

RESEARCH

Long noncoding RNA DLEU2 drives the malignant behaviors of thyroid cancer through mediating the miR-205-5p/TNFAIP8 axis

 Jiwen Yang¹, Yayin Huang², Bohan Dong³ and Yunhai Dai¹
¹Department of Nuclear Medicine, Yijishan Hospital of Wannan Medical College, Wuhu City, Anhui Province, China

²Department of Clinical Laboratory, The Second People's Hospital of Wuhu, Wuhu City, Anhui Province, China

³Department of Biochemistry and Molecular Biology, Wannan Medical College, Wuhu City, Anhui Province, China

 Correspondence should be addressed to Y Dai: kmzzzj@163.com

Abstract

Objective: Considering the plight in thyroid cancer therapy, we aimed to find novel therapeutic targets from a molecular perspective.

Methods: Quantitative real-time PCR (qRT-PCR) and Western blot assay were carried out to determine RNA and protein expression. Cell counting kit-8 (CCK8) assay, flow cytometry, transwell migration assay and aerobic glycolysis analysis were performed to analyze cell proliferation, apoptosis, migration and aerobic glycolysis of thyroid cancer cells. MiRcode and Starbase software were used to search the downstream genes of long noncoding RNA (lncRNA) deleted in lymphocytic leukemia 2 (DLEU2) and microRNA-205-5p (miR-205-5p), and the intermolecular combination was confirmed by dual-luciferase reporter assay. The *in vivo* role of DLEU2 in tumor growth was verified using the murine xenograft model.

Results: DLEU2 and tumor necrosis factor- α -induced protein 8 (TNFAIP8) were highly expressed in thyroid cancer tissues and cell lines. DLEU2 and TNFAIP8 promoted the proliferation, migration and aerobic glycolysis and restrained the apoptosis of thyroid cancer cells. DLEU2/miR-205-5p/TNFAIP8 signaling axis was identified in thyroid cancer cells. TNFAIP8 overexpression largely rescued the malignant phenotypes in DLEU2-silenced thyroid cancer cells. DLEU2 positively regulated TNFAIP8 expression by acting as miR-205-5p sponge in thyroid cancer cells. DLEU2 silencing blocked the growth of xenograft tumors *in vivo*.

Conclusion: lncRNA DLEU2 exerted a pro-tumor role to promote proliferation, migration and aerobic glycolysis while repressing the apoptosis of thyroid cancer cells via miR-205-5p/TNFAIP8 axis.

Key Words

- ▶ thyroid cancer
- ▶ DLEU2
- ▶ miR-205-5p
- ▶ TNFAIP8

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Highlights:

- lncRNA DLEU2 is significantly upregulated in thyroid cancer tissues and cell lines.
- DLEU2 accelerates the proliferation, migration and aerobic glycolysis while inhibits the apoptosis of thyroid cancer cells.
- miR-205-5p is a direct target of DLEU2 in thyroid cancer cells, and TNFAIP8 could bind to miR-205-5p in thyroid cancer cells as well.
- DLEU2 facilitates the development of thyroid cancer through upregulating TNFAIP8 via sponging miR-205-5p *in vivo* and *in vitro*.

Introduction

Thyroid cancer is a common endocrine malignant cancer (1). The survival of patients with thyroid cancer has improved with the combined therapy of surgery and radioactive iodine (RAI) therapy. However, the prognosis of thyroid cancer patients remains dismal due to neoplasm recurrence or other reasons. We aimed to explore the pivotal molecules involved in thyroid cancer progression.

Long noncoding RNAs (lncRNAs) are a set of RNAs that contain more than 200 nucleotides, and they are unable to code proteins. lncRNAs participate in multiple cellular functions, such as tumorigenesis and metastasis (2, 3, 4). lncRNAs exert their functions through sponging miRNAs to suppress their biological functions (5, 6, 7). lncRNA deleted in lymphocytic leukemia 2 (DLEU2) played an oncogenic role in multiple cancers (8, 9, 10). For instance, Xie *et al.* claimed that DLEU2 accelerated the colony formation and metastasis of glioma cells through miR-186-5p/PDK3 signaling (11). DLEU2 was upregulated in thyroid cancer (12). Nevertheless, the biological significance of DLEU2 and its interaction with other factors in thyroid cancer remains poorly understood.

miRNAs are another class of noncoding RNAs. They could modulate diverse cellular processes, such as proliferation and metastasis by reducing the abundance of target messenger RNAs (mRNAs) (13, 14, 15, 16). miR-205-5p was involved in vascularization and epithelial-to-mesenchymal transition (EMT) (17). Li *et al.* found that miR-205-5p suppressed the metastasis of prostatic carcinoma cells via downregulating ZEB1 (18). Tao *et al.* claimed that miR-205-5p inhibited the growth of pulmonary vascular smooth muscle cells via MICAL2/ERK1/2 signal pathway (19). Vosgha *et al.* demonstrated that miR-205 restrained the progression of anaplastic thyroid carcinoma through inhibiting angiogenesis and EMT (20). This study sought to explore the role of miR-205-5p in thyroid cancer.

Tumor necrosis factor- α -induced protein 8 (TNFAIP8) has been identified as a TNF- α -inducible oncogenic molecule (21). Mounting researchers have reported the oncogenic role of TNFAIP8 in a series of cancers (22, 23, 24). Duan *et al.* reported that TNFAIP8 was upregulated in thyroid cancer tissues compared with that in corresponding normal tissues (25). However, the binding molecules and the functional mechanism of TNFAIP8 in thyroid cancer are not fully addressed.

This study explored the target relationship between DLEU2 and miR-205-5p, as well as the target relationship between miR-205-5p and TNFAIP8 in thyroid cancer cells.

Besides, the influences of DLEU2/miR-205-5p/TNFAIP8 signaling on the proliferation, apoptosis, aerobic glycolysis and migration of thyroid cancer cells were illustrated by *in vitro* and *in vivo* experiments.

Materials and methods

Patients

Patients with thyroid cancer ($n = 70$) were recruited in Yijishan Hospital of Wannan Medical College. The abundance of DLEU2 and TNFAIP8 was examined in thyroid cancer tissues and corresponding normal tissues. This study was conducted by the agreement of Yijishan Hospital of Wannan Medical College. Informed consent was provided by patients involved in this study.

Bioinformatic analysis

The expression of DLEU2 and TNFAIP8 in the TCGA database was analyzed using two websites (<http://ualcan.path.uab.edu/index.html>) and (<http://gepia.cancer-pku.cn/>).

Cell culture

Human thyroid normal cell line Nthy-ori 3-1 and thyroid cancer cell lines TPC-1 and BHT-101 were obtained from BeNa Culture Collection (Beijing, China). RPMI-1640 medium (Gibco) added with 10% fetal bovine serum (FBS; Gibco) and 10% penicillin (100 units/mL)–streptomycin (100 μ g/mL) mixed solution was used for the cultivation of Nthy-ori 3-1 and TPC-1 cells. BHT-101 cells were cultivated with DMEM (Gibco) added with 20% FBS (Gibco), 100 units/mL penicillin and 100 μ g/mL streptomycin. Cells were grown in a 37°C, 5% CO₂ humidified incubator.

Quantitative real-time polymerase chain reaction (qRT-PCR)

SYBR Green Mastermix kit (Takara, Tokyo, Japan) was used for the qRT-PCR assay. The expression of DLEU2, miR-205-5p and TNFAIP8 was calculated by $2^{-\Delta\Delta Ct}$ method (26). U6 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) functioned as the internal control. The following primers were used in this study: DLEU2 (forward, 5'-TCTGGAGAACAGCCTCACTTC-3'; reverse, 5'-TGCTGAGCTAAGTAGAGGTCTC-3'), miR-205-5p (forward, 5'-TCCTTCATTCCACCGGAGTCTG-3';

reverse, 5'-GCGAGCACAGAATTAATACGAC-3'), TNFAIP8 (forward, 5'-TGAAGATGGAGCACTGCTGA-3'; reverse, 5'-GGTCTGTTACCCGTTAGGAAG-3') U6 (forward, 5'-CTCGCTTCGGCAGCACATATACTA-3'; reverse, 5'-ACGAATTTGCGTGTGCATCCTTGC-3'), GAPDH (forward, 5'-TGACCACAGTCCATGCCATCAC-3'; reverse, 5'-GCCTGCTTACCACCTTCTTGA-3').

Western blot assay

Thyroid cancer cells were lysed and bicinchoninic acid kit (Beyotime, Shanghai, China) was used for protein concentration determination. The proteins were quantified, and then a total of 40 µg protein samples were separated by SDS-PAGE gel. The proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was blocked by 5% skim milk for 1 h following by incubation with the following antibodies, including Ki67 (ab16667, Abcam), P53 (ab32389, Abcam), matrix metalloproteinase 9 (MMP9; ab219372, Abcam), hexokinase 2 (HK-2; ab104836, Abcam), TNFAIP8 (ab195810, Abcam) and β-actin (ab8226, Abcam). The PVDF membrane was probed with the secondary antibody (ab205718, Abcam). The protein signal was measured and quantified via the enhanced chemiluminescent (ECL) system (Beyotime) and Image J software.

Cell transfection

Transfection was performed using Lipofectamine 3000 (Invitrogen). siRNA negative control (si-NC), siRNA against DLEU2 (si-DLEU2 #1, si-DLEU2 #2 and si-DLEU2 #3), siRNA against TNFAIP8 (si-TNFAIP8 #1, si-TNFAIP8 #2 and si-TNFAIP8 #3), shRNA negative control (sh-NC), shRNA against DLEU2 (sh-DLEU2), Vector, DLEU2 overexpression plasmid (DLEU2) and TNFAIP8 overexpression plasmid (TNFAIP8) were purchased from Genepharma (Shanghai, China). miR-NC and miR-205-5p were synthesized from Ribobio (Guangzhou, China).

Cell counting kit-8 (CCK8) assay

Thyroid cancer cells were seeded into 96-well cell culture plates at a concentration of 1×10^4 cells/well to assess the proliferation with Cell Counting Kit-8 (Sigma), according to the manufacturer's instructions. The absorbance at 450 nm was determined by a microplate reader.

Cell apoptosis analysis

The Annexin V FITC apoptosis detection kit (R&D Systems) was used to measure the apoptosis of thyroid cancer cells. Briefly, thyroid cancer cells were stained with Annexin V combined FITC and propidium iodide (PI) simultaneously. The cells were analyzed through the flow cytometer (BD Biosciences).

Transwell migration assay

Migration assay was carried out to detect the migration ability of TPC-1 and BHT-101 cells. TPC-1 and BHT-101 cells were suspended in the serum-free medium and plated into the upper chambers. Medium supplemented with 10% FBS was used to fill the lower chambers. After cultivation for 48 h, the non-migrated cells were removed with cotton swab and the migrated cells in the basal side of the membrane were stained and counted under a microscope.

Aerobic glycolysis detection

The real-time extracellular acidification rate (ECAR) was detected through an XF96 metabolic flux analyzer (Seahorse Biosciences, Billerica, MA, USA) (27). TPC-1 and BHT-101 cells were seeded into a Seahorse XF 96 cell culture microplate. 10 mM glucose (Glc), 1 mM oligomycin (O), and 80 mM 2-deoxyglucose (2-DG) were injected at specific time points, respectively.

Dual-luciferase reporter assay

MiRcode online software was used for predicting the targets of lncRNA DLEU2. The wild-type or mutant type sequences of DLEU2 were inserted into the reporter vector. TPC-1 and BHT-101 cells were co-transfected with miR-NC or miR-205-5p and WT-DLEU2 or MUT-DLEU2. The Firefly and Renilla luciferase activities in TPC-1 and BHT-101 cells were detected through the Dual-Glo® Luciferase Assay System (Promega). The target genes of miR-205-5p were predicted by using Starbase software. The wild-type or mutant type binding sites of TNFAIP8 3' UTR was constructed into the reporter vector. The validation of the combination between TNFAIP8 and miR-205-5p was conducted following the same approach.

Murine xenograft assay

Nude mice purchased from Beijing Vital River Laboratory (Beijing, China) were divided into two groups:

sh-NC group ($n = 5$) and sh-DLEU2 group ($n = 5$). This experiment was conducted with the agreement of the Animal Research Committee of Yijishan Hospital of Wannan Medical College. TPC-1 cells were stably transfected with sh-NC or sh-DLEU2 and suspended in 150 μ L PBS. The mice were inoculated with the above TPC-1 cells. The dimension of tumors was recorded once a week. The mice were killed following inoculation for 4 weeks, and the tumors were excised from the mice.

Statistical analysis

All outliers have been included in our results. Data were analyzed through GraphPad Prism 7 software and presented as mean \pm s.d. Data were tested for the normality using Shapiro–Wilk test. Student’s t -test or one-way ANOVA followed by Tukey’s test was used to evaluate the differences. Spearman’s correlation coefficient was utilized to analyze the linear relationship between levels of specific genes. P value < 0.05 was considered statistically significant.

Results

The abundance of DLEU2 and TNFAIP8 is upregulated in thyroid cancer

To uncover the molecular mechanism in thyroid cancer progression, we intended to explore the crucial molecules involved in thyroid cancer development. As showed in Fig. 1A, DLEU2 was conspicuously upregulated in thyroid cancer tissues compared with that in matching normal tissues. Meanwhile, the mRNA and protein expression of TNFAIP8 was also higher in thyroid cancer tissues than that in corresponding normal tissues (Fig. 1B and C). The expression of DLEU2 and TNFAIP8 in thyroid cancer in the TCGA database was also analyzed. DLEU2 expression was markedly upregulated in thyroid cancer tissues ($n = 505$) relative to normal tissues ($n = 59$) on the basis of the data in the TCGA database (Supplementary Fig. 1A, see section on supplementary materials given at the end of this article). TNFAIP8 mRNA was slightly upregulated in thyroid cancer tissues ($n = 512$) than that in normal tissues ($n = 337$). However, there was no

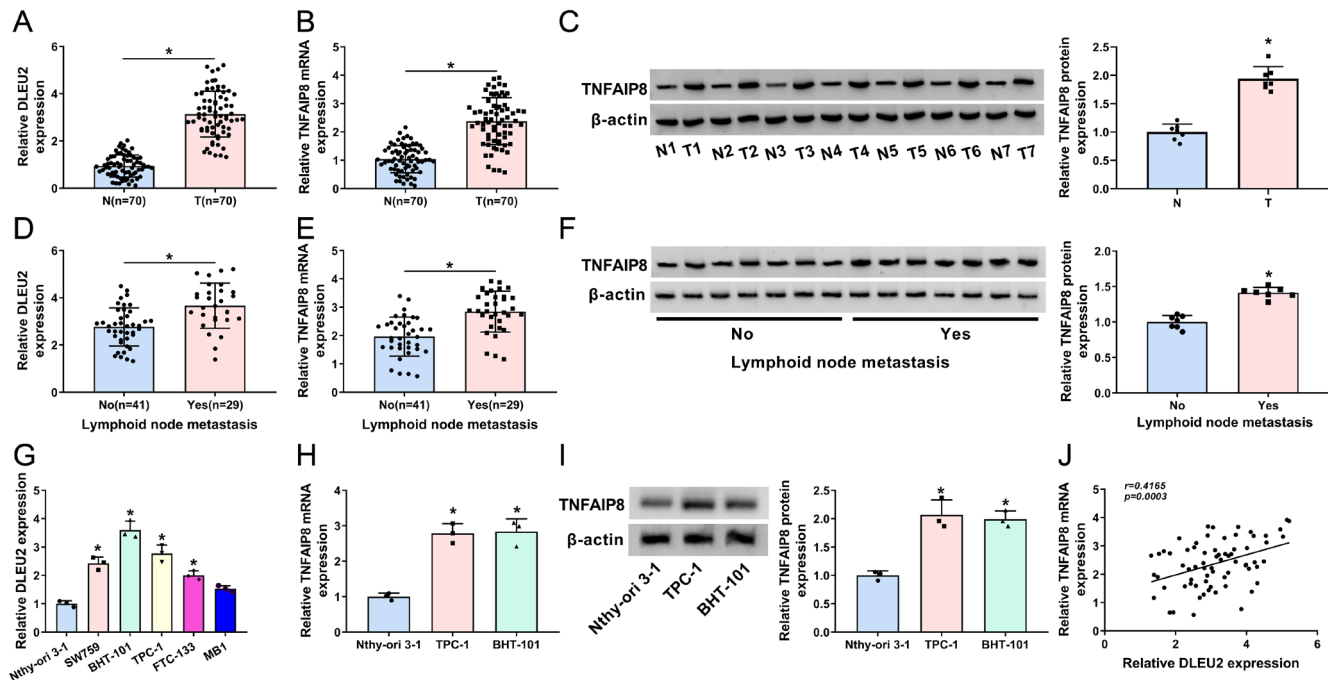


Figure 1

The abundance of DLEU2 and TNFAIP8 is upregulated in thyroid cancer. (A) qRT-PCR was applied to measure the enrichment of DLEU2 in thyroid cancer tissues and corresponding normal tissues. (B and C) The mRNA and protein expression of TNFAIP8 was determined in thyroid cancer tissues and corresponding normal tissues by qRT-PCR and Western blot assay. (D) The expression of DLEU2 was examined in lymphoid node metastatic or non-metastatic thyroid cancer tissues by qRT-PCR. (E and F) qRT-PCR and Western blot assay were conducted to detect the abundance of TNFAIP8 mRNA and protein in lymphoid node metastatic or non-metastatic thyroid cancer tissues. (G) The level of DLEU2 was measured in human thyroid normal cells Nthy-ori 3-1 and thyroid cancer cells TPC-1 and BHT-101 by qRT-PCR. (H and I) The mRNA and protein expression of TNFAIP8 was examined in human thyroid normal cells Nthy-ori 3-1 and thyroid cancer cells TPC-1 and BHT-101 by qRT-PCR and Western blot assay. (J) The correlation between DLEU2 and TNFAIP8 was analyzed in thyroid cancer tissues. $*P < 0.05$.

significant difference in the expression of TNFAIP8 in the two groups (Supplementary Fig. 1B). As indicated in Fig. 1D, E and F, the level of DLEU2 and the abundance of TNFAIP8 mRNA and protein were higher in thyroid cancer patients with lymphoid node metastasis, suggesting that the high expression of DLEU2 and TNFAIP8 was positively related to metastasis of thyroid cancer. Apart from this, we measured the expression of DLEU2 in human thyroid normal cell line Nthy-ori 3-1 and a panel of thyroid cancer cell lines. As mentioned in Fig. 1G, DLEU2 was highly expressed in five thyroid cancer cell lines relative to Nthy-ori 3-1 cell line, especially in BHT-101 and TPC-1 cell lines. Thus TPC-1 and BHT-101 cell lines were selected for further analysis. The mRNA and protein expression of TNFAIP8 was also found to be markedly elevated in

TPC-1 and BHT-101 cell lines compared with Nthy-ori 3-1 cell line (Fig. 1H and I). Correlation analysis showed the expression of DLEU2 was positively correlated with the abundance of TNFAIP8 in thyroid cancer tissues (Fig. 1J). The abnormal upregulation of DLEU2 and TNFAIP8 might imply their important regulatory roles in thyroid cancer progression.

DLEU2 knockdown inhibits the proliferation, migration and aerobic glycolysis while accelerates the apoptosis of thyroid cancer cells

DLEU2 majorly located in the cytoplasmic fraction of thyroid cancer cells (Fig. 2A). To explore the function of DLEU2 in thyroid cancer cells, we conducted

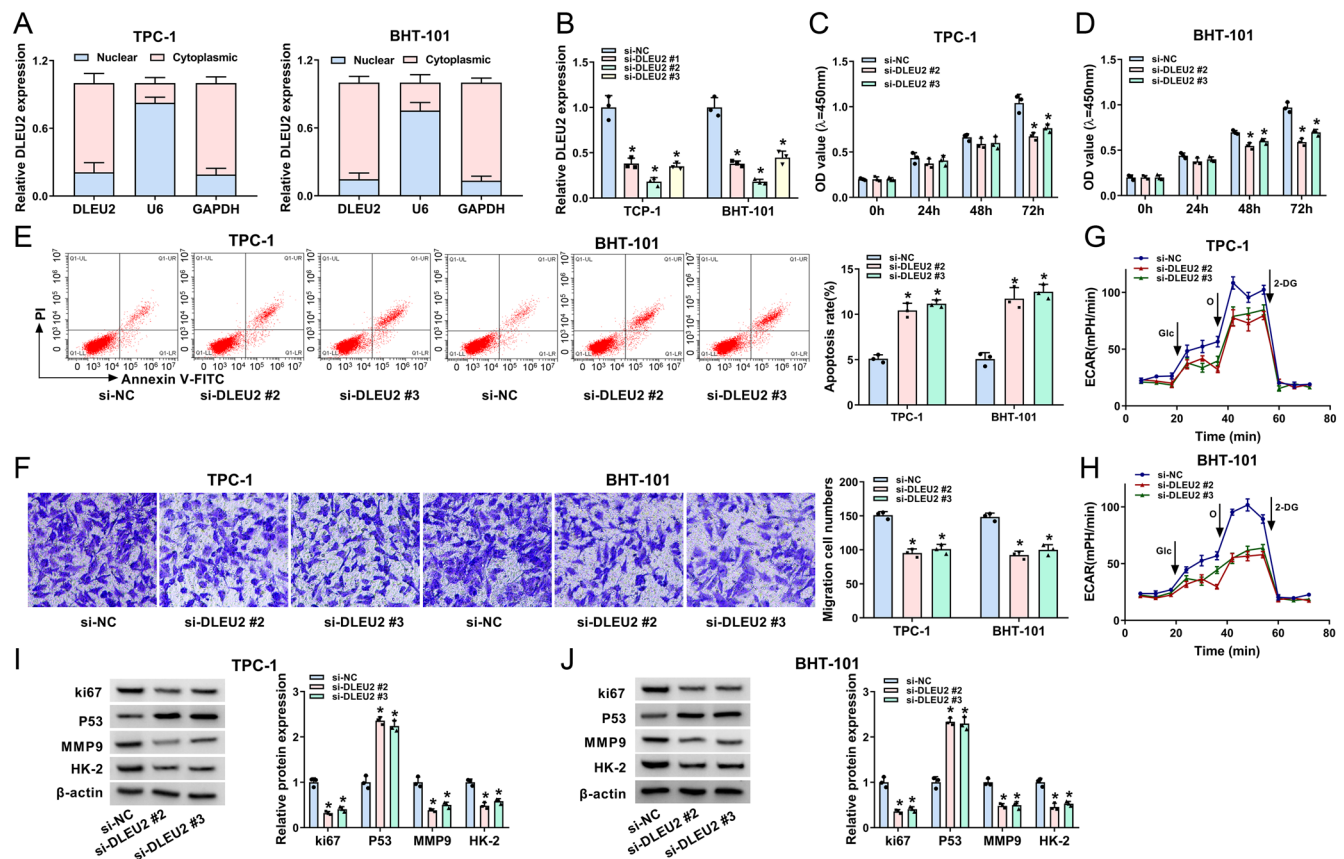


Figure 2

DLEU2 knockdown inhibits the proliferation, migration and aerobic glycolysis while accelerates the apoptosis of thyroid cancer cells. (A) The subcellular distribution of DLEU2 in the nuclear fraction and the cytoplasmic fraction of thyroid cancer cells were analyzed. (B) The enrichment of DLEU2 was determined in TPC-1, and BHT-101 cells transfected with si-NC, si-DLEU2 #1, si-DLEU2 #2 or si-DLEU2 #3 by qRT-PCR. (C and D) The proliferation of si-NC, si-DLEU2 #2 or si-DLEU2 #3 transfected TPC-1 and BHT-101 cells were measured by CCK8 assay. (E) Flow cytometry was performed to detect the apoptosis of TPC-1 and BHT-101 cells transfected with si-NC, si-DLEU2 #2 or si-DLEU2 #3. (F) Transwell migration assay was carried out to examine the migration of si-NC, si-DLEU2 #2 or si-DLEU2 #3 transfected thyroid cancer cells. (G and H) ECAR was analyzed in si-NC, si-DLEU2 #2 or si-DLEU2 #3 transfected TPC-1 and BHT-101 cells by an XF96 metabolic flux analyzer. (I and J) Western blot assay was conducted to measure the expression of proliferation-related protein (Ki67), apoptosis-associated protein (P53), migration-related protein (MMP9) and glycolysis-associated protein (HK-2) in TPC-1 and BHT-101 cells transfected with si-NC, si-DLEU2 #2 or si-DLEU2 #3. *P < 0.05.

loss-of-function experiments. The level of DLEU2 was notably declined in si-DLEU2 #1, si-DLEU2 #2 or si-DLEU2 #3 group compared with that in si-NC group in TPC-1 and BHT-101 cells (Fig. 2B). We selected si-DLEU2 #2 and si-DLEU2 #3 to conduct loss-of-function experiments. As mentioned in Fig. 2C and D, DLEU2 depletion significantly suppressed cell proliferation in thyroid cancer cells. Besides, DLEU2 intervention accelerated the apoptosis of TPC-1 and BHT-101 cells (Fig. 2E). As showed in Fig. 2F, with the silencing of DLEU2, the migration of thyroid cancer cells was markedly restrained. Cancer cells prefer aerobic glycolysis rather than oxidative phosphorylation to obtain energy, and this phenomenon is called the Warburg effect (28). As we all know, the survival of cancer cells is accelerated, while apoptosis is impeded due to the Warburg effect (29). We found that the extracellular acidification rate (ECAR) was reduced in two DLEU2-silenced groups relative to si-NC group, suggesting that DLEU2 silencing suppressed the aerobic glycolysis of thyroid cancer cells (Fig. 2G and H). Additionally, the results of Western blot assay also revealed that the proliferation-related protein (Ki67), migration-associated protein (MMP9) and glycolysis-related protein (HK-2) were downregulated with DLEU2 depletion in thyroid cancer cells (Fig. 2I and J), while the abundance of apoptosis-related protein (P53) was elevated, which further demonstrated that the knockdown of DLEU2 inhibited the proliferation, migration and aerobic glycolysis whereas promoted the apoptosis of thyroid cancer cells.

To further confirm the biological functions of DLEU2 in thyroid cancer cells, we performed gain-of-function experiments. As shown in Supplementary Fig. 2A, B, C, D and E, DLEU2 overexpression facilitated the proliferation, migration and aerobic glycolysis and suppressed the apoptosis of thyroid cancer cells. The results of loss- and gain-of-function experiments together demonstrated that DLEU2 served as an oncogene to promote thyroid cancer progression.

miR-205-5p is a direct target of DLEU2, and miR-205-5p targets TNFAIP8 in thyroid cancer cells as well

miR-205-5p and TNFAIP8 were predicted as targets of DLEU2 and miR-205-5p by miRcode and Starbase software, respectively (Fig. 3A and B). To verify the combination between miR-205-5p and DLEU2 or TNFAIP8 in thyroid cancer cells, we conducted dual-luciferase reporter assay. As showed in Fig. 3C and D, TPC-1 and BHT-101 cells were

transfected with miR-NC or miR-205-5p and WT-DLEU2 or MUT-DLEU2, and luciferase activity was dramatically reduced in miR-205-5p and WT-DLEU2 co-transfected group, demonstrating that miR-205-5p was a direct target of DLEU2 in thyroid cancer cells. Meanwhile, we transfected miR-NC or miR-205-5p and TNFAIP8 3' UTR-WT or TNFAIP8 3' UTR-MUT into TPC-1 and BHT-101 cells. As showed in Fig. 3E and F, the overexpression of miR-205-5p notably decreased the luciferase activity in TNFAIP8 3' UTR-WT group compared with that in TNFAIP8 3' UTR-MUT group, suggesting that TNFAIP8 could bind to miR-205-5p in thyroid cancer cells. To elucidate the regulatory relationship between miR-205-5p and DLEU2 or TNFAIP8, we performed the following experiments. miR-205-5p was negatively regulated by DLEU2 in TPC-1 and BHT-101 cells (Fig. 3G). We assessed the overexpression efficiency of miR-205-5p in TPC-1 and BHT-101 cells, and the expression of miR-205-5p was markedly enhanced by miR-205-5p transfection in TPC-1 and BHT-101 cells (Fig. 3H). The enrichment of TNFAIP8 mRNA and protein was inversely modulated by miR-205-5p in thyroid cancer cells (Fig. 3I and J). Taken together, miR-205-5p was a direct target of DLEU2, and miR-205-5p also could bind to TNFAIP8 in TPC-1 and BHT-101 cells. There was a negative regulatory relationship between miR-205-5p and DLEU2 or TNFAIP8 in thyroid cancer cells.

TNFAIP8 plays an oncogenic role in thyroid cancer cells

To clarify the functions of TNFAIP8 in thyroid cancer cells, we conducted knockdown experiments. As indicated in Fig. 4A, B and C, the expression of TNFAIP8 mRNA and protein was prominently downregulated by TNFAIP8 siRNA transfection in TPC-1 and BHT-101 cells. Si-TNFAIP8 #2 and si-TNFAIP8#3 were selected for the following knockdown experiments. TNFAIP8 depletion impeded the proliferation, migration and aerobic glycolysis while promoted the apoptosis of thyroid cancer cells (Fig. 4D, E, F, G, H and I). Meanwhile, the results of Western blot assay supported the above conclusion. The abundance of proliferation-related protein (Ki67), migration-associated protein (MMP9) and aerobic glycolysis-related protein (HK-2) was downregulated, and the expression of apoptosis-associated protein P53 was elevated in si-TNFAIP8 #2 group and si-TNFAIP8#3 group than that in si-NC group (Fig. 4J). These results suggested that TNFAIP8 silencing suppressed the development of thyroid cancer *in vitro*.

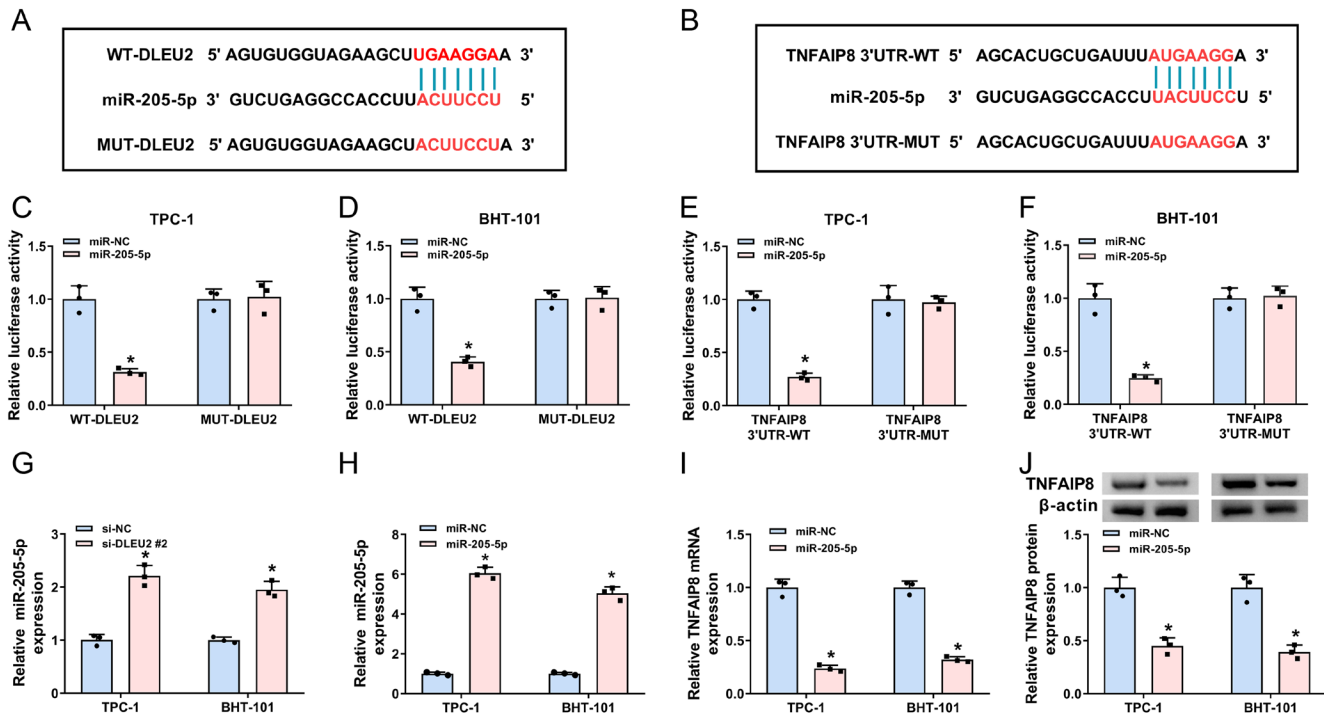


Figure 3

miR-205-5p is a direct target of DLEU2, and miR-205-5p targets TNFAIP8 in thyroid cancer cells as well. (A and B) The binding sites between miR-205-5p and DLEU2 or TNFAIP8 were predicted by miRcode or Starbase software. (C and D) Dual-luciferase reporter assay was conducted to confirm the combination between miR-205-5p and DLEU2 in TPC-1 and BHT-101 cells. (E and F) Luciferase activity was examined in TPC-1 and BHT-101 cells co-transfected with miR-NC or miR-205-5p and TNFAIP8 3' UTR-WT or TNFAIP8 3' UTR-MUT. (G) The level of miR-205-5p was detected in thyroid cancer cells transfected with si-NC or si-DLEU2 #2 by qRT-PCR. (H) The overexpression efficiency of miR-205-5p was assessed in thyroid cancer cells transfected with miR-NC or miR-205-5p by qRT-PCR. (I and J) qRT-PCR and Western blot assay were applied to measure the abundance of TNFAIP8 mRNA and protein in TPC-1 and BHT-101 cells transfected with miR-NC or miR-205-5p. * $P < 0.05$.

Gain-of-function experiments were carried out to further verify the functions of TNFAIP8 in thyroid cancer cells. TNFAIP8 overexpression facilitated the proliferation, migration and glycolysis while suppressed the apoptosis of thyroid cancer cells (Supplementary Fig. 3A, B, C, D and E). The results of loss- and gain-of-function experiments together suggested that TNFAIP8 functioned as a pro-tumor molecule to contribute to thyroid cancer development.

TNFAIP8 overexpression reverses the inhibitory effects of DLEU2 depletion on the proliferation, migration and aerobic glycolysis and the promoting impact on the apoptosis of thyroid cancer cells

The enrichment of TNFAIP8 mRNA and protein was conspicuously enhanced by TNFAIP8 transfection in thyroid cancer cells (Fig. 5A and B). TPC-1 and BHT-101 cells were transfected with si-NC, si-DLEU2 #2, si-DLEU2 #2+Vector or si-DLEU2 #2+TNFAIP8, respectively. As mentioned in Fig. 5C, D and E, the mRNA and protein

expression of TNFAIP8 was declined by si-DLEU2 #2 transfection, and the addition of TNFAIP8 recovered its mRNA and protein expression in TPC-1 and BHT-101 cells. Meanwhile, we measured the proliferation, apoptosis, migration and aerobic glycolysis in the above thyroid cancer cells. The accumulation of TNFAIP8 abrogated the inhibitory effects of DLEU2 silencing on the proliferation, migration and aerobic glycolysis and the promoting impact on the apoptosis of TPC-1 and BHT-101 cells (Fig. 5F, G, H, I and J). Collectively, DLEU2 facilitated the progression of thyroid cancer cells through upregulating TNFAIP8.

TNFAIP8 is modulated by DLEU2/miR-205-5p axis in thyroid cancer cells

To clarify the relationship among DLEU2, miR-205-5p and TNFAIP8 in thyroid cancer cells, we performed the following experiments. The overexpression efficiency of DLEU2 plasmid was assessed in TPC-1 and BHT-101 cells. As showed in Fig. 6A and B, the abundance of DLEU2

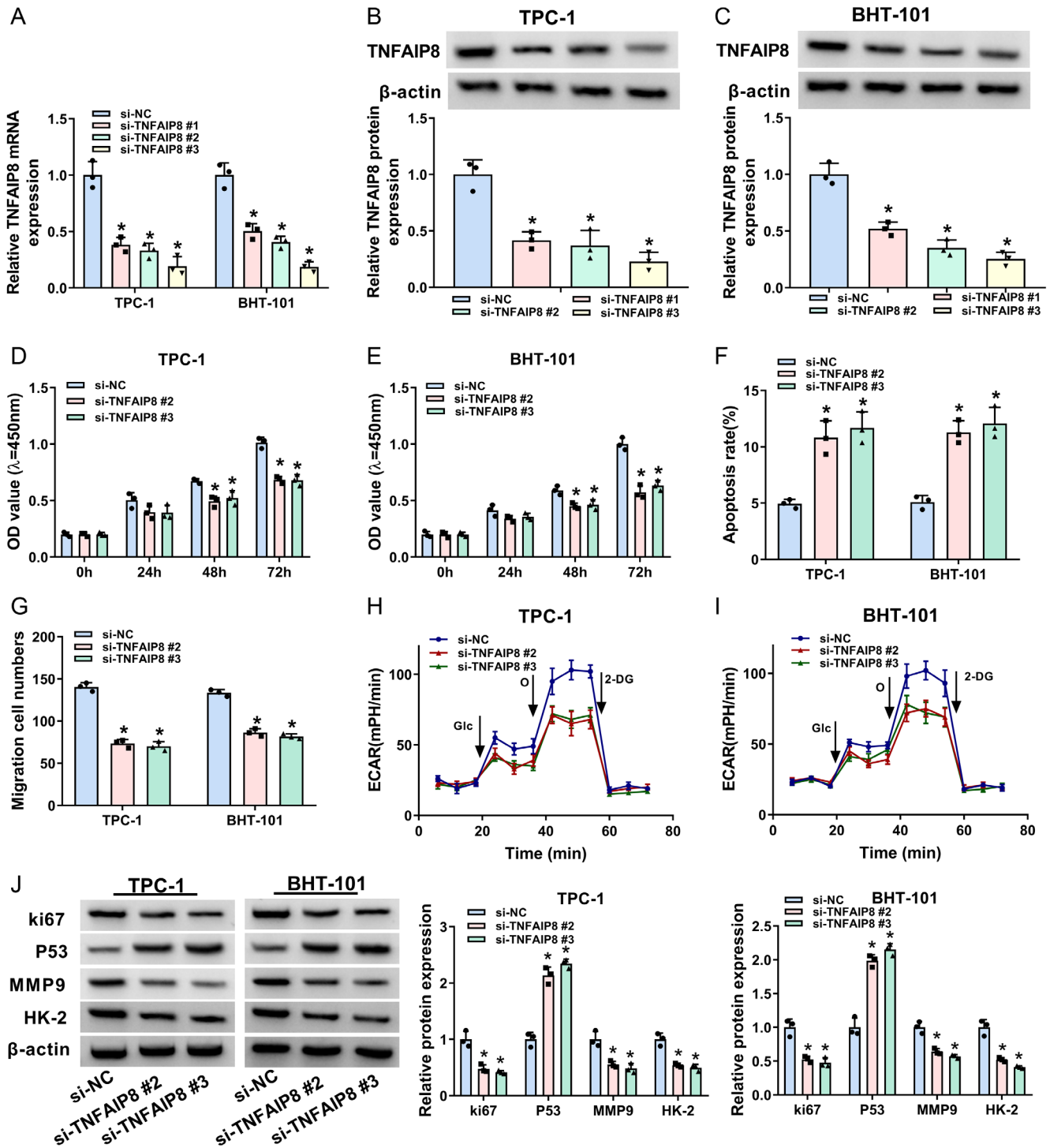


Figure 4 TNFAIP8 plays an oncogenic role in thyroid cancer cells. (A, B and C) The mRNA and protein expression of TNFAIP8 was determined in TPC-1 and BHT-101 cells transfected with si-NC, si-TNFAIP8 #1, si-TNFAIP8 #2 or si-TNFAIP8 #3 by qRT-PCR and Western blot assay. (D and E) The proliferation of thyroid cancer cells transfected with si-NC, si-TNFAIP8 #2 or si-TNFAIP8 #3 was examined by CCK8 assay. (F) Flow cytometry was applied to detect the apoptosis of TPC-1 and BHT-101 cells transfected with si-NC, si-TNFAIP8 #2 or si-TNFAIP8 #3. (G) The migration of si-NC, si-TNFAIP8 #2 or si-TNFAIP8 #3 transfected TPC-1 and BHT-101 cells was measured by transwell migration assay. (H and I) ECAR was analyzed in si-NC, si-TNFAIP8 #2 or si-TNFAIP8 #3 transfected TPC-1 and BHT-101 cells by an XF96 metabolic flux analyzer. (J) The expression of Ki67, P53, MMP9 and HK-2 in si-NC, si-TNFAIP8 #2 or si-TNFAIP8 #3 transfected TPC-1 and BHT-101 cells was determined by Western blot assay. * $P < 0.05$.

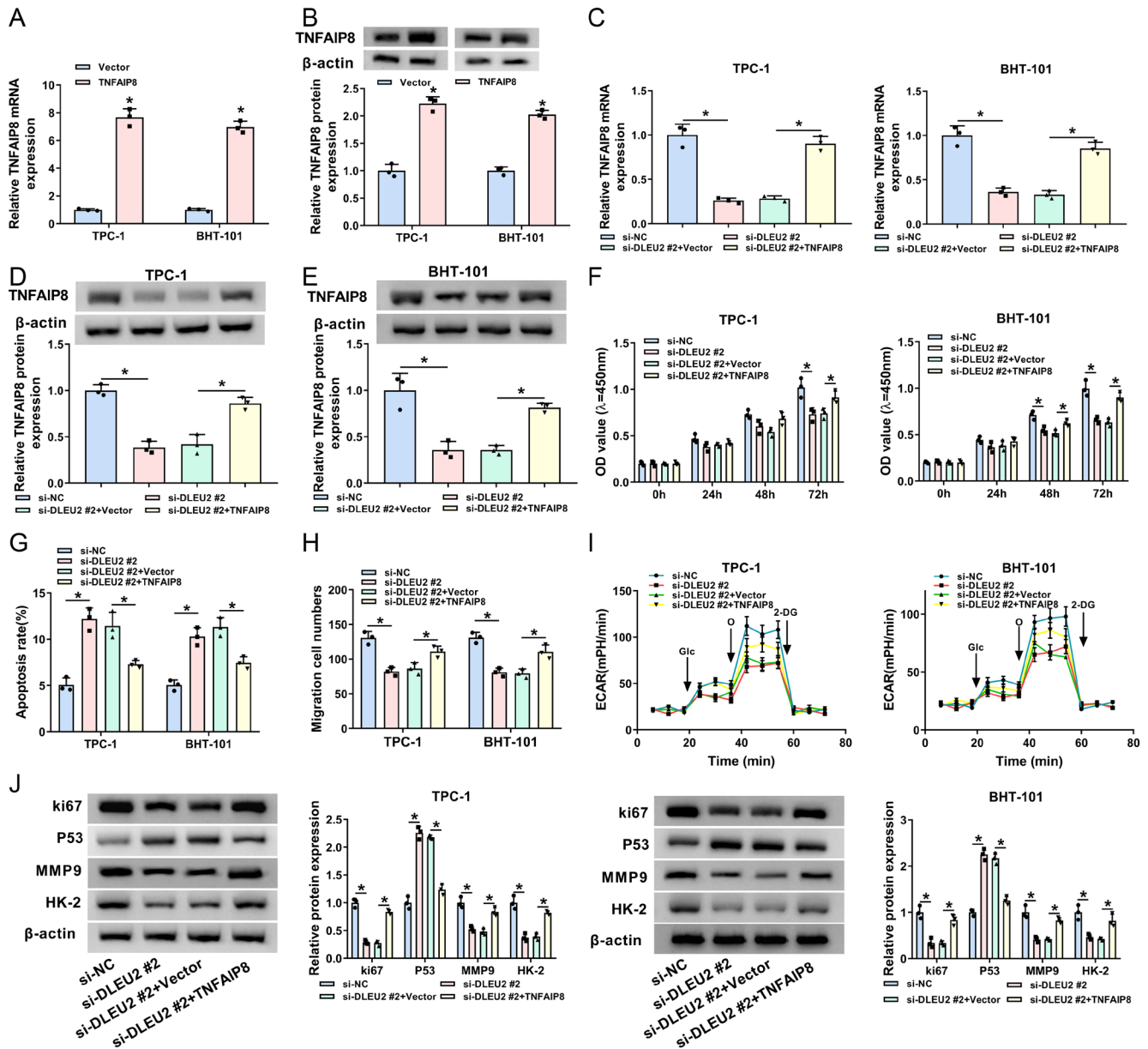


Figure 5

TNFAIP8 overexpression reverses the inhibitory effects of DLEU2 depletion on the proliferation, migration and aerobic glycolysis and the promoting impact on the apoptosis of thyroid cancer cells. (A and B) The abundance of TNFAIP8 mRNA and protein was determined in TPC-1 and BHT-101 cells transfected with Vector or TNFAIP8 by qRT-PCR and Western blot assay. TPC-1 and BHT-101 cells were transfected with si-NC, si-DLEU2 #2, si-DLEU2 #2 + Vector or si-DLEU2 #2 + TNFAIP8, respectively. (C, D and E) The mRNA and protein expression of TNFAIP8 was measured in the above thyroid cancer cells by qRT-PCR and Western blot assay. (F) CCK8 assay was performed to detect the proliferation of the above TPC-1 and BHT-101 cells. (G) The apoptosis of thyroid cancer cells was examined by flow cytometry. (H) Transwell migration assay was applied to determine the migration of TPC-1 and BHT-101 cells. (I) ECAR was measured in TPC-1 and BHT-101 cells by an XF96 metabolic flux analyzer. (J) Western blot assay was performed to detect the levels of proliferation-related protein (Ki67), apoptosis-associated protein (P53), migration-related protein (MMP9) and glycolysis-associated protein (HK-2) in TPC-1 and BHT-101 cells. **P* < 0.05.

was conspicuously elevated in DLEU2-transfected group compared with that in Vector group in TPC-1 and BHT-101 cells. We determined the mRNA and protein expression of TNFAIP8 in TPC-1 and BHT-101 cells transfected with Vector, DLEU2, DLEU2+miR-NC or DLEU2+miR-205-5p.

The abundance of TNFAIP8 mRNA and protein was upregulated with DLEU2 overexpression, while the addition of miR-205-5p decreased the mRNA and protein expression of TNFAIP8 in TPC-1 and BHT-101 cells (Fig. 6C, D, E and F). Taken together, DLEU2 upregulated the

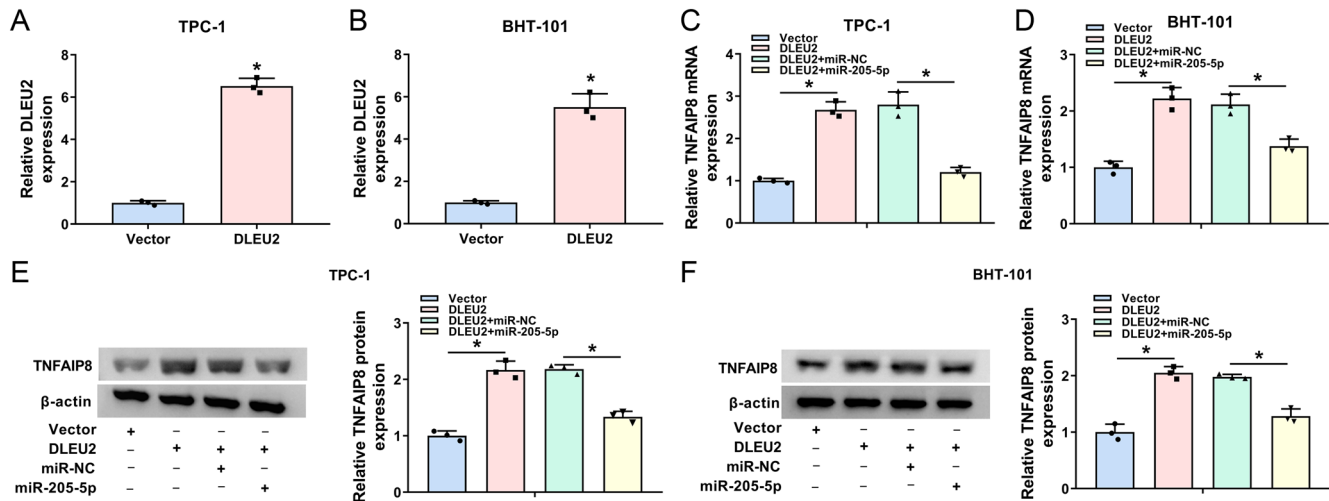


Figure 6

TNFAIP8 is modulated by DLEU2/miR-205-5p axis in thyroid cancer cells. (A and B) The expression of DLEU2 was determined in TPC-1 and BHT-101 cells transfected with Vector or DLEU2 by qRT-PCR. (C, D, E and F) The abundance of TNFAIP8 mRNA and protein was measured in TPC-1 and BHT-101 cells transfected with Vector, DLEU2, DLEU2 + miR-NC or DLEU2 + miR-205-5p by qRT-PCR and Western blot assay. **P* < 0.05.

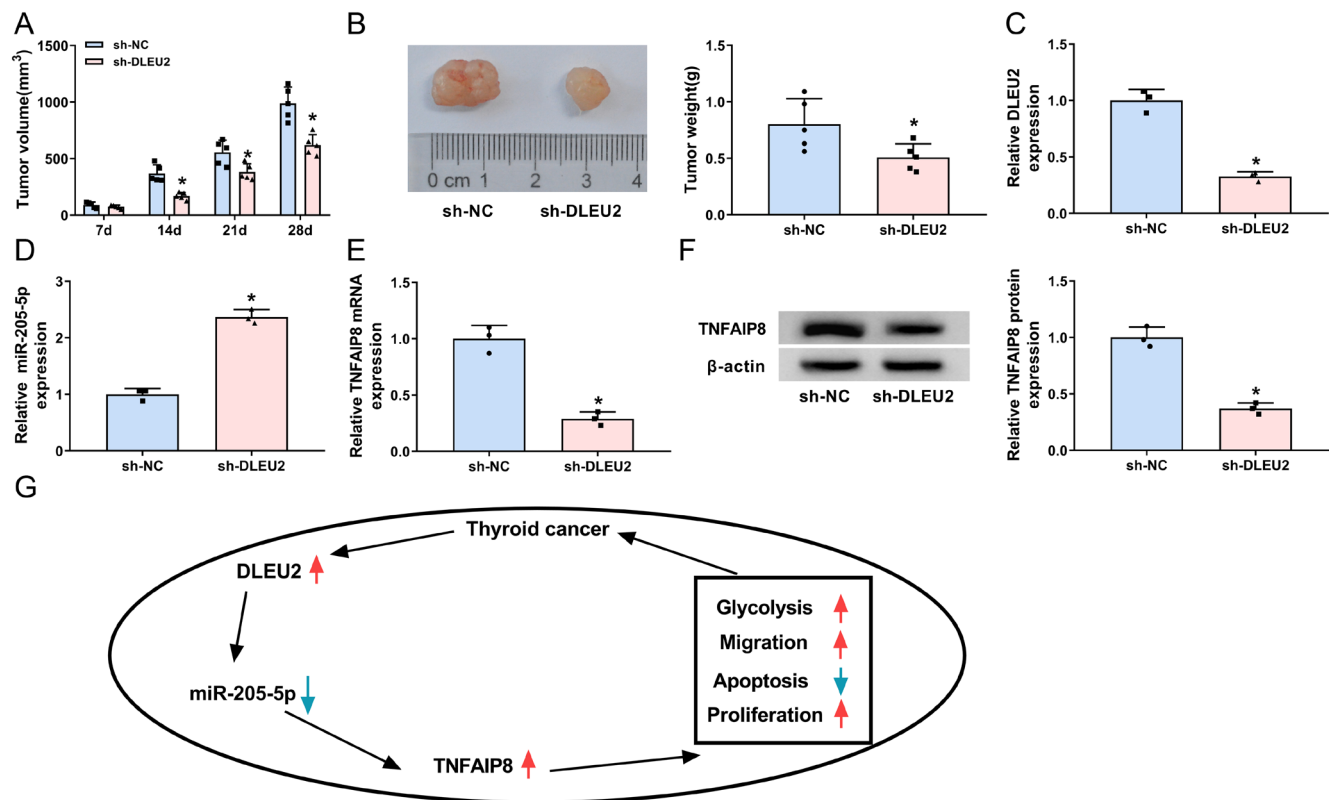


Figure 7

DLEU2 promotes the progression of thyroid cancer through miR-205-5p/TNFAIP8 axis *in vivo*. (A) Tumor volume was measured every 7 days. (B) Tumors were weighed after 4-week injection. (C and D) The expression of DLEU2 and miR-205-5p was detected in resected tumor tissues by qRT-PCR. (E and F) The abundance of TNFAIP8 mRNA and protein was determined in resected tumor tissues by qRT-PCR and Western blot assay. (G) A schematic illustration of our model revealed the molecular mechanism by which DLEU2 promoting the progression of thyroid cancer. **P* < 0.05.

abundance of TNFAIP8 through sponging miR-205-5p in thyroid cancer cells.

DLEU2 promotes the progression of thyroid cancer through miR-205-5p/TNFAIP8 axis *in vivo*

We built murine xenograft model using TPC-1 cells stably transfected with sh-NC or sh-DLEU2 to explore the role of DLEU2 *in vivo*. As indicated in Fig. 7A and B, DLEU2 silencing significantly suppressed tumor growth relative to sh-NC group. The expression of DLEU2 was remarkably decreased in sh-DLEU2 group compared with that in the control group (Fig. 7C). Besides, the level of miR-205-5p was elevated while the abundance of TNFAIP8 mRNA and protein was downregulated in sh-DLEU2 group (Fig. 7D, E and F). Taken together, DLEU2 promoted the progression of thyroid cancer by accelerating the proliferation, migration and aerobic glycolysis while restraining the apoptosis of thyroid cancer cells via miR-205-5p/TNFAIP8 axis (Fig. 7G).

Discussion

This study aimed to investigate the critical molecules and signal pathways involved in thyroid cancer progression. lncRNA DLEU2 was aberrantly upregulated in thyroid cancer tissues and cells compared with that in matching non-tumor tissues and thyroid normal cells Nthy-ori 3-1. DLEU2 promoted the initiation and development of multiple cancers (30, 31, 32). However, the effect of DLEU2 in thyroid cancer is largely unknown. We found that the high expression of DLEU2 was related to the lymphoid node metastasis in patients with thyroid cancer, implying the crucial role of DLEU2 in thyroid cancer progression. Subsequently, we found DLEU2 interference inhibited the proliferation, migration and aerobic glycolysis while accelerated the apoptosis of thyroid cancer cells.

lncRNAs serve as miRNA sponges to exert their functions generally (5, 6, 7). To elucidate the signal pathway of DLEU2 in thyroid cancer, miRcode software was used to search the downstream genes of DLEU2. miR-205-5p was predicted as a target of DLEU2, and dual-luciferase reporter assay confirmed the target relationship between miR-205-5p and DLEU2 in TPC-1 and BHT-101 cells. Subsequently, TNFAIP8 was predicted as a target of miR-205-5p by Starbase software, and the combination between miR-205-5p and TNFAIP8 was verified by dual-luciferase reporter assay.

TNFAIP8 has been reported to be upregulated and acted as an oncogene in thyroid cancer (25). Consistent with the above findings, we found the abundance of TNFAIP8 was conspicuously higher in thyroid cancer tissues and cells than that in adjacent non-tumor tissues and normal thyroid cells. Meanwhile, the enrichment of TNFAIP8 was positively related to lymphoid node metastasis of thyroid cancer patients. As expected, there was a positive relationship between the abundance of TNFAIP8 and DLEU2 in thyroid cancer tissues. TNFAIP8 played an oncogenic role in thyroid cancer through promoting proliferation, migration and aerobic glycolysis and impeding apoptosis of thyroid cancer cells.

The accumulation of TNFAIP8 counteracted the inhibitory effects of DLEU2 intervention on the proliferation, migration and aerobic glycolysis and the promoting impact on the apoptosis of thyroid cancer cells. Additionally, we found that DLEU2 upregulated the level of TNFAIP8 through sponging miR-205-5p in thyroid cancer cells. DLEU2 knockdown also suppressed the growth of thyroid tumors through miR-205-5p/TNFAIP8 axis *in vivo*.

In future, the role of DLEU2 in the invasion of thyroid cancer cells *in vitro* needs to be analyzed. Additionally, the *in vivo* role of DLEU2 in the metastasis of thyroid cancer needs to be assessed in nude mice to better illustrate the biological significance of DLEU2 in the progression of thyroid cancer.

In conclusion, lncRNA DLEU2 facilitated the progression of thyroid cancer through promoting proliferation, migration and aerobic glycolysis and restraining apoptosis of thyroid cancer cells via miR-205-5p/TNFAIP8 signaling.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/EC-21-0046>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Patient consent

Written informed consent was obtained from all enrolled patients.

Ethics approval

The present study was approved by the ethical review committee of The Second Hospital of Hebei Medical University.

Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Author contribution statement

All authors made substantial contribution to conception and design, acquisition of the data, or analysis and interpretation of the data; take part in drafting the article or revising it critically for important intellectual content; gave final approval of the revision to be published; and agree to be accountable for all aspect of the work.

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