Abstract. The incidence of thyroid disorders, which are common endocrine diseases, has rapidly increased in recent years. However, the etiology and pathogenesis of these disorders remain unclear. Phosphatase and tension homolog (PTEN) is a dual-specific phosphatase that is associated with multiple thyroid disorders; however, the role of PTEN in thyroid disorders remains unknown. In the present study, the human thyroid follicular epithelial cell line Nthy-Ori 3-1 was used to determine the role of PTEN in thyroid disorders. PTEN expression was knocked down using a PTEN-specific short hairpin RNA. Western blotting was subsequently used to determine protein expression, the Matrigel tube formation assay and iodide uptake assay were applied for evaluating the morphology and function of thyroid cells. The results showed that PTEN knockdown decreased the protein expression of paired box 8 (PAX8). The morphology and tubular-like growth pattern of thyroid cells were therefore disrupted, and restoration of PAX8 expression reversed these effects. Furthermore, PTEN-knockdown decreased the expression of specific thyroid proteins (thyroglobulin, TG; thyroid peroxidase, TPO; and sodium/iodide symporter, NIS) and inhibited the iodide uptake ability of thyroid cells by downregulating PAX8, suggesting that PTEN deficiency may impair the function of thyroid cells. In conclusion, the present study reported an important function of PTEN in normal thyroid cells and identified the involvement of PAX8. These results may improve understanding of the role of PTEN in the pathogenesis of thyroid disorders.

Introduction

Phosphatase and tension homolog (PTEN), also known as mutated in multiple advanced cancer 1, is a dual-specificity phosphatase that was first identified in 1997 (1). PTEN is one of the most studied tumor suppressors, as it is frequently mutated in various types of cancer, including breast cancer, prostate cancer and brain tumors (2,3). PTEN somatic mutations in sporadic tumors and PTEN germline mutations have also been demonstrated to result in the occurrence of inherited syndromes, including Cowden, Bannayan-Riley-Ruvalcaba and Proteus-like syndromes (4). PTEN somatic mutations in sporadic tumors and PTEN germline mutations have also been demonstrated to result in the occurrence of inherited syndromes, including Cowden, Bannayan-Riley-Ruvalcaba and Proteus-like syndromes (4). PTEN is involved in numerous cellular processes associated with tumorigenesis, including cell proliferation, cell survival, cell migration, genome stability and DNA replication (5-8). In addition to these anti-tumor effects, emerging evidence has demonstrated that PTEN could be associated with other biological functions. For instance, the deletion of PTEN in hepatocytes can increase glycogen and fatty acid synthesis by regulating glycogen synthase kinase-3β, fatty acid synthase, glucose 6-phosphatase as well as phosphoenolpyruvate carboxykinase; resulting in fatty liver and insulin hypersensitivity (9). Furthermore, the inhibition of PTEN can rescue the normal synaptic function in cellular and animal models of Alzheimer's disease and restore cognition in patients with Alzheimer's disease (10). PTEN also effects antiviral innate immunity by activating interferon regulatory factor 3 (11). However, despite numerous evidence demonstrating the multiple functions of PTEN in numerous diseases, the multifaceted roles of PTEN remain unclear.

The thyroid is an important endocrine organ that synthesizes and secretes thyroxine, which is a hormone involved in numerous biological functions, such as metabolism, homeostasis and development, by binding and altering the transcriptional regulatory properties of its receptors (12-14). Thyroid disorders, including hypothyroidism, goiter and thyroid tumor are...
very common, and their incidence has significantly increased in the last decade. It has been estimated that 1.5 billion people will be at risk of thyroid disorders by 2033 worldwide (15). The incidence of thyroid cancer has increased by 211% between 1975 and 2013 in the United States (16). Somatic mutations of PTEN are not common in thyroid disorders; however, loss of heterozygosity at 10q23 is found in 20-60% of all cases of thyroid cancer, although it varies depending on the histological type (17-19). PTEN expression is also reduced in a series of thyroid tumor-derived cell lines and in sporadic human benign and malignant thyroid tumors (20-22). Cowden syndrome, which is an inherited syndrome caused by a germline mutation of PTEN, is also characterized by a high risk of thyroid disorders, including multinodular goiter, thyroid adenoma and carcinoma (23). In addition, loss of PTEN in the thyroid of mice results in goiter and follicular adenomas (24). These findings suggest that PTEN mutation or deficiency may result in thyroid disorders. However, the role of PTEN in thyroid diseases remains unknown.

PAX8 is a member of the paired box gene family of proteins encoding evolutionary conserved transcription factors that control the development of various organs (25). PAX8 is expressed in the developing kidney, neural tube as well as in the developing and adult thyroid (26). Previous studies have indicated that PAX8 is important for the morphogenesis, differentiation and function of the thyroid gland (27,28), which suggested that PAX8 might be a mediator for the function of PTEN in thyroid cells.

To determine the role and underlying mechanism of PTEN in thyroid disorders, PTEN expression was knocked down in the human thyroid follicular epithelial cell line Nthy-Ori 3-1. Western blotting, Matrigel tube formation assay and iodide uptake assay were subsequently performed to determine the role of PTEN in thyroid disorders.

Materials and methods

Cell culture, transfection and chemicals. The human thyroid follicular epithelial cell line Nthy-Ori 3-1 was obtained from the European Collection of Authenticated Cell Cultures. Cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) and penicillin (100 u/ml)-streptomycin (0.1 mg/ml) serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd.) and provided by Suzhou GenePharma Co., Ltd. Nthy-Ori 3-1 cells were seeded into 96-well plates, at a density of 1x10⁴ cells/well, 1 day before viral infection. After the cells had adhered, lentivirus (25 µl) and the transfection agent polybrene (5 µg/ml; Suzhou GenePharma Co., Ltd.) were added to the medium for 12 h. Medium was then replaced by complete medium, and cells were cultured for a further 72 h, prior to subsequent experiments.

MG132, a proteasome inhibitor which can inhibit the degradation of protein mediated by ubiquitin-proteasome pathway, was purchased from Sigma-Aldrich; Merck KGaA and used at the final concentration of 20 µM for 4 h at room temperature. Cycloheximide (CHX; Amresco, LLC) was used at the final concentration of 10 µg/ml.

Immunohistochemistry (IHC). The thyroid tumor tissue microarray (TMA) slides containing 12 thyroid tumor tissues and 12 non-neoplastic tissues were purchased from Alenabio. Slides were deparaffinized in xylene and then rehydrated in decreasing grades of alcohol (100, 100, 95, 90, 80 and 70%). Antigen retrieval was performed by pressure cooker in citrate buffer (0.01 M, pH 6.0) and slides were blocked using 5% goat serum (Vicmed Life Sciences) for 1 h at room temperature. Slides were then incubated with anti-PTEN antibody (1:200; cat. no. 9188; Cell Signaling Technology, Inc.) overnight at 4°C, and with peroxidase-conjugated secondary antibody (ready-to-use; cat. no. PV6001; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) for 2 h at room temperature. Diaminobenzidine (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.) was used to detect positive signals. Images were acquired at x4 and x20 magnifications using a Zeiss light microscope (Carl Zeiss AG). For each specimen, three high-power fields were analyzed. Two independent observers assessed the immunohistochemical score blindly. PTEN staining was scored as 0, 1+, 2+, 3+ or 4+ for 0, <25, 25-49, 50-75 or >75% of positively stained cells, respectively. Positive staining was also graded according to its intensity from 0 to 3+, representing negative, weak, moderate and strong staining, respectively. Subsequently, PTEN protein expression was evaluated by calculating a final score according to the following formula: Score=intensity x positive cell proportion. A final score of 0 to 12 was therefore assigned.

Western blotting. Total cell protein was extracted from Nthy-Ori 3-1 cells using RIPA lysis buffer (Beyotime Institute of Biotechnology). The protein concentration was determined using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Proteins were separated (30 µg per lane) by SDS-PAGE on a 10% gel, and transferred onto polyvinylidene fluoride membranes (Merck KGaA). Membranes were blocked in 5% non-fat milk in TBS-Tween (TBST, 0.05%) for 1 h at room temperature, and incubated at 4°C overnight with the following primary antibodies: Rabbit anti-human PTEN (1:2,000; cat. no. 9188; Cell Signaling Technology, Inc.), rabbit anti-human PAX8 (1:2,000; cat. no. GTX101583; GeneTex, Inc.), rabbit anti-human sodium/iodide symporter (NIS; 1:1,000; cat. no. 83816; Abcam), mouse anti-GAPDH (1:20,000; cat. no. 60004-1-Ig; ProteinTech Group, Inc.), mouse anti-human thyroid peroxidase (TPO; 1:2,000; cat. no. sc-376876; Santa Cruz Biotechnology, Inc.). Membranes were washed three times with TBST and incubated with horseradish peroxidase-conjugated goat
anti-rabbit immunoglobulin G (IgG; 1:10,000; sc-2004; Santa Cruz Biotechnology, Inc.) and goat anti-mouse IgG (1:20,000; cat. no. sc-2005; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. Membranes were washed three times with TBST and the signal was visualized using Pierce Enhanced Chemiluminescent-plus substrate (Thermo Fisher Scientific, Inc.) and analyzed by Image J (version 1.48; National Institute of Health). GAPDH was used as an internal control.

**Matrigel tube formation assay.** Nthy-Ori 3-1 cells were seeded at the density of 1x10⁴ cells per well in 96-well plates that were precoated with 50% Matrigel (BD Biosciences) at 37˚C for 30 min. After 8 h incubation, the tubular-like structures were imaged and evaluated under a light microscope (magnification, x100). The capillary tubes in each image were counted and analyzed for statistical significance.

**Iodide uptake assay.** Cells were seeded at a density of 1x10⁵ cells per well in 24-well plates 24 h prior to the assay. Once cells had reached 75% confluence, the medium was discarded and cells were washed twice with ice-cold PBS. Subsequently, 0.5 ml medium containing 3.7 kBq ¹²⁵I (Xuzhou Atomic High Tech Pharmaceutical Co., Ltd.) was added into each well and cells were cultured at 37˚C. After 15, 30, 60 or 120 min, cells in each well were separately lysed with 0.5 ml of 0.3 mol/l NaOH and iodide uptake was measured using a γ radioimmunoassay counter. A parallel set of cells treated with the same method were applied for cell counting assay with the Cell Counting Kit-8 (Nanjing KeyGen Biotech Co., Ltd.) according to the manufacturer's instructions, and used for normalization of iodide uptake.

**Statistical analysis.** Statistical analyses were performed using SPSS software version 16.0 (SPSS, Inc.). The data are presented as the mean ± standard deviation of three independent repetitions. Statistical comparisons were performed using a two-tailed Student's t-test when comparing two groups, or ANOVA for multiple comparisons, along with Newman-Keuls test, were used for pairwise comparisons in multiple groups. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**PTEN expression is decreased in thyroid tumors.** To study the association between PTEN and thyroid diseases, the present study determined whether PTEN expression was different in human thyroid tumors compared with normal thyroid tissues. IHC staining was performed with TMA slides containing 12 thyroid tumor tissues and 12 non-neoplastic tissues. As presented in Fig. 1A, non-neoplastic thyroid tissue exhibited a uniform strong nuclear signal whereas the cytoplasmic staining was less strong. Furthermore, a significantly lower PTEN expression was observed in thyroid tumor tissue (P<0.01; Fig. 1B) compared with non-neoplastic tissues. These data indicated that PTEN was usually expressed in normal thyroid tissues but was downregulated in thyroid tumor tissues, suggesting that PTEN deficiency may lead to thyroid tumors.

**PTEN-knockdown modifies the morphology and growth pattern of Nthy-Ori 3-1 cells.** To determine the function of PTEN expression in thyroid cells, PTEN-specific shRNA was transfected into Nthy-Ori 3-1 to knockdown PTEN expression. As presented in Fig. 2A, PTEN protein expression was significantly decreased following 72 h of transfection. In addition, Nthy-Ori 3-1 cell morphology had changed. Cells transfected with PTEN-shRNA appeared rounder and flatter compared with control cells (Fig. 2B). Cells were then cultured on Matrigel to

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Figure 1. PTEN expression was decreased in thyroid tumors. (A) Representative images showing immunohistochemical staining of PTEN in non-neoplastic and neoplastic thyroid tissues. Top panel: Magnification, x100; scale bar, 200 μm. Bottom panel: Magnification, x200; scale bar, 100 μm. (B) Distribution of PTEN immunohistochemical score in non-neoplastic and neoplastic thyroid tissues. *P<0.05; **P<0.01. PTEN, phosphatase and tensin homolog.
Figure 2. PTEN-knockdown alters the morphology and growth pattern of Nthy-Ori 3-1 cells. Nthy-Ori 3-1 cells were transfected with control or PTEN-specific shRNA. (A) Cell lysates were harvested 72 h following transfection and PTEN protein expression was detected using western blotting. GAPDH served as an internal control. (B) Cell morphology of the different groups of cells. Scale bar, 50 µm; magnification, x200. (C) Cells were cultured on Matrigel for 8 h and imaged under the microscope. Scale bar, 100 µm; magnification, x100. The number of capillary tubes in each field were counted and used for subsequent analysis. ***P<0.001 vs. control. PTEN, phosphatase and tensin homolog; sh, short hairpin.

Figure 3. PTEN-knockdown decreases PAX8 protein expression. (A) Cell lysates were harvested 72 h following transfection and PAX8 protein expression was detected by western blotting. (B) PTEN-knockdown Nthy-Ori 3-1 cells were cultured with or without CHX (10 µg/ml) and were harvested at 0, 3 or 6 h following treatment. PAX8 protein expression was then evaluated by western blotting. (C) shControl and PTEN-knockdown cells were cultured with or without MG132 (20 µM) for 4 h and PAX8 expression was analyzed by western blotting. GAPDH served as an internal control. **P<0.01, ***P<0.001 vs. control. #P<0.01, ##P<0.001 vs. shPTEN. PAX8, paired box 8; PTEN, phosphatase and tensin homolog; CHX, cycloheximide.
evaluate their growth pattern. Cells in the control group grew on the Matrigel with a tubular-like structure; however, cells in the PTEN-knockdown group presented a significantly reduced number of tubular-like structures (Fig. 2C). These results suggested that PTEN-knockdown may induce Nthy-Ori 3-1 cell morphological changes and affect their growth pattern.

PTEN-knockdown decreases PAX8 protein level. Since PAX8 is important for the morphogenesis, differentiation and function of the thyroid gland, it was hypothesized that PTEN may affect the morphology and growth pattern of thyroid cells by regulating PAX8. To validate this hypothesis, PAX8 protein expression levels in the control and PTEN-knockdown cells were determined using western blotting. The results indicated that PAX8 protein expression protein was significantly reduced in PTEN-knockdown cells compared with the control group (Fig. 3A).

To examine the underlying mechanisms, cells were treated with MG132 (a proteasome inhibitor) and CHX (a protein synthesis inhibitor) to evaluate the stability of PAX8 protein. The results from western blotting demonstrated that, following CHX treatment, PAX8 protein expression was increased in the control group compared with cells transfected with PTEN-shRNA (Fig. 3B). Following treatment with MG132, PAX8 protein expression was significantly recovered in the PTEN-shRNA group (Fig. 3C). Together, these results suggested that PAX8 stability may be regulated by PTEN, as PTEN-knockdown increased PAX8 degradation.

Reintroduction of PAX8 in PTEN-knockdown cells restores normal morphology and growth pattern of thyroid cells. PAX8 overexpression in PTEN knocked down Nthy-Ori 3-1 cells. (A) PAX8 was overexpressed in PTEN knocked down Nthy-Ori 3-1 cells. Cell lysates were harvested 72 h following transfection and PAX8 protein expression in the different groups was detected by western blotting. (B) Nthy-Ori 3-1 cells were transfected with control or PAX8-specific shRNA. Cell lysates were collected 72 h following transfection to detect PAX8 expression by western blotting. GAPDH served as an internal control. (C) Cell morphology of the different groups of cells. Scale bar, 50 µm; magnification, x200. (D) Cells were cultured on Matrigel for 8 h and imaged under the microscope. Scale bar, 100 µm; magnification, x100. The number of capillary tubes in each field was counted and used for subsequent analysis. **P<0.01, ***P<0.001 vs. control. ###P<0.001 vs. shPTEN. PAX8, paired box 8; PTEN, phosphatase and tensin homolog; sh, short hairpin.
To further validate the hypothesis that a PTEN-PAX8 pathway may exist in the thyroid cells, PAX8 was overexpressed in PTEN knocked down Nthy-Ori 3-1 cells (Fig. 4A) and PAX8 was knocked down in Nthy-Ori 3-1 cells (Fig. 4B). The cell morphology and growth pattern were then evaluated. As presented in Fig. 4C, PTEN or PAX8 knockdown resulted in a rounder and flatter cell morphology (Fig. 4C-a and d). However, PAX8 overexpression in PTEN knocked down cells resulted in a thin and long cell morphology (Fig. 4C-c). The results from the Matrigel assay demonstrated that PTEN or PAX8-knockdown alone decreased the tubular-like structures formed by Nthy-Ori 3-1 cells (Fig. 4D-b and d), whereas PAX8 reintroduction significantly increased the formation of the tubular-like structures (Fig. 4D-c). These results suggested that PAX8 may serve a crucial role in the effect of PTEN on Nthy-Ori 3-1 cell morphology and growth pattern.

PTEN affects Nthy-Ori 3-1 cell function by regulating PAX8. PAX8 is a key transcription factor that controls the expression of numerous thyroid-specific proteins, including TG, TPO and NIS, which are essential for physiological functioning of the thyroid gland (29). As PAX8 was downregulated following PTEN-knockdown, the expression levels of TG, TPO and NIS were also determined. As presented in Fig. 5A, compared with the control cells, TG, TPO and NIS expression levels in PTEN-knockdown cells were all decreased, whereas PAX8 overexpression restored the expression of these proteins. These results suggested that PTEN-knockdown may also affect Nthy-Ori 3-1 cell function by regulating PAX8.

Iodide uptake is an important characteristic of thyroid follicular cells and is pivotal for its normal function (30). Iodide uptake was therefore assessed in Nthy-Ori 3-1 cells by using an iodide uptake assay. The results demonstrated that PTEN-knockdown decreased the peak value of iodide uptake and increased the time period before peak uptake was reached from 30 to 120 min. Reintroduction of PAX8 into the PTEN knockdown cells restored the iodide uptake ability of Nthy-Ori 3-1 cells. The peak value of iodide uptake was increased and...
the time period before peak uptake was reached decreased from 120 to 60 min (Fig. 5B). These results indicated that PTEN may disrupt thyroid cell function by regulating PAX8 expression.

Discussion

PTEN is a dual specificity phosphatase that affects cell proliferation, cell apoptosis, DNA replication, cell metabolism and organ development. Numerous studies have reported an association between PTEN and thyroid disorders (23,24,31); however, most of these studies demonstrated the inhibitory effects of PTEN only in thyroid neoplasms. The present study investigated the non-antitumor effect of PTEN in Nthy-Ori 3-1 cells, and demonstrated that PTEN-knockdown induced a change in the morphology, growth pattern and function of thyroid cells. These results increased understanding of PTEN function and highlighted how PTEN insufficiency may result in thyroid diseases.

Follicle formation is a typical characteristic of thyrocytes that is crucial for the physiological functioning of the thyroid gland. Numerous studies demonstrated by using 3D Matrigel and tubular-like structure in 2D in vitro cultures that thyroid cells grow with a follicular-like structure (32-34). Similarly, Nthy-Ori 3-1 cells were cultured as a monolayer in Matrigel and the formation of tubular-like structures was observed in the present study. Following PTEN-knockdown, formation of the tubular-like structures was partially disrupted, suggesting that PTEN may be involved in maintaining thyroid cell structure. PAX8 has been demonstrated as a key regulator of follicle formation both in vivo and in vitro. PAX8-knockout in mice results in a lack of follicular cells in the thyroid gland (27), and mutations of PAX8 gene in humans are associated with congenital hypothyroidism (35). Furthermore, it has been reported that PAX8 can regulate the morphology, differentiation and polarity of thyrocytes cultured in vitro (28,33,36). Therefore, the present study investigated PAX8 expression in Nthy-Ori 3-1 cells. The results demonstrated that PAX8 expression was decreased in PTEN knocked down cells, which may be responsible for the aberrant morphology and growth pattern observed in these cells.

Although PAX8 has been demonstrated to have a pivotal role and to be involved in the development and differentiation of thyroid tissue, how PAX8 is regulated remains unclear. Thyroid-stimulating hormone may regulate PAX8 synthesis at the transcriptional level (37). Furthermore, de Cristofaro et al (38) demonstrated that sumoylation could control PAX8 protein stability. The present study determined another potential PAX8 regulatory mechanism involving PTEN. PTEN-knockdown facilitated PAX8 degradation and decreased its protein expression. However, the role of PTEN in the regulation of PAX8 stability requires further investigation.

PAX8 is a member of the paired box gene family of transcription factors, which regulate the expression of several thyroid-specific proteins, including TG, TPO and NIS (39). These thyroid-specific proteins are crucial for normal thyroid function, and their decreased secretion results in hypothyroidism in humans (40,41). In the present study, PAX8 was downregulated in PTEN knocked down cells. As a result, TG, TPO and NIS were also downregulated, suggesting that PTEN-knockdown may impair the function of thyroid cells. Iodide uptake is a crucial function of the thyroid (30). Subsequently, an iodide uptake assay was used in the present study to evaluate the function of thyroid cells in vitro. The results from the iodide uptake assay analyzed two important indexes, the peak value of iodide uptake, which represents the iodide uptake capacity, and the time when the peak value appeared, which represents the iodide uptake speed. The results demonstrated that the peak value and peak time were both disrupted following knockdown of PTEN in Nthy-Ori 3-1 cells. In addition, reintroduction of PAX8 reversed the effects of PTEN-knockdown, suggesting that PAX8 may be an important mediator. No significant difference was observed in the peak value of iodide uptake between shControl and shPTEN at 60 min, which may be due to the fact that iodide uptake is a dynamic process. Indeed, when at 60 min, iodide uptake in shControl group had reached its peak and decreased with time, the iodide uptake in shPTEN group had not yet reached its peak and was still increasing. These observations may explain that the difference observed between shControl and shPTEN at 60 min was smaller than at 15 or 30 min. In addition, the peak value measured in the shControl group during the three repetitions at 60 min varied a lot (2,672,4,652 and 4,080 cpm/10^5 cells), suggesting a higher intra-group variance. Subsequently, smaller inter-group difference and higher intra-group variance induced no significant difference at 60 min. However, this didn't affect the final conclusion of the present study. Since it was observed that the shControl group had a higher peak value and shorter peak time compared with shPTEN group, this demonstrated that PTEN-knockdown may impair the iodide uptake ability of Nthy-Ori 3-1 cells. These results suggested that PTEN may have a crucial role in the physiological functioning of thyroid cells.

In conclusion, the present study investigated the role of PTEN in the morphology and function of thyroid cells by using the normal thyroid follicular epithelial cell line Nthy-Ori 3-1. The results demonstrated that PTEN-knockdown in Nthy-Ori 3-1 cells induced important morphological changes, which were determined to be mediated by PAX8 downregulation. It was also reported that PTEN-knockdown decreased the protein expression of several thyroid-specific proteins and attenuated the iodide uptake ability of thyroid cells. These findings highlighted the importance of PTEN in the regulation of thyroid cell morphology and function, and reported that PTEN function may be mediated by PAX8.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YPW and ZDS conceived and designed the experiments. ZS, JQL and MYW performed the experiments, collected the data and analyzed the results. CLO and XCH contributed to the iodide uptake assay. YPX assisted with western blotting and Matrigel assay. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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