and Use Committee of Huazhong University of Science and Technology. Four-week-old male BALB/C mice were randomly divided into two groups (n = 5 in each group): pCDNA and pCDNA-MEG3. 5 × 10^6 PC3 cells (0.2 ml) that stably transfected with pCDNA or pCDNA-MEG3 were injected subcutaneously on the right flanks of the mice. The diameters of the tumors were measured every 3 days. Three weeks after inoculation, the mice were sacrificed by cervical dislocation, and the tumors were resected, measured and weighed. Tumor volume was calculated using the equation: volume = 0.5 × W^2 × L (W, width; L, length). Then, the tumor tissues were fixed in 10% formalin solution for immunostaining analysis.

Statistical analysis

Data were analyzed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The measurement data were expressed as the mean ± SD. For the statistical analysis of clinicopathological features and mRNA expression of MEG3, MEG3 expression in cancer tissues was divided by that in matched normal tissues (T/N expression ratio). Samples were then classified into two groups, with the low expression group having a T/N ≤ 0.5 and the high expression group having a T/N >0.5. Two-tailed Student’s t-test and one-way ANOVA were performed to analyze the in vitro and in vivo data. Statistically significant differences were established at p < 0.05.

Results

MEG3 expression is down-regulated in human prostate cancer tissues

To find whether lncRNA MEG3 was differentially expressed in the prostate cancer tissues, a total of 21 paired prostate cancer tissues and adjacent normal tissues were analyzed for MEG3 expression using qRT-PCR and normalized to GAPDH. Our results showed that MEG3 expression was significantly downregulated in cancer tissues compared with normal tissues (P < 0.05) (Fig. 1). Moreover, we evaluated the correlation of MEG3 expression with clinicopathological parameters. As presented in Table 2, there was no significant relationship between MEG3 expression and clinical characteristics.
MEG3 impairs viability of PC3 and DU145 cells

To explore the effect of MEG3 in prostate cancer in vitro, we first measured its expression in prostate cancer cell lines. The expression levels of MEG3 in PC3 and DU145 cells were compared with that in HepG2 cells, one of the cell lines lacking the expression of MEG3 [24]. As shown in Fig. 2A, PC3 and DU145 cells expressed higher levels of MEG3 compared with HepG2 cells. Next, we transfected pCDNA, pCDNA-MEG3, si-NC and si-MEG3 into PC3 and DU145 cell lines. qRT-PCR analysis of MEG3 expression was performed 48 hours after transfection. Compared with control cells, MEG3 expression was increased 166 and 102 fold by pCDNA-MEG3 (Fig. 2B), and reduced by 64% and 61% by si-MEG3-1 in PC3 (Fig. 2C) and DU145 (Fig. 2D) cells respectively. To confirm the validity of MEG3 reduction, si-MEG3-1 was used in follow-on experiments and abbreviated to si-MEG3. MTT assays were performed to determine the viability of PC3 (E and F) and DU145 (G and H) cells. (* represents p < 0.05).
MEG3 suppresses proliferation of PC3 and DU145 cells

Then, we attempted to determine whether MEG3 affects prostate cancer cell proliferation \textit{in vitro}. Colony formation assays were performed and date showed that overexpression of MEG3 suppressed the colony formation capacity of prostate cancer cells (Fig. 3A and 3B). To further confirm this, flow cytometry analysis of cell cycle was performed. Results demonstrated that overexpression of MEG3 increased the proportion of cells in G0/G1 phase and decreased the proportion of cells in S phase compared with the control (Fig. 3C and 3D). After knockdown of MEG3, the G0/G1 phase cell rate declined and the S phase rate ascended (Fig. 3E and 3F). These findings suggested that MEG3 could inhibit the proliferation of prostate cancer cells \textit{in vitro}.

Fig. 3. The effects of MEG3 on cell proliferation. Representative micrographs (A) and quantifications (B) of crystal violet stained colonies formed by the indicated cells. (C) PC3 and DU145 cells were treated with pCDNA or pCDNA-MEG3 and cell cycle distribution was detected. (D) The histograms were analyzed and the percentage of cells in each phase of the cell cycle was shown. (E) MEG3 expression was inhibited by specific siRNAs and cell cycle distribution was analyzed by flow cytometry. (F) The respective proportion of cells in the G0/G1 phase, S phase, and G2/M phase. Results were expressed as mean ± SD for 3 replicates. (*) represents p < 0.05.
MEG3 induces apoptosis of PC3 and DU145 cells

As defects in apoptosis could lead to tumorigenesis, we further explored whether MEG3 affected apoptosis of prostate cancer cells. Flow cytometry analysis was performed and results showed that the apoptotic rates of PC3 and DU145 cells transfected with pCDNA-MEG3 increased significantly compared to the control (Fig. 4A-4D). After knockdown of MEG3, the apoptotic rate of PC3 cells decreased (Fig. 4E and 4F).

MEG3 induces Bax protein expression, inhibits Bcl-2 and Cyclin D1 protein expression and activates caspase 3

To explore the mechanism by which MEG3 induced growth arrest and apoptosis, western blot assay was carried out to examine the expression of cell cycle and apoptosis associated proteins Cyclin A, Cyclin D1, Cyclin E, P21, CDK2, Bax, Bcl-2 and Cleaved caspase 3. As shown in Fig. 5A and 5B, Bax was upregulated and Cyclin D1 and Bcl-2 were downregulated in pCDNA-MEG3-transfected cells compared with pCDNA-transfected cells. Meanwhile, the activation of caspase 3 (as indicated by Cleaved caspase 3) increased in pCDNA-MEG3 group. Conversely, Bax was downregulated and Cyclin D1 and Bcl-2 were upregulated in si-MEG3-transfected cells compared with si-NC-transfected cells. In addition, the activation of caspase 3 decreased in si-MEG3 group. However, the variation in MEG3 expression did not affect the expression of Cyclin A, Cyclin E, P21 and CDK2 protein. Next, we performed qRT-PCR assay.
to examine the expression of Cyclin D1, Bax and Cleaved caspase 3, and results showed that Cyclin D1 mRNA was negatively regulated by MEG3. However, there was no significant change in Bcl-2 and Bax mRNA (Fig. 5C).

To further verify the role of Cyclin D1 in cell cycle changes, pCDNA-Cyclin D1 were co-transfected with pCDNA-MEG3 into PC3 cells. As shown in Fig. 5D, compared to the pCDNA-MEG3 group, the expression of Cyclin D1 protein increased in cells co-transfected with pCDNA-Cyclin D1. In addition, transfection of pCDNA-Cyclin D1 effectively reversed the G0/G1 phase arrest induced by pCDNA-MEG3 (Fig. 5E and 5F).

**MEG3 inhibits prostate cancer cells tumorigenesis in vivo**

To investigate whether the upregulation of MEG3 could suppress the tumor formation in vivo, pCDNA-MEG3 or empty vector stably-transfected PC3 cells were inoculated into male nude mice. Consistent with the results, the tumors formed in the pCDNA-MEG3 group were obviously smaller than those in the pCDNA group (Fig. 6B and 6C), and the tumor weights derived from cells transfected with pCDNA-MEG3 were also significantly less than those in the pCDNA group (Fig. 6D). Similar to the results of the western blot assay, the immunohistochemistry analysis revealed that Bax was upregulated and Cyclin D1 and Bcl-2 were downregulated in pCDNA-MEG3 group compared to the pCDNA group. Likewise, the activation of caspase 3 increased in pCDNA-MEG3 group (Fig. 6E). These results suggested that upregulation of MEG3 could significantly inhibit prostate cancer cell growth in vivo and then resulted in delayed tumor progression.
Discussion

LncRNAs were once considered to be nucleic acids without any functions. But recent studies have indicated that lncRNAs are important players in the carcinogenesis and aggressive progression of human malignancies [25, 26]. To date, many lncRNAs have been identified, and their involvement in prostate cancer has been reported. Prostate cancer upregulated long noncoding RNA 1 (PlncRNA-1) is upregulated in prostate cancer and silencing PlncRNA-1 expression inhibits the proliferation and promotes the apoptosis of prostate cancer cells [27]. LncRNA H19 plays a tumor-suppressive role in metastatic prostate cancer by repressing the effects of TGFβ1 [28]. Additionally, MALAT1, PCAT-1 and SchLAP1 are found to exert oncogenic functions in prostate cancer [29-31].

In this study, we found that the expression of MEG3 decreased in prostate cancer tissues compared to normal tissues. There was no significant relationship between MEG3 expression and clinical characteristics, and more studies with larger samples were required to confirm this. We further demonstrated that upregulation of MEG3 in prostate cancer cell lines induced cell apoptosis and G0/G1 phase arrest. On the contrary, knockdown of MEG3 increased cell viability and prompted the G1/S cell cycle transition. Similar to these findings, our in vivo data revealed that the average tumor weight and volume decreased markedly in mice injected with MEG3-transfected PC3 cells compared to the control. All of these suggested
that MEG3 played an important role in the occurrence and development of prostate cancer. Failure to induce apoptosis is a crucial factor that leads to the formation of cancer [32]. The Bcl-2 protein plays an important role in preventing cancer cell apoptosis and Bax is known for its pro-apoptotic activity [33-36]. Our results showed that MEG3-induced apoptosis of PC3 and DU145 cells was associated with the downregulation of the Bcl-2 protein and the upregulation of the Bax protein. However, the expression of Bcl-2 and Bax mRNA did not change markedly and we speculated that MEG3 functioned at the post-transcriptional level. Additionally, caspase 3, the key mediator in the apoptotic pathway [37-39], was observed to be activated by MEG3. So we deduced that MEG3 induced apoptosis possibly through targeting those proteins. Cyclin D1 is a key cell cycle regulator during the G1/S transition and abrogation of its expression leads to G1 cell cycle arrest [40-43]. Here, we showed that MEG3 induced G0/G1 arrest possibly by downregulation of Cyclin D1 in PC3 and DU145 cells.

Collectively, our study presents that MEG3 is significantly downregulated in prostate cancer tissues and exerts tumor-suppressive functions in the genesis and progression of prostate cancer, providing a potential attractive therapeutic approach for this malignancy.

Disclosure Statement

The authors declare no conflict of interest.

References

Luo et al.: MEG3 Suppresses Prostate Cancer


