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Original Article

Tuft1 promotes thyroid carcinoma cell invasion and proliferation and suppresses apoptosis through the Akt-mTOR/GSK3β signaling pathway

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Abstract: In this study, we aimed to investigate the biological functions of Tuftelin 1 (Tuft1) in thyroid carcinoma (TC) and determine its underlying molecular mechanism. We found that the expression of Tuft1 was significantly upregulated in TC tissues. Using TC tissue microarrays (n = 154), we found that Tuft1 expression was closely related with the overall survival (OS) and disease-free survival (DFS) of TC patients. Knockdown of Tuft1 in TPC-1 and SW579 cells suppressed the invasion and proliferation of TC cells and increased the apoptosis of TC cells. In vivo, knockdown of Tuft1 attenuated tumor growth and suppressed the phosphorylation of Akt, mTOR, and GSK3β signaling. Addition of recombinant Tuft1 protein (rTuft1) to TC cells increased the phosphorylation of Akt, mTOR, and GSK3β signaling. An mTOR inhibitor (Dactolisib) abrogated rTuft1 protein-induced TC cell invasion, proliferation, and apoptosis inhibition, whereas a GSK3β inhibitor (CHIR-98014) only abrogated rTuft1 protein-induced proliferation and apoptosis inhibition. These results suggest that Tuft1 promotes TC cell invasion and proliferation, and suppresses apoptosis through the Akt-mTOR or Akt-GSK3β signaling pathway. In the future, Tuft1 may serve as a potential therapeutic target for TC.

Keywords: Tuft1, thyroid carcinoma, invasion, proliferation, apoptosis, Akt-mTOR/GSK3β

Introduction

Thyroid carcinoma (TC) is the most common malignancy of the endocrine system, and its incidence has recently dramatically increased worldwide [1-4]. TC is generated by multi-step carcinogenesis, in which thyroid follicular cells undergo multiple incidences of damage to their oncogenes or anti-oncogenes, accelerating proliferation or fostering malignant phenotypes [5, 6]. The initiation of TC occurs in infancy. The risk of radiation-induced thyroid cancer is evident in children under the age of 5 years, but not in adults [7, 8]. In mice, the induction of thyroid cancer by expression of oncogenes in the thyroid was only possible when they were introduced at the fetal stage [9-11]. Based on clinically evident TC, the first event of carcinogenesis is thought to occur in middle age, and some cancer cells acquire a more aggressive phenotype after repeated proliferation [12, 13].

Tuftelin 1 (Tuft1) was originally identified as a molecule involved in the growth and maturation of extracellular enamel, leading to the mineralization of the tooth epithelial tissue in vertebrates [14]. Tuft1 is expressed in the morula, embryonic stem cells, brain, kidneys, adrenal gland, liver, testis, and tumor cells. Tuft1 may perform certain functions in mesenchymal stem cells, and is involved in the differentiation of neural cells [15, 16]. Further, hypoxic environments induce Tuft1 expression in human HepG2 and MCF-7 cell lines, which is necessary for tumorigenesis [17]. Together, these studies demonstrated that Tuft1 is likely to have various functions in different tissues, and the alterations in these normal functions may promote the development of certain diseases and tumors [18]. However, the role of Tuft1 in tumors has rarely been reported, except for in breast cancer [19] and pancreatic cancer [20].

Here, we found that Tuft1 expression was significantly upregulated in TC tissues and closely related with patient prognoses. Knockdown of Tuft1 significantly suppressed the invasion and proliferation and promoted the apoptosis of TC

cells. Further, the effects of Tuft1 on the invasion, proliferation, and apoptosis of TC cells were dependent on Akt-mTOR or Akt-GSK3β signaling.

Materials and methods

Cell culture

Human TC cell lines (TPC-1, SW579, K1, BCPAP, TT) and human normal thyroid cell line HT-ori3 were purchased from the Cell Bank of the Chinese Academy of Sciences. Cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum and 1% antibiotics. Cells were grown at 37°C in a humidified incubator under 5% CO_2 conditions.

Clinical samples

Human TC and normal tissues and tissue microarrays containing TC samples from 154 cases were purchased from Alenabio (Beijing, China). All human materials were obtained with informed consent, and the protocols were approved by the ethical review committee of the World Health Organization Collaborating Center for Research in Human Production.

Quantitative real-time PCR

Total RNA was extracted by TRIzol (Takara, Tokyo, Japan), and reverse transcribed using a PrimeScript RT-PCR kit (Perfect Real Time; Takara). Quantitative PCR analyses were performed with SYBR Premix Ex Taq (Takara) on a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA).

Western blotting

Cells were lysed in lysis buffer and proteins were separated by SDS-PAGE under reducing conditions. The membrane was blocked in phosphate-buffered saline (PBS)/Tween-20 containing 5% bovine serum albumin. Then, the antibodies for Tuft1 (Abcam, Burlingame, CA, USA), phospho-Akt (Cell Signaling Technology, Danvers, MA, USA), total-Akt (Cell Signaling Technology), phospho-mTOR (Cell Signaling Technology), total-mTOR (Cell Signaling Technology), phospho-GSK3β (Cell Signaling Technology), total-GSK3β (Cell Signaling Technology), GAPDH (Sigma-Aldrich, St. Louis, MO, USA), and species-specific secondary antibodies were used to incubate the membrane separately. The secondary antibodies were detected by an Odyssey imaging system (LI-COR, Lincoln, NE, USA).

siRNA transfection

Small interfering RNA (siRNA) duplexes for Tuft1 were produced by Genepharma (Shanghai, China). Transfection steps were performed following the manufacturer's protocols.

rTuft1 protein and inhibitors

Recombinant Tuft1 (rTuft1) protein was purchased from Abnova (Taipei, Taiwan). The inhibitor of mTOR (Dactolisib) and the inhibitor of GSK3β (CHIR-98014) were purchased from Selleck Chemicals (New York, NY, USA).

Invasion assay

TC cells were detached and resuspended in serum-free RPMI-1640 medium. A total of 2×10^4 cells in 0.1 mL were placed in Matrigel (BD)-coated inserts (Millipore, Hayward, CA, USA) seated in the 24-well plates. RPMI-1640 medium supplemented with 5% FBS was added to the bottom chamber. Cells were incubated at 37°C. After 48 hours, filters were fixed and stained with 0.1% (w/v) Crystal Violet. Non-invading cells were removed, and invading cells were counted under a microscope at 400 × magnification. Three grids per field were counted and the experiments were repeated twice.

Cell viability assay

Cell viability was detected using a standard Cell Counting Kit-8 (CCK-8 assay; Dojindo, Tokyo, Japan). TC cells were seeded into 96-well plates (100 μL per well) at a density of 2×10^4 cells per mL. We then added 10 μL of reagent from the CCK-8 kit to each well for detection at 12, 24, 48, and 72 hours of culture, respectively. After 2 hours of incubation at 37°C, the optical density was measured using a microplate reader at a wavelength of 450 nm.

Apoptosis assay

Apoptosis was assessed by placing 5×10^5 cells/well in 12-well plates and serum starving for 48 hours at 37°C. After 48 hours, adherent cells were detached and harvested in complete RPMI-1640 medium. After centrifuging at 1,000 rpm for 5 minutes, the cells were washed

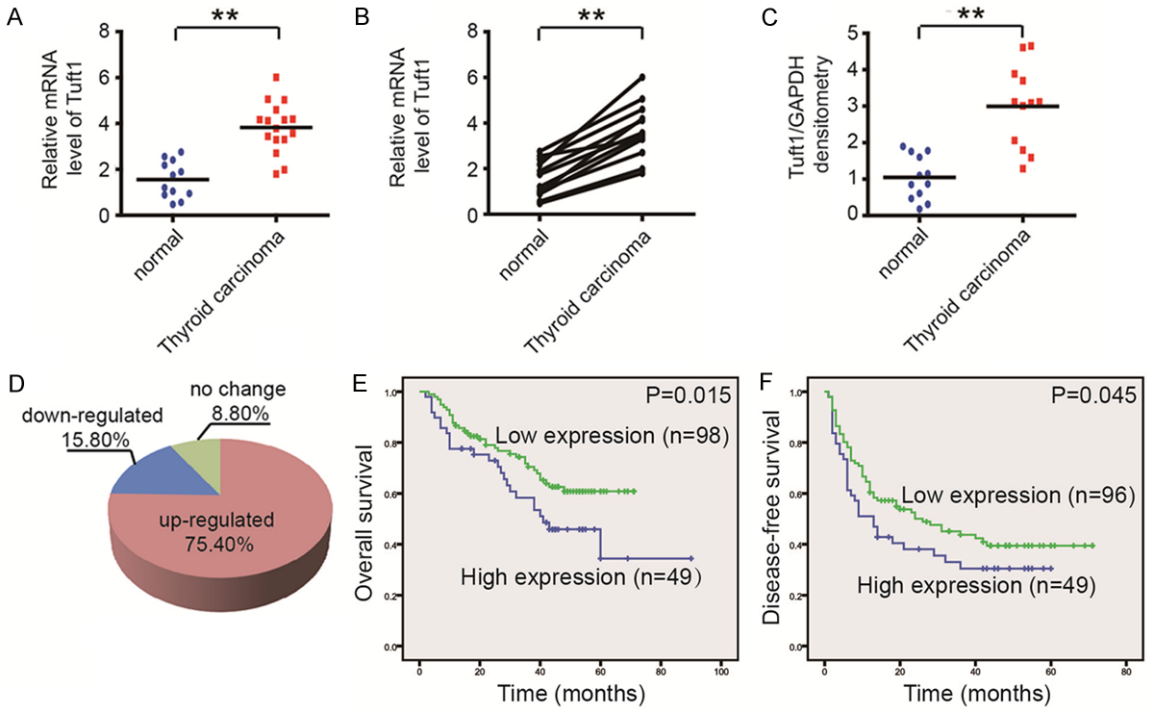

Figure 1. The expression of Tuft1 is upregulated in TC and closely related with patient prognoses. (A) The mRNA expression level of Tuft1 in 16 cases TC and 12 cases normal tissues. (B) The mRNA expression level of Tuft1 in 12 paired TC and normal tissues. (C) The protein expression level of Tuft1 in 12 paired TC and normal tissues. $^{**}P < 0.01$. (D) The expression of Tuft1 is upregulated in 75.40% TC tissues by using TC tissue microarray (n = 154). (E and F) Kaplan-Meier analysis of overall survival (OS) (E, $P = 0.015$) and disease-free survival (DFS) (F, $P = 0.045$) for the expression of Tuft1.

with 1 × PBS and stained with 100 μL binding buffer containing 3 μL Annexin V and 3 μL propidium iodide. After incubating at room temperature for 15 minutes, the cells were analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA).

Animal experiments

Mice were housed and manipulated according to protocols approved by the Shanghai Jiao Tong University School of Medicine Animal Care Commission. All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (USA). Male NU/NU mice (6 weeks, n = 5, every group) were kept on a 12-hour day/night cycle with free access to food and water. TPC-1 cells were trypsinized, washed in PBS, and resuspended in serum-free Dulbecco's Modified Eagle's medium (DMEM). A total of 2 × 10^6 TPC-1 cells in 200 μL DMEM were injected subcutaneously in the lower back. Tumor growth was monitored for 2 weeks.

Statistical analysis

Data were presented as the mean ± standard error of the mean (SEM). Statistical analyses were performed using SPSS 16.0 for windows (SPSS, Inc., Chicago, IL, USA). Survival time was analyzed by the Kaplan-Meier method. One-way analysis of variance was used for comparison between groups. Multiple comparison between the groups was performed using Student-Newman-Keuls post-hoc method. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Tuft1 is upregulated in TC tissues and closely related with patient prognoses

To investigate the expression of *TUFT1* in TC tissues, we used 16 TC cases and 12 normal tissue cases. By quantitative PCR, we found that the expression level of *TUFT1* was significantly upregulated in TC tissues (**Figure 1A**). In 12 paired TC and normal tissues, *TUFT1* mRNA expression was consistently higher in TC tis-

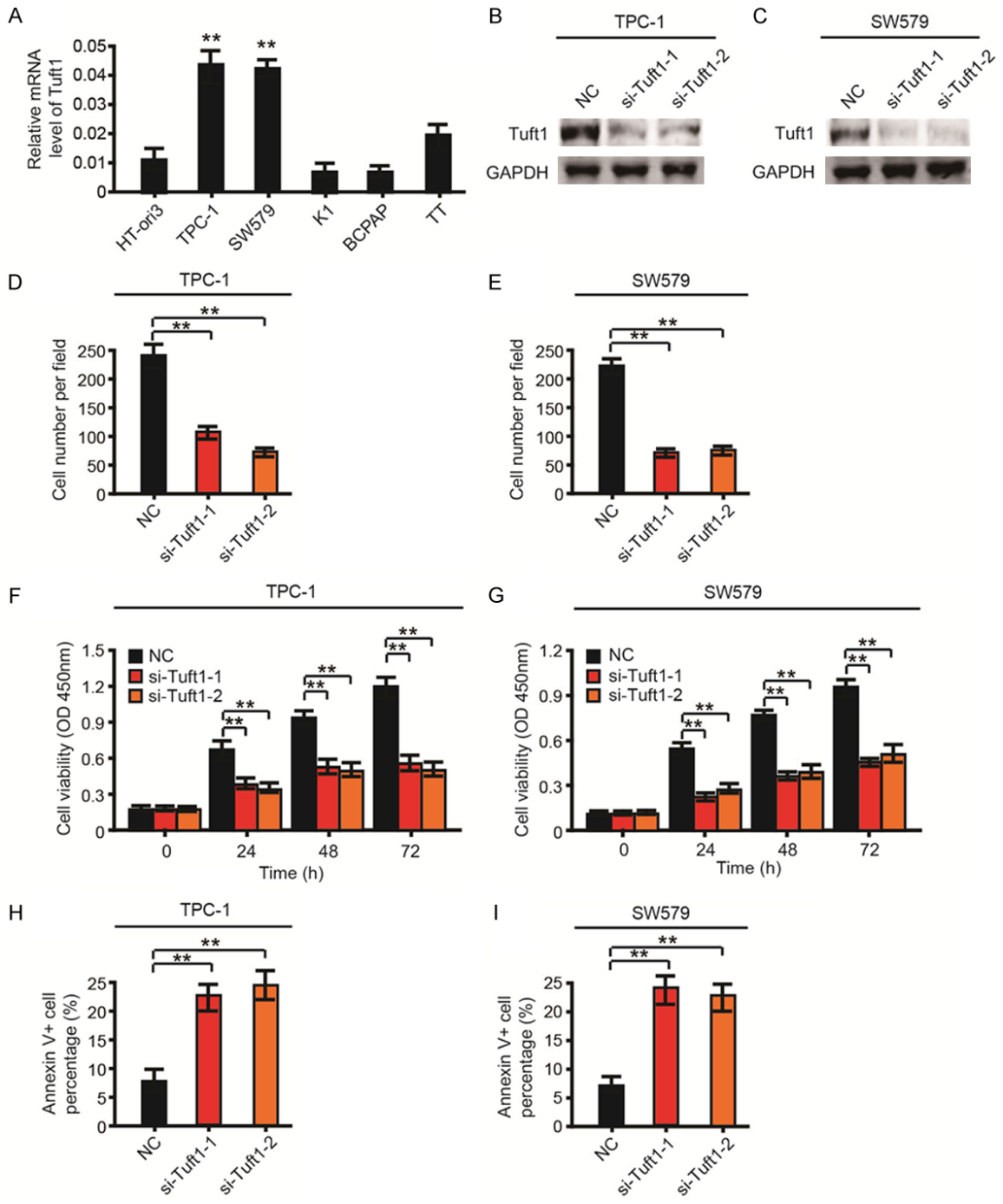

Figure 2. Knockdown of Tuft1 suppresses the invasion, proliferation and promotes the apoptosis of TC cells. (A) Expression of Tuft1 in five human TC cell lines, including TPC-1, SW579, K1, BCPAP, TT cells, and human normal thyroid cell line HT-ori3. (B and C) The protein expression level of Tuft1 in TPC-1 (B) and SW579 (C) cells, which were infected with siRNA or negative control (NC) of Tuft1. (D and E) Statistical analysis of invaded TPC-1 (D) and SW579 (E) cells infected with siRNA or NC of Tuft1. (F and G) CCK8 cell viability assay of TPC-1 (F) and SW579 (G) cells infected with siRNA or NC of Tuft1 at 0, 24, 48 and 72 hour time points respectively. (H and I) Statistical analysis of apoptotic TPC-1 (H) and SW579 (I) cells infected with siRNA or NC of Tuft1. $^{**}P < 0.01$.

sues than in paired normal tissues (**Figure 1B**). The protein expression of Tuft1 was also found to be significantly upregulated in TC tissues in 12 of these tissue sample pairs (**Figure 1C**).

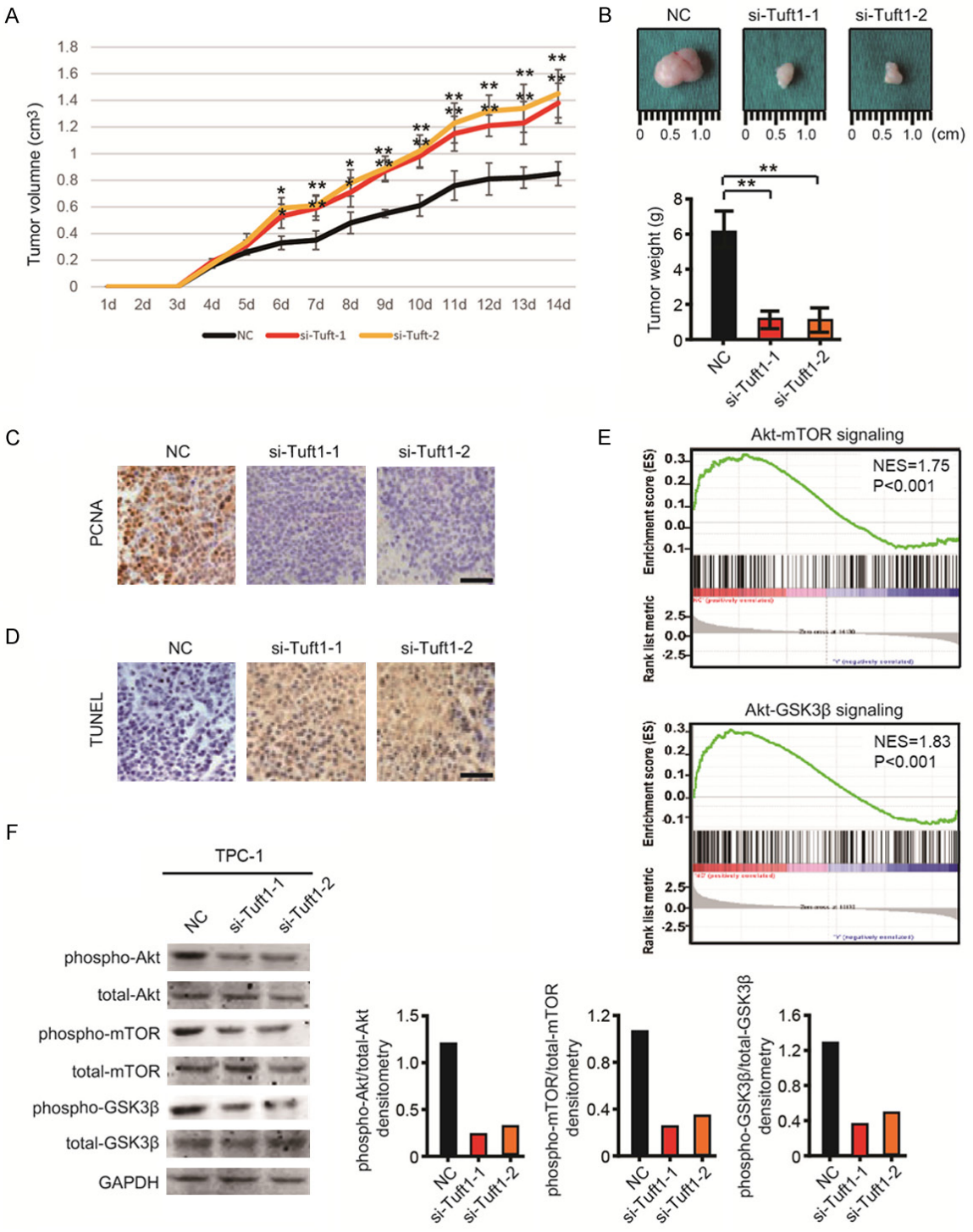

Figure 3. Knockdown of Tuft1 attenuates tumor growth *in vivo* and decreases the phosphorylation of Akt, mTOR and GSK3β signaling. A. The tumor volumes of mice in NC, si-Tuft1-1 and si-Tuft1-2 groups during two weeks. B. Mice in si-Tuft1-1 and si-Tuft1-2 groups show relatively larger tumors compared with that in NC group after two weeks. The tumor weights of mice in NC, si-Tuft1-1 and si-Tuft1-2 groups are shown below. $^{**}P < 0.01$. C. The expression of PCNA in the tumors of NC, si-Tuft1-1 and si-Tuft1-2 group mice by IHC staining. D. The apoptosis in the tumors of NC, si-Tuft1-1 and si-Tuft1-2 group mice by TUNEL staining. E. Tuft1 is closely related with Akt-mTOR and Akt-GSK3β signaling by using GESA analysis. F. Western blotting analysis of phospho-Akt, total-Akt, phospho-mTOR, total-mTOR, phospho-GSK3β and total-GSK3β in Tuft1 silenced and control MGC-803 cells. Statistical analysis of phospho-Akt/total-Akt, phospho-mTOR/total-mTOR and phospho-GSK3β/total-GSK3β densitometry are shown right.

We then used a TC tissue microarray (n = 154) to investigate the correlation between Tuft1 expression and patient prognoses. Consistent with our other findings, the expression of Tuft1 was upregulated in 75.40% of TC tissues (**Figure 1D**). The high expression of Tuft1 was positively correlated with poor overall survival (OS) ($P = 0.044$) and disease-free survival (DFS) ($P = 0.045$) (**Figure 1E** and **1F**).

Tuft1 knockdown suppresses the invasion and proliferation and promotes the apoptosis of TC cells

To further investigate the biological functions of Tuft1 in TC, we examined the expression level of *TUFT1* in five human TC cell lines, including TPC-1, SW579, K1, BCPAP, and TT cells, and the human normal thyroid cell line HT-ori3. As shown in **Figure 2A**, *TUFT1* expression was high in TPC-1 and SW579 cells. We therefore silenced Tuft1 using siRNA (si-Tuft1-1 and si-Tuft1-2) or transfected negative control (NC) siRNA in TPC-1 and SW579 cells. Through western blotting analysis, we found that Tuft1 was successfully silenced in TPC-1 (**Figure 2B**) and SW579 (**Figure 2C**) cells.

We then investigated the biological function of Tuft1 in the invasion of TC cells. By using Transwell® Matrigel invasion assays, we found that knockdown of Tuft1 for 48 hours suppressed the invasiveness of TPC-1 (**Figure 2D**) and SW579 (**Figure 2E**) cells. We then investigated the role of Tuft1 in the proliferation of TC cells. Using the CCK-8 cell viability assay, we found that the cell viability of TPC-1 and SW579 cells was significantly suppressed by knockdown of Tuft1 for 24, 48, and 72 hours (**Figure 2F** and **2G**). Moreover, by annexin V detection, we found that knockdown of Tuft1 promoted the apoptosis of TPC-1 (**Figure 2H**) and SW579 (**Figure 2I**) cells after 48 hours.

Knockdown of Tuft1 attenuates tumor growth in vivo and decreases the phosphorylation of Akt and mTOR, and GSK3β signaling

TPC-1 cells were subcutaneously injected into the lower back of male NU/NU mice. After 6 days, the tumor volumes in the si-Tuft1-1 and si-Tuft1-2 group mice were significantly larger than those of the NC group mice (**Figure 3A**). After 2 weeks, all mice were sacrificed. The tumors in the si-Tuft1-1 and si-Tuft1-2 groups were larger with greater weights than those of the NC group (**Figure 3B**). By IHC and TUNEL staining, it was found that the PCNA expression of NC group was higher than si-Tuft1-1 and si-Tuft1-2 groups, while the apoptosis of NC group was lower than si-Tuft1-1 and si-Tuft1-2 groups (**Figure 3C** and **3D**).

To investigate the underlying mechanism of the association of Tuft1 with TC, we performed GESA analysis and found that Tuft1 was closely related with Akt-mTOR and Akt-GSK3β signaling (**Figure 3E**). Then we detected Akt-mTOR and Akt-GSK3β signaling pathway proteins in TPC-1 cells by western blotting. Interestingly, Tuft1 knockdown significantly decreased the phosphorylation of Akt (**Figure 3F**). Likewise, silencing Tuft1 decreased phosphorylation of signaling proteins downstream of Akt, including mTOR and GSK3β (**Figure 3F**).

To further confirm our results, we added recombinant Tuft1 (rTuft1) protein to K1 cells, which have a low Tuft1 expression level. It was found that the phosphorylation levels of Akt, mTOR, and GSK3β were increased by rTuft1 administration (**Figure 4A**).

Tuft1-induced TC cell invasion is dependent on Akt-mTOR signaling, whereas proliferation is dependent on both Akt-mTOR and Akt-GSK3β signaling

Because mTOR and GSK3β are different downstream signaling proteins of Akt, we investigated the effects of these pathways on cell invasion and proliferation using Dactolisib, an inhibitor of mTOR, and CHIR-98014, an inhibitor of GSK3β. The rTuft1 protein was added to K1 and BCPAP cells with low Tuft1 expression levels, then Dactolisib and CHIR-98014 were added 2 hours later. Dactolisib abrogated rTuft1 protein-induced TC cell invasion (**Figure 4B** and **4C**). Both Dactolisib and CHIR-98014 abrogated rTuft1 protein-induced TC cell proliferation and apoptosis inhibition (**Figure 4D-G**).

These results indicated that Tuft1-induced TC cell invasion was dependent on Akt-mTOR signaling. Tuft1-induced TC cell proliferation and apoptosis inhibition, however, were dependent on both Akt-mTOR and Akt-GSK3β signaling (**Figure 4H**).

Discussion

In recent years, studies regarding Tuft1 in cancers have been rare, and the detailed biological functions and underlying mechanism of Tuft1

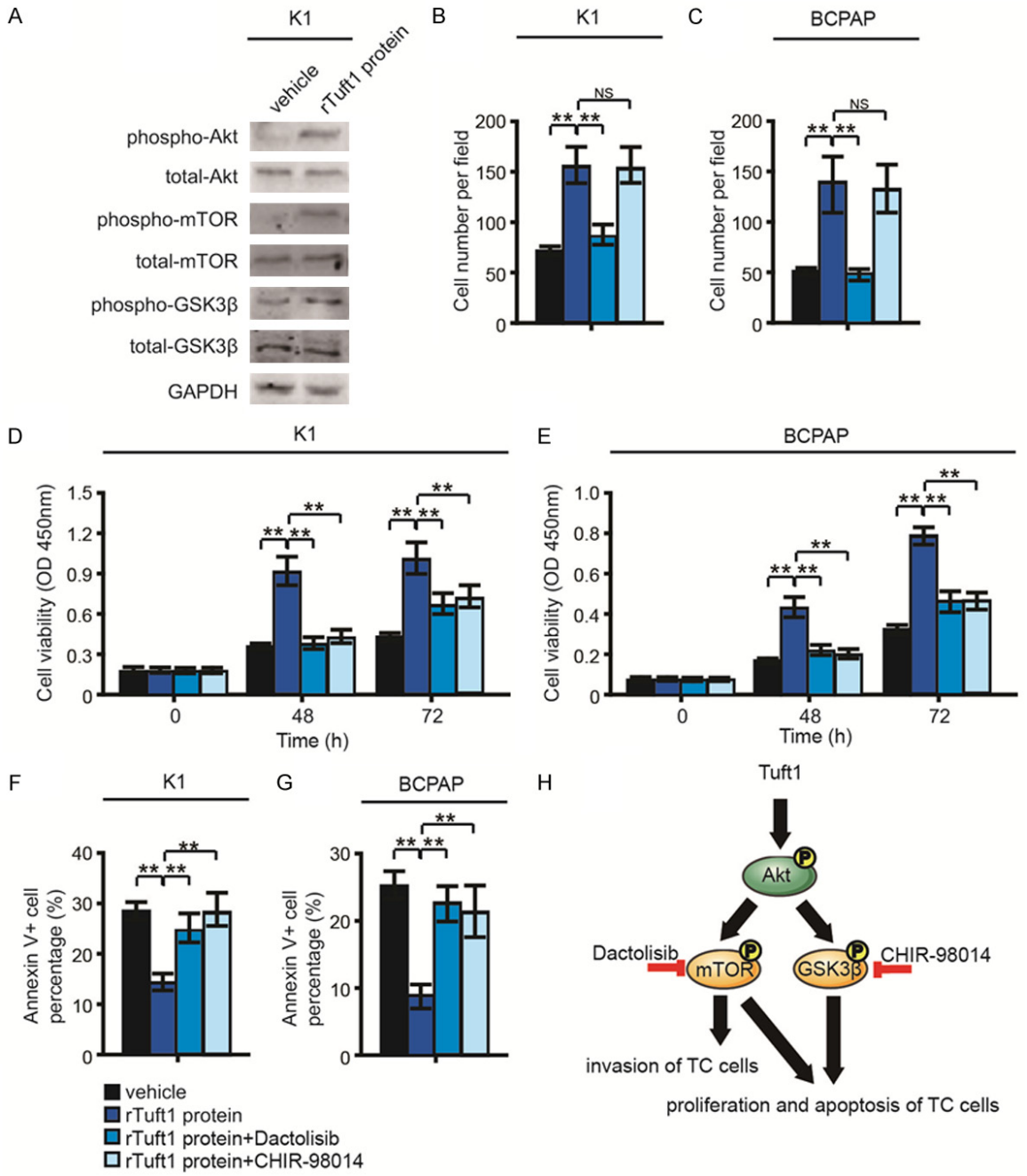

Figure 4. Tuft1-induced TC cell invasion is dependent on Akt-mTOR signaling, whereas proliferation is dependent on both Akt-mTOR and Akt-GSK3β signaling. (A) Western blotting analysis of phospho-Akt, total-Akt, phospho-mTOR, total-mTOR, phospho-GSK3β and total-GSK3β in rTuft1 treated and vehicle BGC-823 cells. (B and C) K1 and BCPAP cells were treated with 50 nM rTuft1 protein, 50 nM rTuft1 protein plus 50 nM Dactolisib (the inhibitor of mTOR), 50 nM rTuft1 protein plus 50 nM CHIR-98014 (the inhibitor of GSK3β) respectively. The invaded K1 (B) and BCPAP (C) cells were analyzed after 48 hours. (D and E) K1 and BCPAP cells were treated with 50 nM rTuft1 protein, 50 nM rTuft1 protein plus 50 nM Dactolisib, 50 nM rTuft1 protein plus 50 nM CHIR-98014 respectively. CCK8 cell viability of K1 (D) and BCPAP (E) cells were detected at 0, 48, 72 hour time points. (F and G) K1 and BCPAP cells were treated with 50 nM rTuft1 protein, 50 nM rTuft1 protein plus 50 nM Dactolisib, 50 nM rTuft1 protein plus 50 nM CHIR-98014 respectively. The apoptotic K1 (F) and BCPAP (G) cells were analyzed after 48 hours. $^{**}P < 0.01$, NS, no significant. (H) Tuft1-induced TC cell invasion is dependent on Akt-mTOR signaling. Whereas, Tuft1 induced TC cell proliferation and apoptosis inhibition are dependent on both Akt-mTOR and Akt-GSK3β signaling.

in TC had not been mentioned until the present study. By analyzing TC tissue microarrays, we found that the expression of Tuft1 was closely related with poor patient prognosis. These data

suggested that Tuft1 may play important roles during the development of TC. We further revealed that the invasion and cell viability of TC cells were significantly suppressed by Tuft1 knockdown, and apoptosis was increased. These results indicated that Tuft1 is involved in the invasion, proliferation, and apoptosis of TC cells.

In this study, we found that the knockdown of Tuft1 was correlated with decreased Akt and mTOR phosphorylation. The Akt-mTOR pathway is pivotal in modulating the invasion and migration of tumor cells [21, 22]. By using the inhibitor of mTOR, Dactolisib, we found that Tuft1-induced TC cell invasion, proliferation, and apoptosis inhibition were dependent on Akt-mTOR signaling.

PI3K/Akt-GSK3β signaling plays key roles in the regulation of many biological functions in various cancers, such as cell migration, invasion, proliferation, or apoptosis. Herein, we found that Tuft1 knockdown decreased the phosphorylation of GSK3β, and Tuft1 induced TC cell proliferation and apoptosis inhibition were also dependent on the Akt-GSK3β pathway.

In conclusion, we found that Tuft1 played an important role in TC cell invasion and proliferation. Tuft1 promoted TC cell invasion and proliferation through the Akt-mTOR or Akt-GSK3β signaling pathways. Tuft1 may therefore be useful as a potential therapeutic strategy for TC in the future.

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Disclosure of conflict of interest

None.

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