

Original Article

Crosstalk between Meg3 and miR-1297 regulates growth of testicular germ cell tumor through PTEN/PI3K/AKT pathway

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Abstract: Maternally Expressed Gene 3 (Meg3) encodes a long non-coding RNA that has been recently shown to regulate tumorigenesis through its interaction with microRNA (miR). We have recently reported that miR-1297 might play a role in the regulation of PTEN/PI3k/Akt signaling pathway in testicular germ cell tumor (TGCT). However, a crosstalk between Meg3 and miR-1297 in TGCT has not been appreciated. Here, we analyzed the levels of Meg3, miR-1297 and PTEN in TGCT specimens, compared to paired adjacent non-tumor tissue (NT), and found that Meg3 levels were significantly decreased and miR-1297 levels were unchanged in TGCT. PTEN protein but not mRNA levels significantly decreased in TGCT. Bioinformatics analyses showed that miR-1297 bound to 3'-UTR of PTEN mRNA, while miR-1297 also bound to Meg3. Luciferase report assay showed that Meg3 overexpression abolished the effects of miR-1297 on 3'-UTR of PTEN mRNA, possibly through competitive binding, which was supported by double fluorescent in situ hybridization showing co-localization of intracellular Meg3 and miR-1297 signals in TGCT cells. Moreover, Meg3 overexpression abolished the inhibitory effects of miR-1297 on PTEN, resulting in deactivation of Akt and decreases in cell growth. Together, these data demonstrate a previous unappreciated pathway in which Crosstalk between Meg3 and miR-1297 regulates growth of TGCT through PTEN/PI3K/AKT signaling. Re-expression of Meg3 may be an attractive strategy for TGCT therapy.

Keywords: Testicular germ cell tumor (TGCT), Maternally Expressed Gene 3 (Meg3), miR-1297, PTEN/PI3K/AKT

Introduction

Testicular germ cell tumor (TGCT) is predominantly detected in young men aged from 25 to 34 years. TGCT originates from transformed primordial germ cells, while most patients with newly diagnosed TGCT are highly treatable [1]. However, some TGCTs have already reached advanced stage, and the therapeutic outcome of these tumors is not satisfactory [2]. Therefore, a better understanding of the molecular mechanisms underlying the carcinogenesis, tumor growth and progression is important for generating novel methods to treat TGCT [3-5].

MicroRNAs (miRNAs) are small, endogenous, non-coding RNAs of about 22 nucleotides. The miRNAs have been extensively studied recently and their potential roles in regulation of protein translation through targeting the 3'-untranslat-

ed region (3'-UTR) of the mRNA have been well established [6, 7]. It is noteworthy that miRNAs regulate many biological events including cancer initiation, progression, outgrowth and metastases [8-11]. Among all miRNAs, the involvement of miR-1297 in development of various cancers has been also reported [12-15]. Specifically, we and other recently found that miR-1297 might regulate TGCT cell growth [16, 17].

Phosphatase and tensin homolog (PTEN) gene has been identified as a tumor suppressor that is mutated in a large number of cancers at high frequency [18-28]. PTEN encodes a protein called phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase, which is comprised of a tensin-like domain and a catalytic domain, like the dual specificity protein tyrosine phosphatases [29-31]. PTEN preferentially dephosphorylates phosphoinositide substrates, and negatively

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regulates intracellular levels of Phosphoinositide 3-kinase (PI3k) to inhibit PI3k/Akt signaling pathway, activation of which is critical for growth and survival of various types of cancers [29-31]. Previous reports have shown that suppression of PTEN may play a role in TGCT pathogenesis [32-34]. Most recently, we reported miR-1297 as a potential oncogene that may induce TGCT cell proliferation by targeting PTEN and inhibiting its protein translation [16]. However, the exact molecular mechanisms remain undetermined. Moreover, the clinical relevance is uncertain.

Maternally expressed gene 3 (Meg3) is an imprinted DLK1-MEG3 locus gene that is located at human chromosome 14q32.3 [35-37]. The Meg3 gene encodes a long noncoding RNA (lncRNA) that is widely expressed in various tissues, and is often absent in cancer cells [38-41]. The loss of Meg3 expression has been shown to be a consequence of gene deletion and promoter hypermethylation, while re-expression of Meg3 has been shown to suppress in cultured tumor cell growth [38-41]. Nevertheless, a possible role of Meg3 in the tumorigenesis of TGCT has not been appreciated.

Here, we found that Meg3 levels were significantly decreased and miR-1297 levels were unchanged in TGCT specimens. Moreover, PTEN protein but not mRNA levels significantly decreased. Bioinformatics analyses showed that miR-1297 bound to 3'-UTR of PTEN mRNA, while miR-1297 also bound to Meg3. Luciferase report assay showed that Meg3 overexpression abolished the effects of miR-1297 on 3'-UTR of PTEN mRNA, possibly through competitive binding, which was supported by double fluorescent in situ hybridization showing co-localization of intracellular Meg3 and miR-1297 signals in TGCT cells. Moreover, Meg3 overexpression abolished the inhibitory effects of miR-1297 on PTEN, resulting in deactivation of Akt and decreases in cell growth.

Materials and methods

Patient specimens

Resected TGCT specimens from 33 patients were obtained together with the paired adjacent non-tumor tissues (NT) from 2009 to 2013 at Zhongshan Hospital of Fudan Univer-

sity. All patients provided signed, informed consent for their tissues to be used for scientific research. Ethical approval for the current study was obtained from Zhongshan Hospital of Fudan University. All diagnoses were based on pathological and/or cytological evidence. The histological features of the specimens were evaluated by senior pathologists according to the World Health Organization classification criteria. Obtained tissues were immediately frozen and stored at -70°C prior to RT-qPCR and Western blot.

TGCT cell lines

Human TGCT cell line NCCIT was established by Shinichi Teshima (National Cancer Institute, Tokyo, Japan) in 1985 from a mediastinal mixed germ cell tumor [42]. NCCIT was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in RPMI 1640 medium supplemented with 20% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) in a humidified atmosphere of 5% CO₂ at 37°C.

Transfection

Meg3 transgene, short-hairpin interfering RNA for Meg3 (shMeg3), miR-1297 mimics (miR-1297), and control null sequence (null) were obtained from Origene (Beijing, China). These plasmids were used to modify either Meg3 levels or miR-1297 levels, and transfected into NCCIT cells at a concentration of 50 nmol/l using Lipofectamine-2000 (Invitrogen), according to the manufacturer's instructions. Transfection efficiency was more than 95%, based on expression of a co-transgene, green fluorescent protein (GFP).

MTT assay

For assay of cell viability, cancer cells were seeded into 24 well-plate at 10000 cells per well and subjected to a MTT Kit (MTT, Roche, Indianapolis, IN, USA), according to the instruction of the manufacturer. The MTT assay is a colorimetric assay for assessing viable cell number, taking advantage that NADPH-dependent cellular oxidoreductase enzymes in viable cells reduce the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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(MTT) to its insoluble formazan in purple readily being quantified by absorbance value (OD) at 570 nm. Experiments were performed 5 times.

MicroRNA target prediction and 3'-UTR luciferase-reporter assay

MiRNAs targets were predicted with the algorithms TargetScan (<https://www.targetscan.org>) [43]. The PTEN 3'-UTR reporter plasmid and mutant were both purchased from Creative Biogene (Shirley, NY, USA). NCCIT cells were co-transfected with plasmids using Lipofectamine 2000 (5×10^4 cells per well). Cells were collected 48 hours after transfection for assay using the dual-luciferase reporter assay system gene assay kit (Promega), according to the manufacturer's instructions.

In situ hybridization

In situ hybridization analysis of Meg3 and miR-1297 mRNA in NCCIT cells was performed as has been previously described [44]. Briefly, the digoxigenin (DIG)-labeled and biotin-labeled riboprobes for Meg3 and miR-1297 were synthesized by in vitro transcription, using the fragments of Meg3 and miR-1297 amplified from NCCIT cells by PCR with the Expand High Fidelity PCR system (Roche Applied Science, Nutley, NJ, USA), followed by cloning into pGEM-T vector (Promega). The amplicons were confirmed by DNA sequencing.

Western blot

Protein was extracted from the resected specimens or cultured cells with RIPA lysis buffer (1% NP40, 0.1% Sodium dodecyl sulfate (SDS), 100 μ g/ml phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, in PBS) on ice. The supernatants were collected after centrifugation at $12000 \times g$ at 4°C for 20 min. Protein concentration was determined using a BCA protein assay kit (Bio-rad, China), and whole lysates were mixed with 4 \times SDS loading buffer (125 mmol/l Tris-HCl, 4% SDS, 20% glycerol, 100 mmol/l Dithiothreitol (DTT), and 0.2% bromophenol blue) at a ratio of 1:3. Samples were heated at 100°C for 5 min and were separated on SDS-polyacrylamide gels. The separated proteins were then transferred to a PVDF membrane. The membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated second antibody,

autoradiograms were prepared using the enhanced chemiluminescent system to visualize the protein antigen. The signals were recorded using X-ray film. Primary antibodies were rabbit anti-PTEN, anti-Akt and anti- α -tubulin (Cell Signaling, San Jose, CA, USA). Secondary antibody is HRP-conjugated anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). α -tubulin was used as a protein loading control. The protein levels were first normalized to α -tubulin, and then normalized to the experimental control.

Quantitative real-time PCR (RT-qPCR)

Total RNA were extracted with miRNeasy mini kit (Qiagen, Hilden, Germany). For cDNA synthesis, complementary DNA (cDNA) was randomly primed from 2 μ g of total RNA using the Omniscript reverse transcription kit (Qiagen). RT-qPCR was subsequently performed in triplicate with a 1:4 dilution of cDNA. All primers were purchased from Qiagen. Data were collected and analyzed using $2^{-\Delta\Delta\text{Ct}}$ method. Values of genes were first normalized against α -tubulin, and then compared to controls.

Statistical analysis

All statistical analyses were carried out using the SPSS 18.0 statistical software package. All data were statistically analyzed using one-way ANOVA with a Bonferroni correction, followed by Fisher's exact test. All values are depicted as mean \pm standard deviation and are considered significant if $p < 0.05$.

Results

Meg3 levels and PTEN protein decrease, while PTEN mRNA and miR-1297 levels are unchanged in TGCT

We recently reported miR-1297 as a potential oncogene that may induce TGCT cell proliferation by targeting PTEN and inhibiting its protein translation. In order to figure out clinical relevance and further elucidate the underlying mechanisms, we analyzed the levels of PTEN in the TGCT specimens from the patients, and compared to the paired adjacent non-tumor tissue (NT). We found that the mRNA levels of PTEN in TGCT specimens were not significantly changed (**Figure 1A**), but the protein levels of PTEN in TGCT were significantly lower than NT

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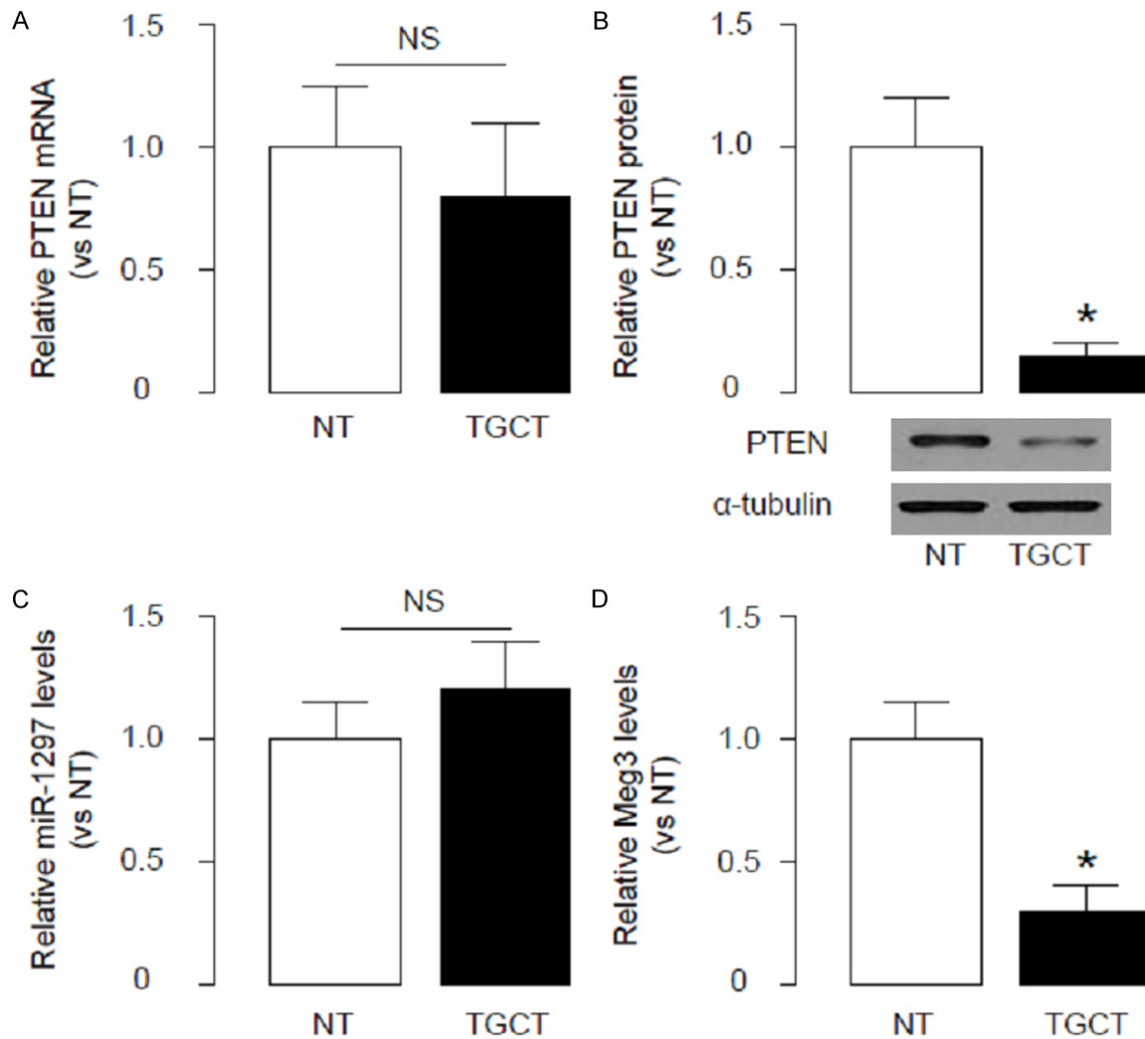


Figure 1. Meg3 levels and PTEN protein decrease, while PTEN mRNA and miR-1297 levels are unchanged in TGCT. We analyzed the levels of PTEN in the TGCT specimens from the patients, and compared to the paired adjacent non-tumor tissue (NT). (A, B) We found that the mRNA levels of PTEN in TGCT specimens were not significantly changed (A), but the protein levels of PTEN were significantly lower than NT (B). (C) We did not detect significant changes in miR-1297 levels in TGCT. (D) We found that another non-coding RNA, Meg3, significantly decreased in TGCT, compared to NT. * $p < 0.05$. NS: non-significant. N=33.

A predicted binding of miR-1297 to 3'-UTR of PTEN mRNA



B predicted binding of miR-1297 to Meg3

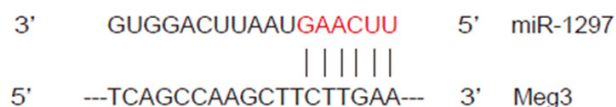


Figure 2. MiR-1297 binds to both PTEN mRNA and Meg3 in TGCT cells. (A, B) We performed bioinformatics analyses that showed that miR-1297 bound to 3'-UTR of PTEN mRNA (A), while miR-1297 also bound to Meg3 (B).

(Figure 1B), suggesting presence of the post-transcriptional control of PTEN. We hypothesized that miR-1297 may be a regulator of PTEN in TGCT based on our previous studies. However, we did not detect significant changes in miR-1297 levels in TGCT (Figure 1C). Interestingly, we found that another non-coding RNA, Meg3, significantly decreased in TGCT, compared to NT (Figure 1D).

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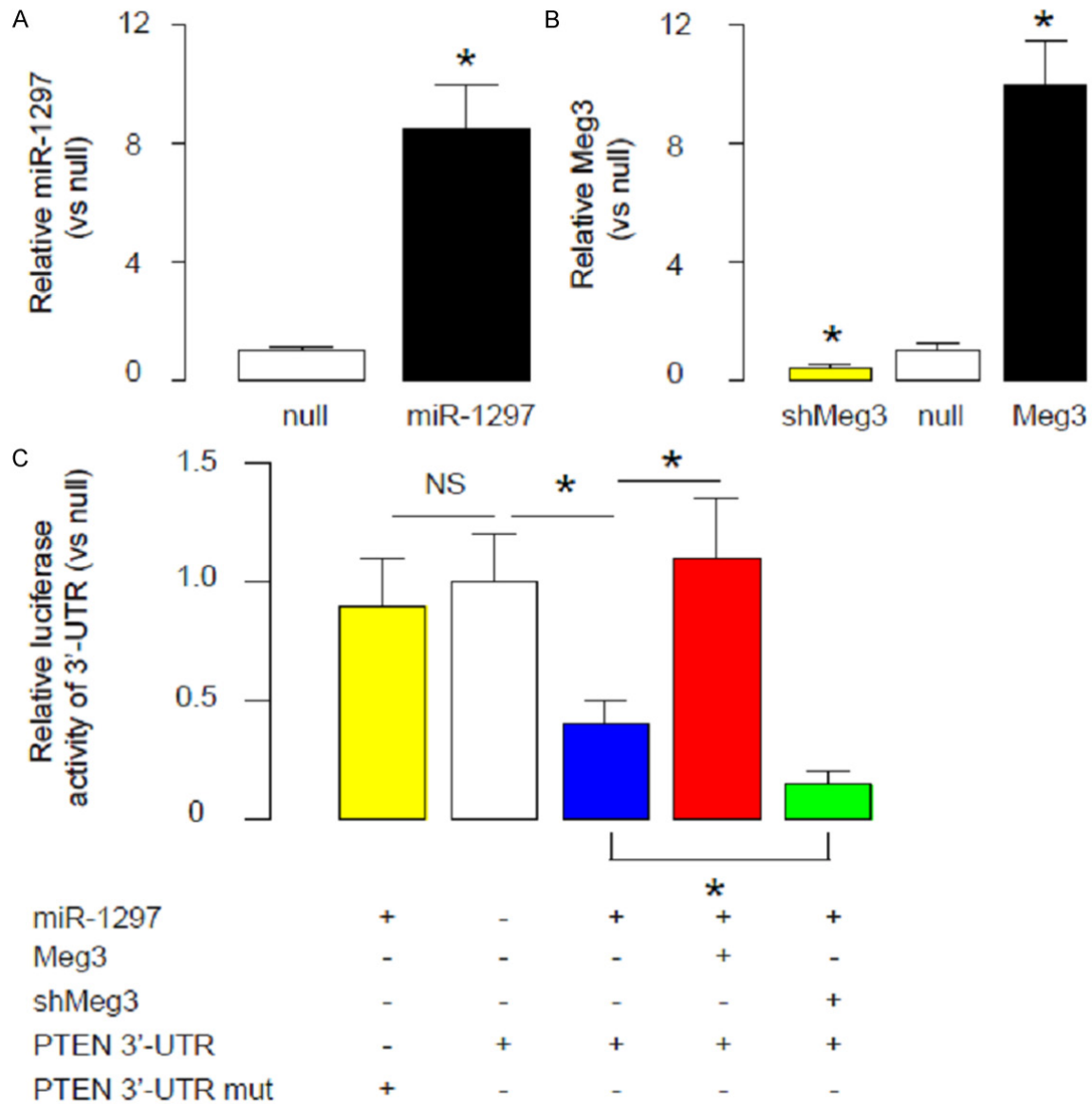


Figure 3. Meg3 competes with PTEN mRNA for binding to miR-1297. We prepared miR-1297-expressing plasmids, Meg3-expressing plasmids and Meg3-depleting plasmids (shMeg3), as well as plasmids carrying 3'-UTR of PTEN mRNA and plasmids carrying 3'-UTR of PTEN mRNA with spot mutation at miR-1297-binding sites. (A, B) The modulation of miR-1297 levels by miR-1297-expressing plasmids in a human TGCT cell line NCCIT was confirmed by RT-qPCR (A), and the modulation of Meg3 levels by either Meg3-expressing plasmids or Meg3-depleting plasmids was also confirmed by RT-qPCR (B). (C) We performed Luciferase report assay. We found that miR-1297 overexpression significantly decreased the bioluminescence of NCCIT cells transfected with 3'-UTR of PTEN mRNA, but not changed the bioluminescence of NCCIT cells transfected with 3'-UTR of PTEN mRNA mutation. Moreover, Overexpression of Meg3 abolished the suppressive effects of miR-1297 on bioluminescence, while depletion of Meg3 augmented it. * $p < 0.05$. NS: non-significant. N=5.

MiR-1297 binds to both PTEN mRNA and Meg3 in TGCT cells

Then, we performed bioinformatics analyses that showed that miR-1297 bound to 3'-UTR of PTEN mRNA (Figure 2A), while miR-1297 also bound to Meg3 (Figure 2B). Hence, we hypothesized that Meg3 may affect the binding between miR-1297 and 3'-UTR of PTEN mRNA.

Meg3 competes with PTEN mRNA for binding to miR-1297

Thus, we prepared miR-1297-expressing plasmids, Meg3-expressing plasmids and Meg3-depleting plasmids (shMeg3), as well as plasmids carrying 3'-UTR of PTEN mRNA and plasmids carrying 3'-UTR of PTEN mRNA with spot mutation at miR-1297-binding sites. First, the

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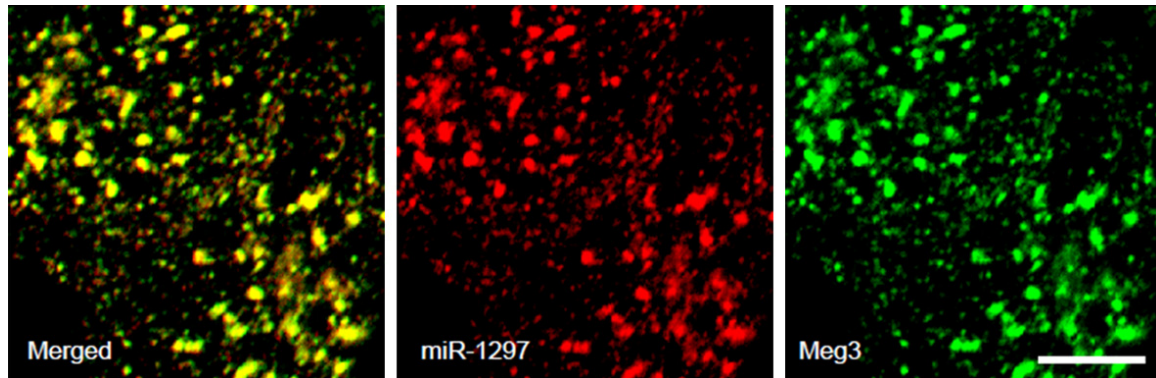


Figure 4. Co-localization of Meg3 and miR-1297 in NCCIT cells. We performed double fluorescent in situ hybridization to study the association of miR-1297 and Meg3 in NCCIT cells. We found that the signals of Meg3 and miR-1297 co-localized in NCCIT cells that were co-transfected with Meg3 and miR-1297. Scale bars are 1 μ m.

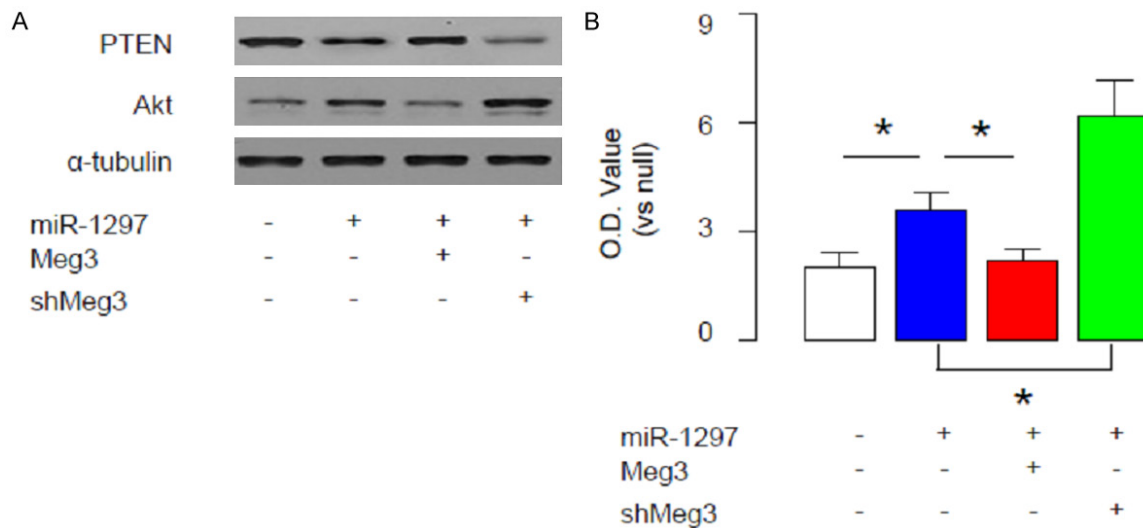


Figure 5. Meg3 contradicts the inhibitory effects of miR-1297 on PTEN to regulate Akt-mediated cell growth. We examined the NCCIT cells transfected with miR-1297, or miR-1297 and Meg3, or miR-1297 and shMeg3 for PTEN and Akt. A. We found that miR-1297 significantly decreased PTEN levels, and increased Akt. The effects of miR-1297 on PTEN and Akt were abolished by Meg3 overexpression, and augmented by Meg depletion. B. In an MTT assay, miR-1297 significantly increased cell growth. The effects of miR-1297 on cell growth were abolished by Meg3 overexpression, and augmented by Meg depletion. * $p < 0.05$. N=5.

modulation of miR-1297 levels by miR-1297-expressing plasmids in a human TGCT cell line NCCIT was confirmed by RT-qPCR (**Figure 3A**), and the modulation of Meg3 levels by either Meg3-expressing plasmids or Meg3-depleting plasmids was also confirmed by RT-qPCR (**Figure 3B**). Then we performed Luciferase report assay. We found that miR-1297 overexpression significantly decreased the bioluminescence of NCCIT cells transfected with 3'-UTR of PTEN mRNA, but not changed the bioluminescence of NCCIT cells transfected with 3'-UTR of PTEN mRNA mutation (**Figure 3C**).

Moreover, Overexpression of Meg3 abolished the suppressive effects of miR-1297 on bioluminescence, while depletion of Meg3 augmented it (**Figure 3C**). All these data suggest that Meg3 may compete with PTEN mRNA for binding to miR-1297.

Co-localization of Meg3 and miR-1297 in NCCIT cells

To confirm the physical binding between Meg3 and miR-1297, we performed double fluorescent in situ hybridization to study the associa-

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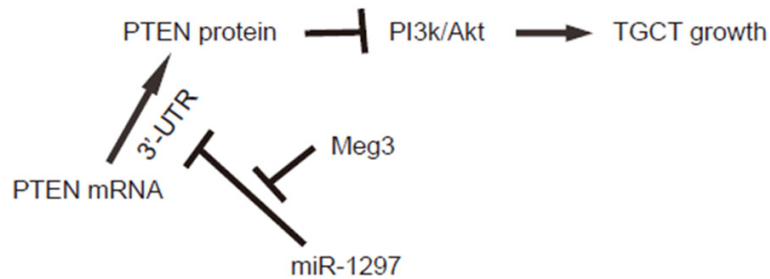


Figure 6. Schematic of the model. Meg3 may contradict the inhibitory effects of miR-1297 on PTEN to regulate Akt-mediated cell growth in TGCT.

tion of miR-1297 and Meg3 in NCCIT cells. We found that the signals of Meg3 and miR-1297 co-localized in NCCIT cells that were co-transfected with Meg3 and miR-1297 (**Figure 4**). Together, these data suggest that Meg3 and miR-1297 indeed physically bind to each other.

Meg3 contradicts the inhibitory effects of miR-1297 on PTEN to regulate Akt-mediated cell growth

Finally, we examined the NCCIT cells transfected with miR-1297, or miR-1297 and Meg3, or miR-1297 and shMeg3 for PTEN and Akt. We found that miR-1297 significantly decreased PTEN levels, and increased Akt. The effects of miR-1297 on PTEN and Akt were abolished by Meg3 overexpression, and augmented by Meg3 depletion (**Figure 5A**). Moreover, in an MTT assay, miR-1297 significantly increased cell growth. The effects of miR-1297 on cell growth were abolished by Meg3 overexpression, and augmented by Meg3 depletion (**Figure 5B**). Together, these data confirm our findings using luciferase reporter assay, and demonstrate that Meg3 may contradict the inhibitory effects of miR-1297 on PTEN to regulate Akt-mediated cell growth in TGCT (**Figure 6**).

Discussion

Identification of novel molecular regulatory pathways involved in the carcinogenesis of TGCT may provide novel strategies for clinical treatment of TGCT. Meg3 is an lncRNA and is often lost in various cancers [38-41]. Multiple mechanisms have been predicted to explain the regulation of cell growth by Meg3, e.g. through induction of apoptosis by p53. Maternal deletion of the Meg3 gene in mice has been studied and shown that the mice are suffered from skeletal muscle defects and perinatal death. Moreover, inactivation of Meg3 has

been reported to result in a significant increase in angiogenesis-promoting genes and microvessel formation in the brain. These evidence highlights Meg3 as a novel lncRNA tumor suppressor [37].

In the current study, by using bioinformatics analyses, luciferase report assay implanted with loss-of-function and gain-of-function experiments,

and double in situ hybridization, we provided strong evidence showing that Meg3 may contradict the effects of miR-1297 on the PTEN mRNA. The net effects on PTEN, which is a potential inhibitor of PI3k/Akt pathway, decide the activating levels of this signaling pathway for cell growth control. These in vitro data well explained our clinical findings, showing that miR-1297 did not alter in TGCT tissue, compared to NT. The loss of Meg3 in TGCT may weaken the suppressive effects on the binding of miR-1297 to PTEN mRNA, resulting in amplification of miR-1297 effects and the consequent decreases in PTEN protein translation.

Although here we describe a model in which Meg3 competes with PTEN mRNA for binding to miR-1297 in TGCT cells, we do not exclude the possibility of other mechanisms may also contribute to the difference in the changes in mRNA and protein levels of PTEN in TGCT cells, e.g. miRNAs other than miR-1297, or modification of protein degradation through phosphorylation, SUMOylation, acetylation, and ubiquitination. These possibilities may be analyzed in future studies.

To summarize, here we reported a novel molecular regulatory pathway among miR-1297, Meg3, and PTEN mRNA. We propose that Meg3 may be a promising target to treat TGCT and deserves further investigation.

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Disclosure of conflict of interest

None.

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