Article

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KLHDC8A Regulates M1/M2 Macrophage Polarization Through the PD-1/STAT3 Pathway to Promote Papillary Thyroid Cancer Development

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Background: Papillary thyroid cancer (PTC) is one of the most frequent endocrine malignancies. Kelch domain containing 8A (KLHDC8A) is reported as an epigenetically driven oncogene, but the role of KLHDC8A in PTC is still unclear. This study aimed to explore the function of KLHDC8A in PTC progression.

Methods: KLHDC8A expression was analyzed by the Gene Expression Profiling Interactive Analysis (GEPIA) website, quantitative real-time PCR (qRT-PCR), and western blot. The viability of PTC cells (TPC-1 and BCPAP) was assessed by cell counting kit-8 (CCK-8) kit. A transwell assay was carried out to evaluate the invasion and migration of PTC cells. Macrophage polarization-associated markers were determined by qRT-PCR and western blot. Mice tumor xenograft models were established to analyze the role of KLHDC8A *in vivo*. Pathway-related proteins (programmed cell death protein 1 (PD-1) and signal transducer and activator of transcription 3 (STAT3)) were determined by western blot.

Results: GEPIA demonstrated that KLHDC8A was highly expressed in PTC ($p < 0.05$). Knockdown of KLHDC8A hindered cell viability, invasion, and migration of PTC cells ($p < 0.0001$). Additionally, KLHDC8A knockdown inhibited M2 polarization while promoting M1 polarization ($p < 0.0001$). Meanwhile, KLHDC8A silencing inhibited tumor growth in mice xenografted models ($p < 0.0001$). Moreover, the PD-1/STAT3 pathway was suppressed by KLHDC8A silencing ($p < 0.01$), and the STAT3 activator (colivelin) attenuated the inhibitory effects of KLHDC8A silencing on PTC progression ($p < 0.01$).

Conclusions: Through *in vivo* and *in vitro* experiments, KLHDC8A silencing could restrain PTC cell viability, migration, and invasion, inhibit tumor growth, and promote M1 polarization via the PD-1/STAT3 axis, providing a new therapeutic idea for PTC clinical treatment.

Keywords: KLHDC8A; papillary thyroid cancer; PD-1; STAT3; macrophage polarization

Introduction

As estimated by Global Cancer Statistics 2020, thyroid cancer has been ranked as the ninth most common malignant tumor in the world [1,2]. Approximately 90% of patients with papillary thyroid cancer (PTC) can be cured with standard treatment, such as surgery and radioactive iodine therapy [3,4]. In cases of low-risk thyroid cancer, minimally invasive intervention and active surveillance are generally adopted [5]. However, due to geographical and social differences, PTC treatment is inconsistent across countries, and some new targeted therapies are not well publicized [5]. While most PTCs are well differentiated and have low rates of local invasion, recurrence, or metastasis, a small subset of tumors exhibit heterogeneous and more aggressive variants with distinct pathological, clinical, and molecular features [6]. This remains a challenge for PTC therapy as approximately 10% of patients develop remote metastasis or locoregional recurrence [7]. Currently, the pathogenesis of PTC development is not fully understood. Consequently, it is vital to inquire into the mechanisms that inhibit PTC progression and offer new strategies for treating PTC.

Kelch domain containing 8A (KLHDC8A) is a member of the Kelch superfamily, which is identified to be upregulated in cancers [8]. Kelch family members are essential in cell migration, gene expression, protein degradation, and extracellular communication/interaction [9,10]. KLHDC8A plays key roles in the developmental progression of several cancers. KLHDC8A is highly expressed in high-grade glioma tissues and is closely associated with poor prognosis [8]. Knockdown of KLHDC8A blocks the cell cycle, accelerates apoptosis, and obstacles proliferation, invasion, and migration of glioma cells [8]. KLHDC8A expression is increased in human glioma, and knockdown of KLHDC8A expression inhibits tumor growth in DeltaEGFR-independent “escaper” tumors in glioma [11].

Nevertheless, the effects of KLHDC8A on PTC development are still unclear.

The tumor microenvironment (TME) has a crucial effect on tumor metastasis and progression [12]. Tumor-associated macrophages (TAMs) are substantial components of TME. The M2 phenotype is closely associated with invasion, metastasis, and immune suppression [13]. Previous research has demonstrated that the density of TAMs is significantly higher in PTC tumors and is positively associated with tumor-node-metastasis [14]. However, the correlation between KLHDC8A and TAMs phenotype is unclear.

Programmed cell death protein 1 (PD-1) and signal transducer and activator of transcription 3 (STAT3) play pivotal roles in the progress of the tumor. The inhibition of PD-L1 reinvigorates T cells, thereby further promoting the M1 polarization of macrophages [15]. Yuan *et al*. [16] have reported that in non-small cell lung cancer, the PD-1/STAT3 pathway is associated with the regulation of immune escape of tumor cells, cancer development, and metastasis. Additionally, STAT3 is identified to regulate glycolysis, growth, and lung metastasis in PTC [17]. However, whether the PD-1/STAT3 pathway participates in regulating KLHDC8A on PTC development is unclear.

Herein, the influence and underlying mechanism of KLHDC8A on PTC were investigated through the *in vivo* and *in vitro* assays. KLHDC8A was silenced to explore its role in cell viability, migration, invasion, macrophage polarization, and tumor growth, which may provide a promising target for PTC treatment.

Materials and Methods

Gene Expression Profiling Analysis

Gene Expression Profiling Interactive Analysis (GEPIA) (http://gepia.cancer-pku.cn/index.html) was carried out to process the gene expression.

Cell Culture and Transfection

The human normal thyroid cells (Nthy-ori3-1, iCell-h335) and human PTC cell lines (TPC-1, iCell-h309, and BCPAP, iCell-h022) were obtained from iCell (Shanghai, China) with their short tandem repeat (STR) validations. All cells have been detected by Mycolor One-Step Mycoplasma Detector (Vazyme, Nanjing, China) to confirm the absence of mycoplasma infection. Cells were grown in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented by 10% FBS, 1% streptomycin/penicillin at 37 °C, and 5% CO_2. siRNA targeting *KLHDC8A* (*si-KLHDC8A*) and *si-NC* were purchased from RiboBio (Shanghai, China). *si-KLHDC8A* or *si-NC* were transfected into TPC-1 and BCPAP cells at 37 °C for 48 h using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA). The siRNA sequences are listed in Table 1.

Macrophage Polarization

THP-1 (iCell) was incubated with phorbol 12-myristate 13-acetate (200 nM) (Sigma-Aldrich, St. Louis, MO, USA) for 24 h and differentiated into macrophages. PTC cells (TPC-1 and BCPAP) were grown in a serum-free medium for 24 h and centrifuged at 1500 rpm for 20 min. The transwell system with 0.4 µm pore size (Corning, Cambridge, MA, USA) was applied to co-cultivate macrophages (the upper chamber) and PTC cells (the lower chamber). Macrophages communicated with comparable PTC cells for 48 h, and then the PTC cells were removed and further analyzed. M1 markers (inducible nitric oxide synthase (iNOS), interleukin (IL)-12, and CD80), and M2 markers (arginase-1 (Arg-1), IL-10, and CD163) were detected by quantitative real-time PCR (qRT-PCR) and western blot.

Western Blot

Protein samples from PTC cells were extracted and transferred to the PVDF membranes after being resolved by 10% SDS-PAGE. The membranes were blocked with Skim milk (5%) and then incubated with the primary antibodies (Abcam, Cambridge, UK) against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000, ab9485), STAT3 (ab68153, 1:1000), KLHDC8A (1:1000, ab235419), p-STAT3 (ab76315, 1:2000), iNOS (ab178945, 1:1000), and PD-1 (ab237728, 1:50) at 4 °C all night. Then, after incubation with goat anti-rabbit IgG secondary antibody (1:5000, ab6721, Abcam) for 1 h at 25 °C, the ECL solution was added for development. Finally, results were observed and quantified by a Gel-Pro analyzer (Media Cybernetics, Silver Springs, MD, USA).

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from PTC cells using TRIzol reagent (Takara, Otsu, Japan). Based on the manufacturer's instructions, RNA was reverse transcribed using a Reverse Transcriptase kit (Takara). Subsequently, SYBR® Green Master Mix Kit (Takara) was employed to run qRT-PCR on ABI7500 quantitative PCR (Applied Biosystems, Foster City, CA, USA). β-actin was chosen as the internal control. The $2^{-\Delta\Delta Ct}$ method was performed to calculate the results. The sequences of primers are exhibited in Table 1.

Cell Counting Kit-8 (CCK-8) Assay

Cell viability of PTC cells was evaluated by cell counting kit-8 (CCK-8) (Beyotime, Shanghai, China). Cells were sown in 96-well plates (1×10^4cells/well) for 24 h at 37 °C. Afterward, the wells were added with CCK-8 solution (10 µL) and incubated at 37 °C for 4 h. The optical density (OD) value (450 nm) was determined by a microplate reader (Multiskan SkyHigh, Thermo Fisher Scientific, Waltham, MA, USA) at 0, 24, 48, and 72 h, respectively.

Table 1. siRNA and primer sequences in this study.

Gene	Forward (5′-3′)	Reverse (5′-3′)
si-KLHDC8A-1	GGACGUGUUCGACAUGGAACA	UUCCAUGUCGAACACGUCCAU
si-KLHDC8A-2	GCUCCAGCAUAGUCGUCAAGA	UUGACGACUAUGCUGGAGCAG
si-NC	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
KLHDC8A	TGTGACCCTGGACAACCACT	GTCGAACACGTCCATCGTCC
CD80	CTCACTTCTGTTCAGGTGTTATCCA	TCCTTTTGCCAGTAGATGCGA
iNOS	TCCAAGGTATCCTGGAGCGA	CAGGGACGGGAACTCCTCTA
IL-12	GCACAGTGGAGGCCTGTTTA	GCCAGGCAACTCCCATTAGT
CD163	GGGGACATTCCCTGTTCTGG	CAGGCGAAGTTGACCACTCT
Arg-1	ACGGAAGAATCAGCCTGGTG	GTCCACGTCTCTCAAGCCAA
IL-10	GAGAACAGCTGCACCCACTT	TCACATGCGCCTTGATGTCT
β-actin	AGGATTCCTATGTGGGCGAC	ATAGCACAGCCTGGATAGCAA

KLHDC8A, Kelch domain containing 8A; *iNOS*, inducible nitric oxide synthase; *IL-12*, interleukin-12; *Arg-1*, arginase-1.

Transwell Assay

The invasion and migration of TPC-1 and BCPAP cells were analyzed by transwell (8 µm pore, Corning, Inc.). Matrigel (1 µg/µL, BD Biosciences, Sparks, NV, USA) was applied to pre-coat the upper chamber for 5 h, and then cells (5×10^4 cells) were supplemented into the upper chambers and cultured at 37 °C overnight. The lower chamber was added into a medium containing 10% FBS. Cells were fixed with paraformaldehyde (4%) for 30 min, and afterward, they were stained with 0.1% crystal violet for 10 min at 25 °C in the lower chamber. The invasion of cells was observed by the light microscope (Olympus, Tokyo, Japan). Concerning cell migration, the upper chamber was not pre-coated with Matrigel, and other steps show no difference from the above descriptions.

Animals

Five-week-old BALB/c nude mice (20~22 g) were obtained from SPF (Beijing, China). This study was approved by the Ethics Committee of Ganzhou Hospital, Affiliated with Nanchang University (20220305), and in compliance with China Animal Welfare Legislation. PTC cells (TPC-1, 1×10^6 cells) were subcutaneously injected into mice to conduct PTC mice models. Mice were randomized into LV-si-NC, LV-si-KLHDC8A-1, and LV-si-KLHDC8A-1+colivelin groups on average (n = 6 for each group). Mice of different groups were locally injected with lentivirus-delivered KLHDC8A-1 siRNA and si-NC. Mice of the LV-si-KLHDC8A-1+colivelin group were treated with intratracheal instillation of colivelin (STAT3 activator, 10 nmol). After 28 days, mice were anesthetized by isoflurane inhalation and sacrificed by cervical dislocation. The tumor was obtained and weighed, and tumor volume was measured every seven days and calculated by (length $\times$ width^2)/2.

Statistical Analysis

Data were processed by GraphPad Prism 8.0 (GraphPad, San Diego, CA, USA) and exhibited as the mean $\pm$ SD. Student's *t*-test was performed to analyze comparisons between two groups. Comparisons between multiple groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test. $p < 0.05$ demonstrated a statistically significant difference.

Results

KLHDC8A is Increased in PTC Tissue and Cells

As shown in Fig. 1A, GEPIA revealed that KLHDC8A was notably higher in tumor tissues than in normal tissues ($p < 0.05$). Meanwhile, the expression of *KLHDC8A* was increased in PTC cells (TPC-1 and BCPAP) compared with Nthy-ori3-1 cells ($p < 0.0001$, Fig. 1B,C).

KLHDC8A Silencing Curbs Cell Viability, Migration, and Invasion of PTC Cells

To explore the influence of KLHDC8A on PTC cells, KLHDC8A was knocked down in PTC cells (TPC-1 and BCPAP). KLHDC8A was inhibited after being transfected with si-KLHDC8A-1 and si-KLHDC8A-2 in TPC-1 ($p < 0.001$) and BCPAP cells ($p < 0.0001$) (Fig. 2A). The knockout of KLHDC8A hindered the viability of TPC-1 and BCPAP cells compared with the si-NC group ($p < 0.01$, Fig. 2B). Additionally, si-KLHDC8A-1 inhibited the migratory and invasive abilities of TPC-1 and BCPAP cells ($p < 0.001$, Fig. 2C,D). These data indicated that the silencing of KLHDC8A reduced cell viability and inhibited PTC cells' invasive and migratory abilities.

KLHDC8A Silencing Regulates M1 and M2 Macrophage Polarization

The impact of KLHDC8A silencing on macrophage polarization was evaluated in PTC cells (TPC-1 and BCPAP) co-cultured with TAMs. In comparison with the

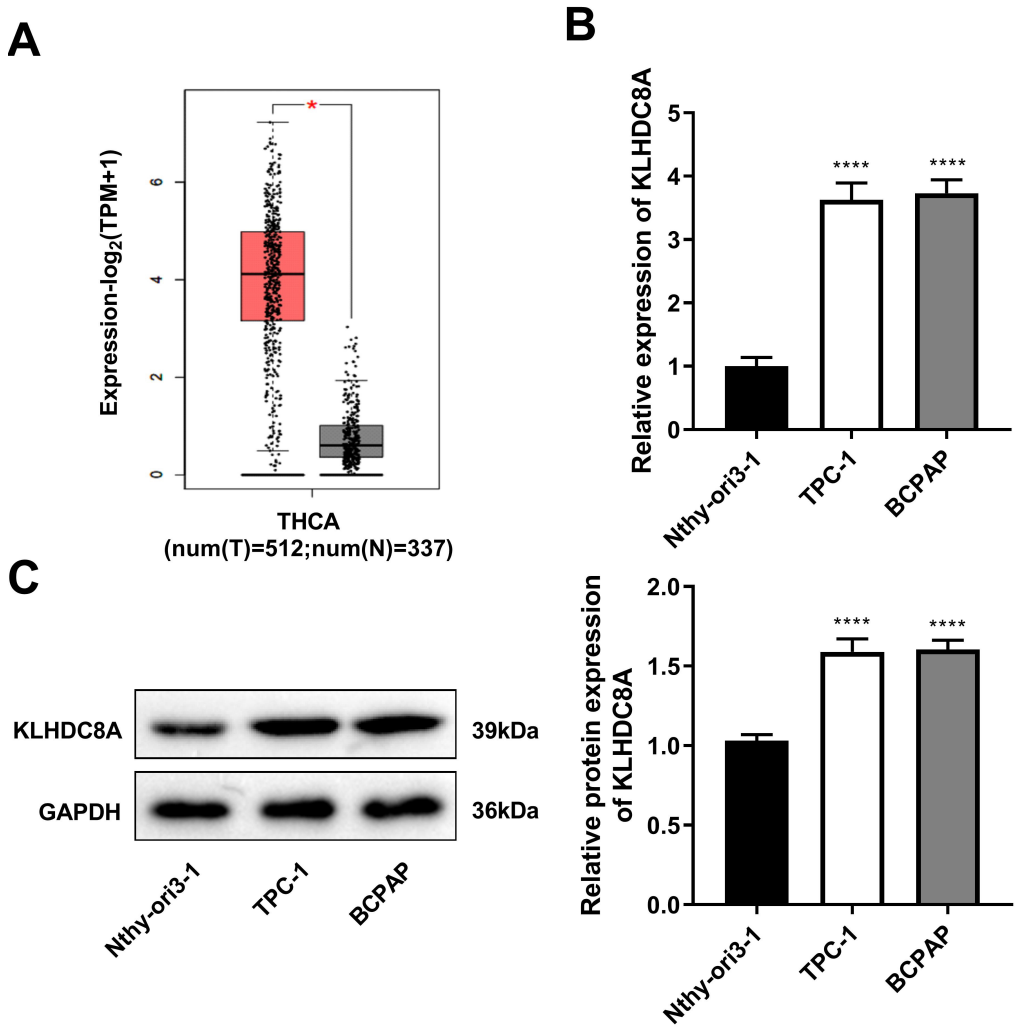

Fig. 1. KLHDC8A is increased in PTC tissues and cells. (A) Levels of KLHDC8A in PTC patients and normal individuals were analyzed by the GEPIA database. (B,C) The expression of KLHDC8A was detected by quantitative real-time PCR (qRT-PCR) and western blot in PTC cells (TPC-1 and BCPAP). $^{*}p < 0.05$ *vs.* normal (num(T) = 512, num(N) = 337); $^{****}p < 0.0001$ *vs.* Nthy-ori3-1 (n = 3). PTC, papillary thyroid cancer; GEPIA, Gene Expression Profiling Interactive Analysis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

si-NC group, the M1 markers (*CD80*, *IL-12*, and *iNOS*) showed a higher expression ($p < 0.0001$, Fig. 3A), while M2 markers (*Arg1*, *IL-10*, and *CD163*) were reduced in macrophages after transfected with si-KLHDC8A ($p < 0.01$, Fig. 3B). Meanwhile, si-KLHDC8A-1 promoted iNOS protein expression, while Arg1 expression was reduced compared with the si-NC group ($p < 0.001$, Fig. 3C).

Silencing of KLHDC8A Inhibits PD-1/STAT3 Pathway in PTC Cells

Studies have reported that PD-1 and STAT3 are substantial in cancer progression, so PD-1 and STAT3 were detected after silencing KLHDC8A in PTC cells. In the si-KLHDC8A-1 group, PD-1 and phosphorylation of STAT3 were depressed compared with the si-NC group ($p < 0.01$, Fig. 4). The above results suggested that KLHDC8A regulated the PD-1/STAT3 pathway.

Colivelin Reverses the Effects of KLHDC8A Knockdown on the Viability, Migration, Invasion, and Macrophage Polarization

To inquiry the regulatory mechanism of KLHDC8A on PTC development, TPC-1 cells received STAT3 activator (colivelin) treatment, demonstrating that colivelin weakened the inhibitory effects of KLHDC8A silencing on the cell viability, migration, and invasion of TPC-1 cells ($p < 0.01$, Fig. 5A,B). In addition, the si-KLHDC8A-1+colivelin group has lower levels of M1 markers and higher levels of M2 markers than the si-KLHDC8A-1 group ($p < 0.001$, Fig. 5C,D). The above consequences illustrated that KLHDC8A silencing suppressed PTC progression by inhibiting the PD-1/STAT3 pathway.

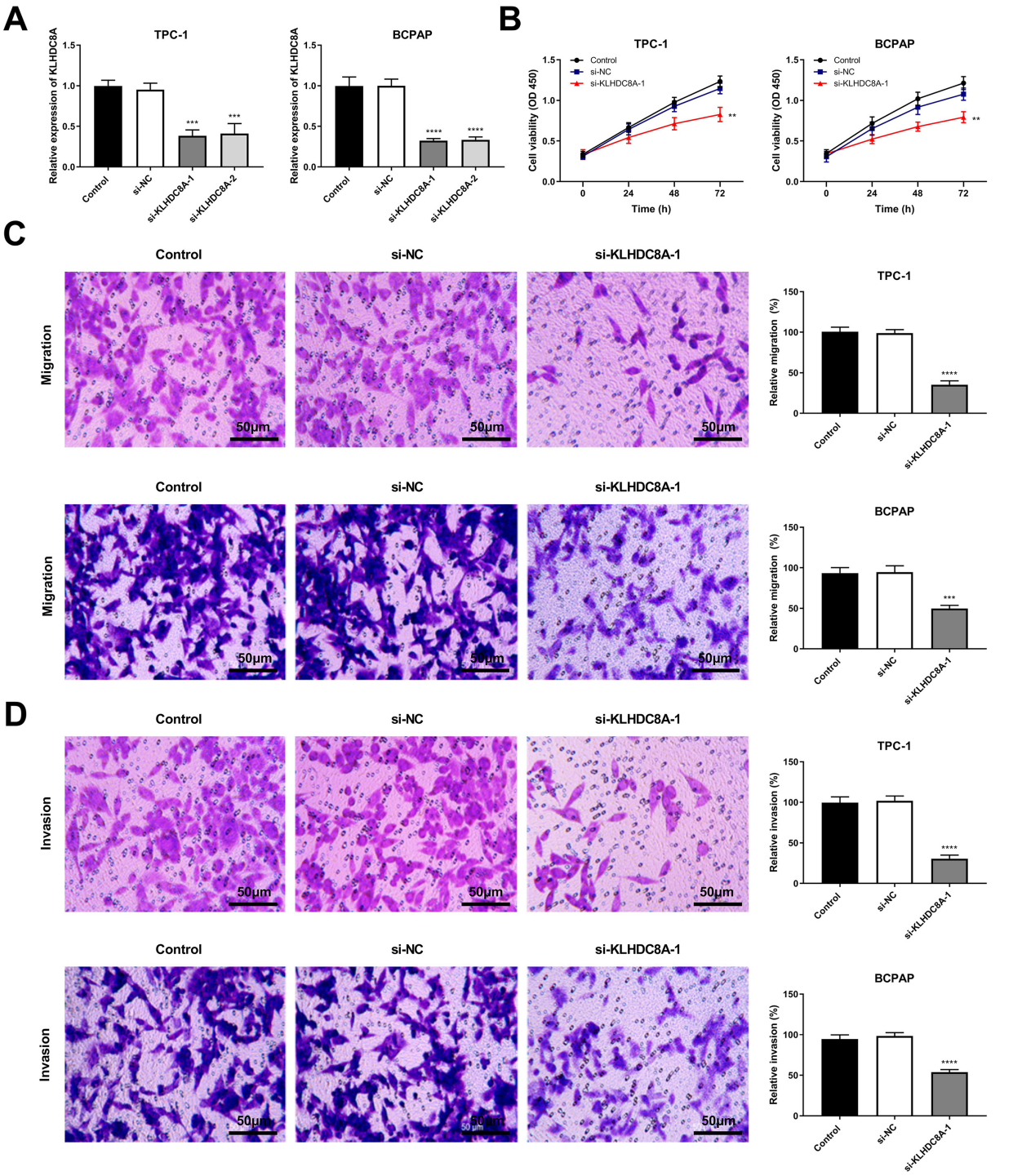

Fig. 2. KLHDC8A silencing hinders cell viability, migration, and invasion of PTC cells. (A) KLHDC8A expression was detected by qRT-PCR in PTC cells. (B) Cell viability was evaluated by cell counting kit-8. (C,D) PTC cells' migratory and invasive abilities were evaluated by transwell assay (scale bar: 50 μm, magnification: 100×). $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ *vs.* si-NC (n = 3).

KLHDC8A Silencing Inhibits Tumor Growth by PD-1/STAT3 Axis

The role of KLHDC8A was further analyzed *in vivo*. The knockdown of KLHDC8A suppressed tumor volume and weight ($p < 0.0001$), while colivelin attenuated the inhibitory effects of KLHDC8A knockdown on tumor growth ($p < 0.001$, Fig. 6A–C). Additionally, KLHDC8A expression was higher in the LV-si-KLHDC8A-1+colivelin group than in the LV-si-KLHDC8A-1 group ($p < 0.001$, Fig. 6D). *In vivo*, tests concluded that KLHDC8A silencing suppresses tumor growth by regulating the PD-1/STAT3 pathway.

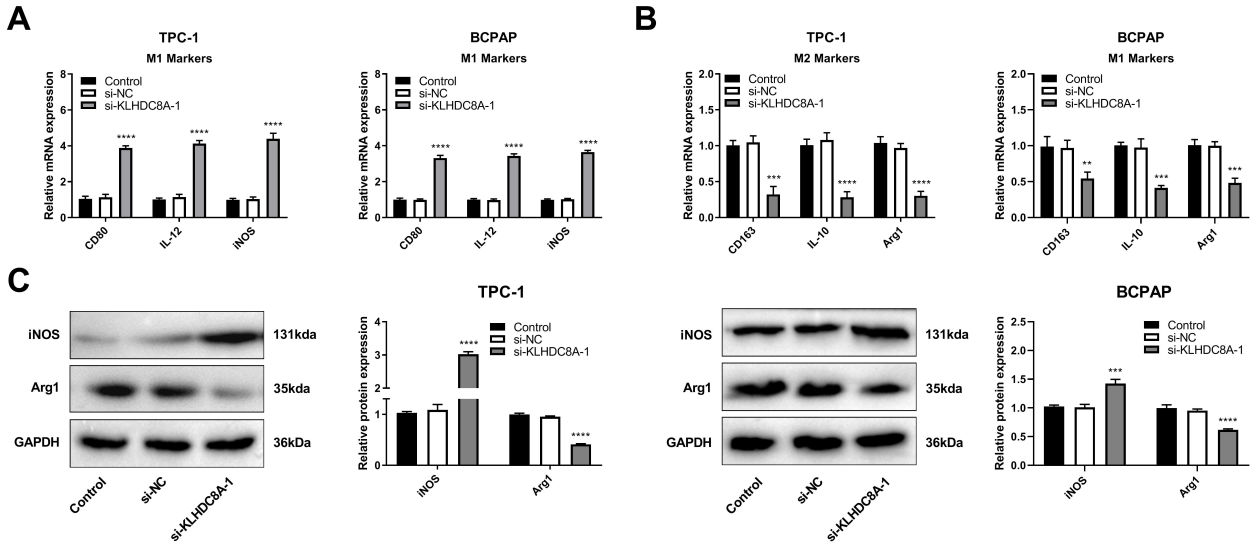

Fig. 3. Knockdown of KLHDC8A restrains M2 polarization and contributes to M1 polarization of macrophages. (A,B) M1 markers (*CD80*, *IL-12*, and *iNOS*) and M2 markers (*CD163*, *IL-10*, and *Arg1*) were determined by qRT-PCR. (C) The expression of iNOS and Arg1 was inspected by western blot. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ *vs.* si-NC (n = 3).

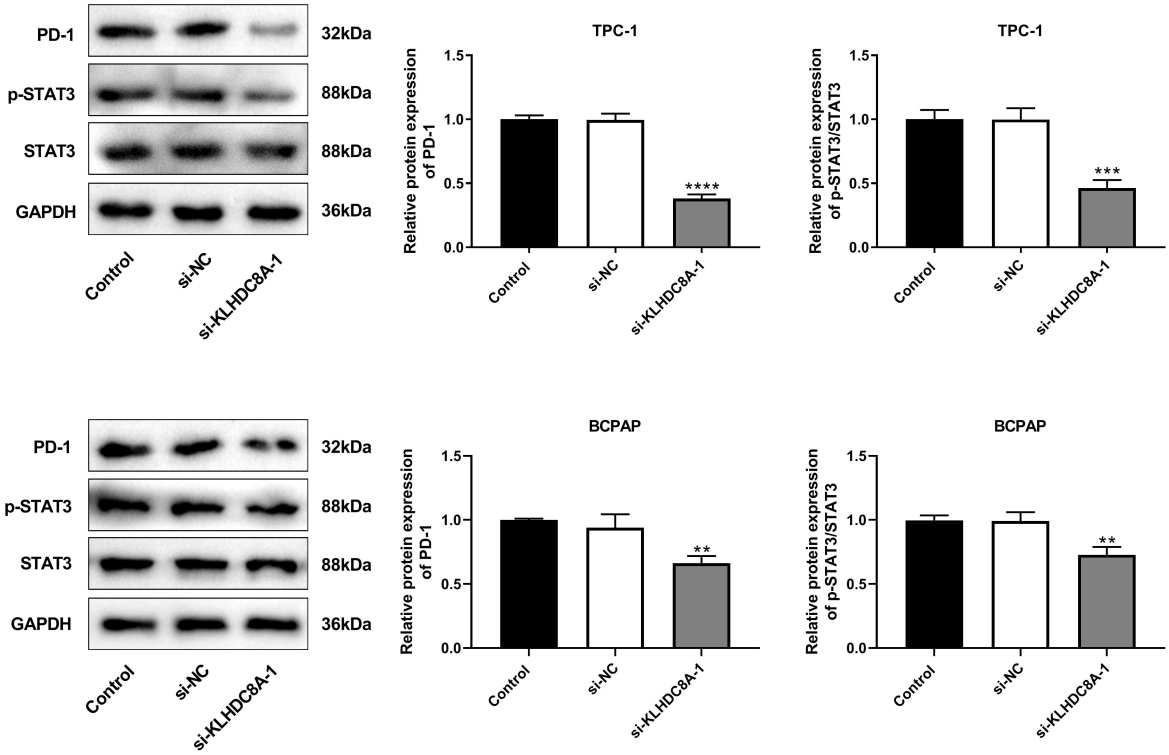

Fig. 4. Silencing of KLHDC8A inhibits the PD-1/STAT3 pathway in PTC cells. The levels of PD-1, p-STAT3, and STAT3 were detected by western blot. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ *vs.* si-NC (n = 3). PD-1, programmed cell death protein 1; STAT3, signal transducer and activator of transcription 3.

Colivelin Abates the Impacts of KLHDC8A Knockdown on Macrophage Polarization-Related Markers

Moreover, colivelin reduced the expression of M1 markers (*CD80*, *IL-12*, and *iNOS*) and increased the levels of M2 markers (*Arg1*, *IL-10*, and *CD163*) after KLHDC8A knockdown *in vivo* compared to the LV-si-KLHDC8A-1 group ($p < 0.01$, Fig. 7A). Meanwhile, the western blot exhibited that iNOS was down-regulated ($p < 0.0001$) and Arg-1 was up-regulated ($p < 0.01$) in the LV-si-KLHDC8A-1+colivelin group compared with the LV-si-KLHDC8A-1 group (Fig. 7B).

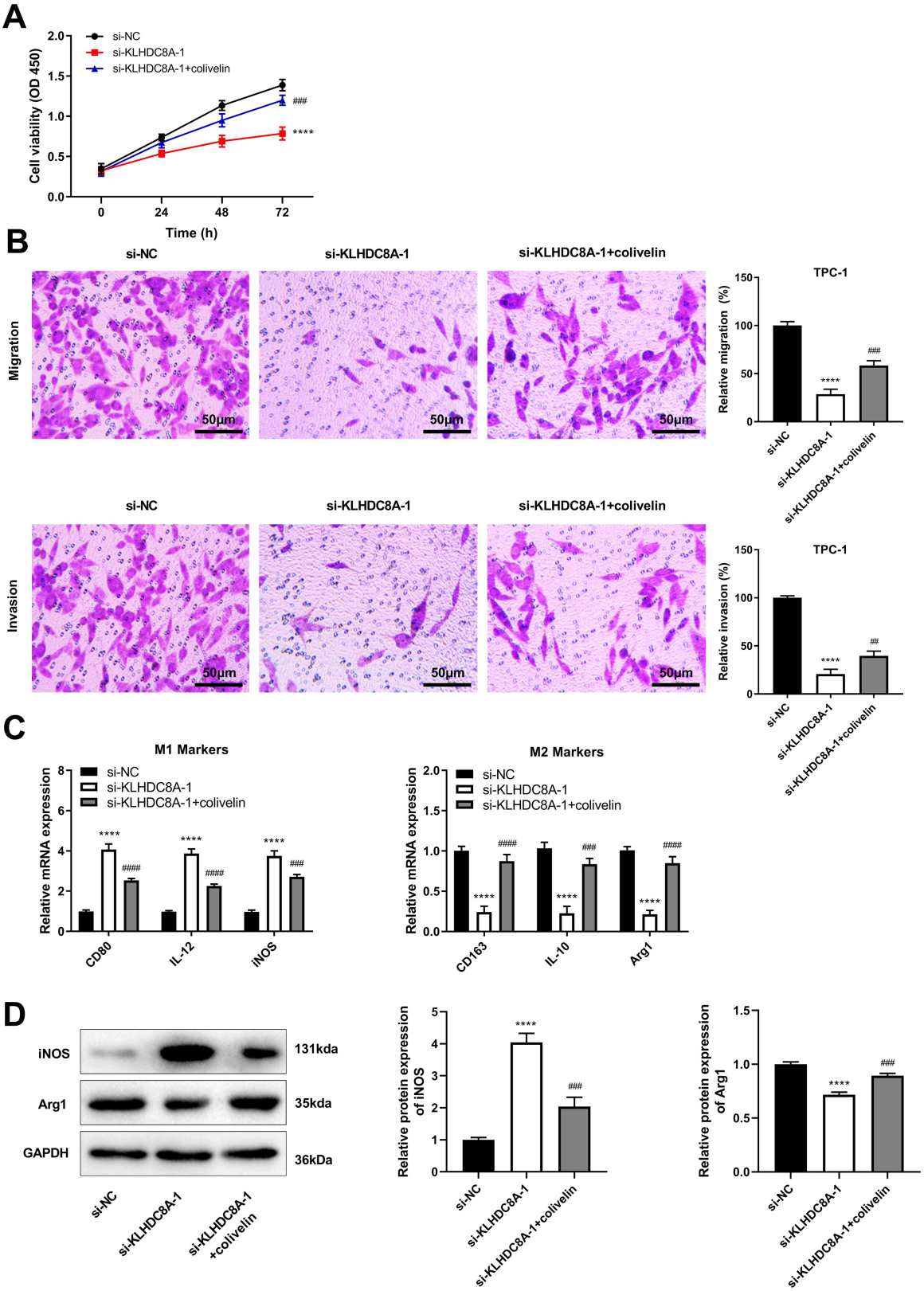

Fig. 5. Colivelin weakens the impacts of KLHDC8A silencing on viability, migration, invasion, and macrophage polarization. (A) Cell viability was inspected by cell counting kit-8. (B) Transwell assay was carried out to detect cell migration and invasion (scale bar: 50 μm, magnification: 100×). (C) The levels of M1 markers and M2 markers were determined by qRT-PCR. (D) The expression of iNOS and Arg1 was inspected by western blot. $^{****}p < 0.0001$ *vs.* si-NC; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ *vs.* si-KLHDC8A-1 (n = 3).

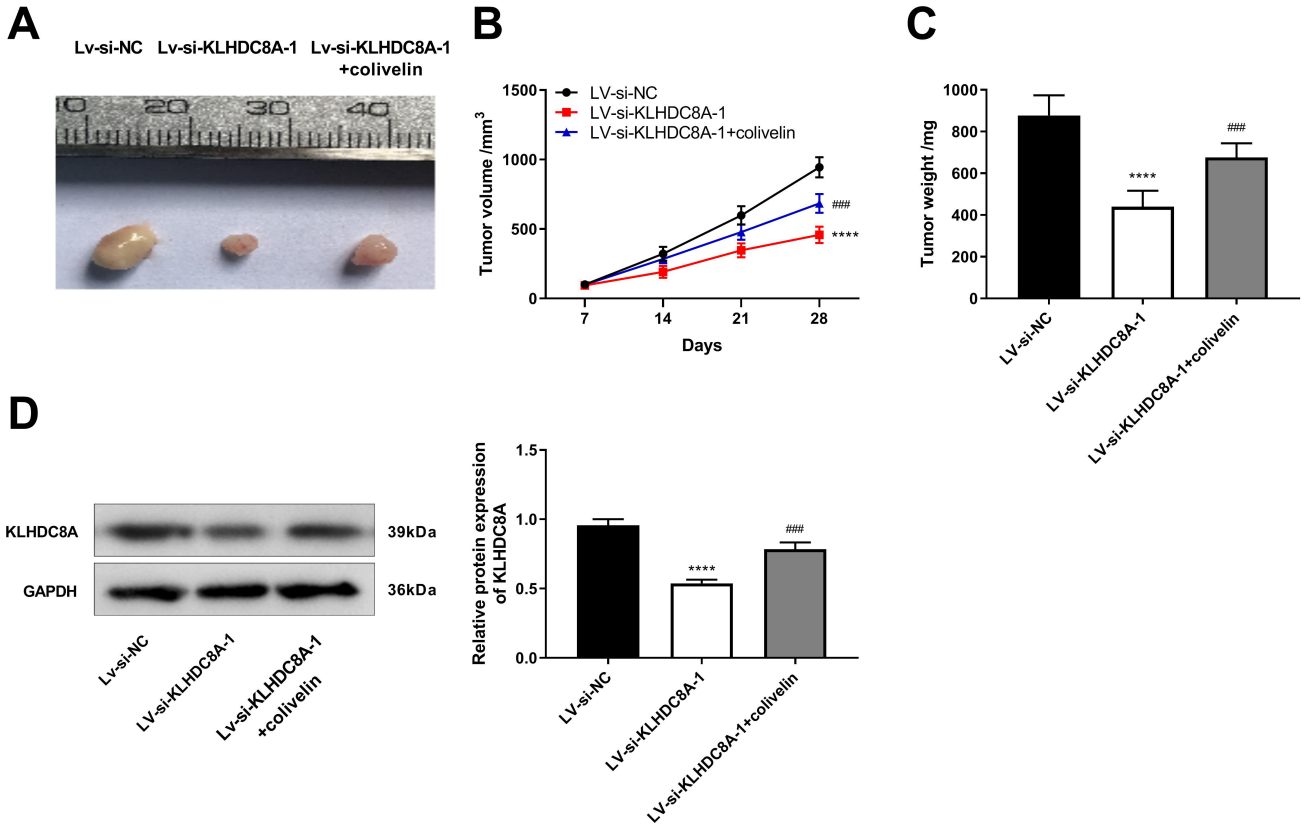

Fig. 6. KLHDC8A silencing inhibits tumor growth by PD-1/STAT3 axis. (A–C) Tumor volume and weight were calculated. (D) KLHDC8A expression was inspected by western blot. $^{****}p < 0.0001$ vs. LV-si-NC; $^{\#\#\#}p < 0.001$ *vs*. LV-si-KLHDC8A-1 (n = 6).

Discussion

The incidence of PTC continues to increase worldwide [18,19]. However, 15–20% of PTC patients have a relatively poor outcome after standard treatment [20]. Therefore, it is necessary to develop effective PTC treatment strategies. In this study, the knockdown of KLHDC8A hindered PTC cells' proliferative, invasive, and migratory abilities. KLHDC8A silencing hindered M2 polarization and accelerated M1 polarization. Furthermore, the mechanism of KLHDC8A in PTC progression was further explored (**Supplementary Fig. 1**).

Previous research has reported that the KLHDC subfamily proteins are pivotal in the progression of tumors, such as glioma and nasopharyngeal carcinoma. For instance, KLHDC8A is highly expressed in human gliomas, which is closely linked to the grade of glioma and promotes malignant metastasis of glioma cells [8]. KLHDC subfamily protein KLHDC4 induces the carcinogenesis of nasopharyngeal carcinoma by inhibiting apoptosis, a promising target for the prognosis of nasopharyngeal carcinoma [21]. Based on previous reports, we speculate that KLHDC8A is closely associated with the malignant development of PTC. In this study, KLHDC8A expression was increased in PTC tissues and cells. Silencing of KLHDC8A reduced cell viability and inhibited migration, invasion, and tumor growth, indicating that KLHDC8A promoted PTC development. Therefore, these findings suggested that KLHDC8A exerted a tumor-promoting role in developing PTC.

M1 macrophage polarization can inhibit the development of tumor cells, while M2 polarization promotes tumor progression and survival, and induces an anti-inflammatory effect [22,23]. Previous evidence supports a substantial role for macrophage polarization in tumor development, such as PTC [24–26]. M2-like macrophages are increased, and M1-like macrophages are decreased in advanced PTC, which have been associated with poor prognosis in thyroid cancer [27,28]. Bleomycin exerts suppressive effects on TPC-1 cell invasion and migration by reversing M2 to M1 macrophage polarization, thereby inhibiting PTC progression [26]. The ratio of suppressive M2-type macrophages to pro-inflammatory M1-type macrophages increases with advanced stages of PTC [24]. TAMs play a pro-tumorigenic role in advanced PTC [25]. Therefore, it may be a novel strategy for PTC treatment by regulating TAM polarization to M1. In this study, silencing of KLHDC8A promoted the markers of M1 macrophage polarization while inhibiting M2 macrophage markers. Taken together, our results suggested that KLHDC8A silencing may suppress PTC development by inhibiting M2 macrophage polarization. Targeting KLHDC8A may be a potential therapeu-

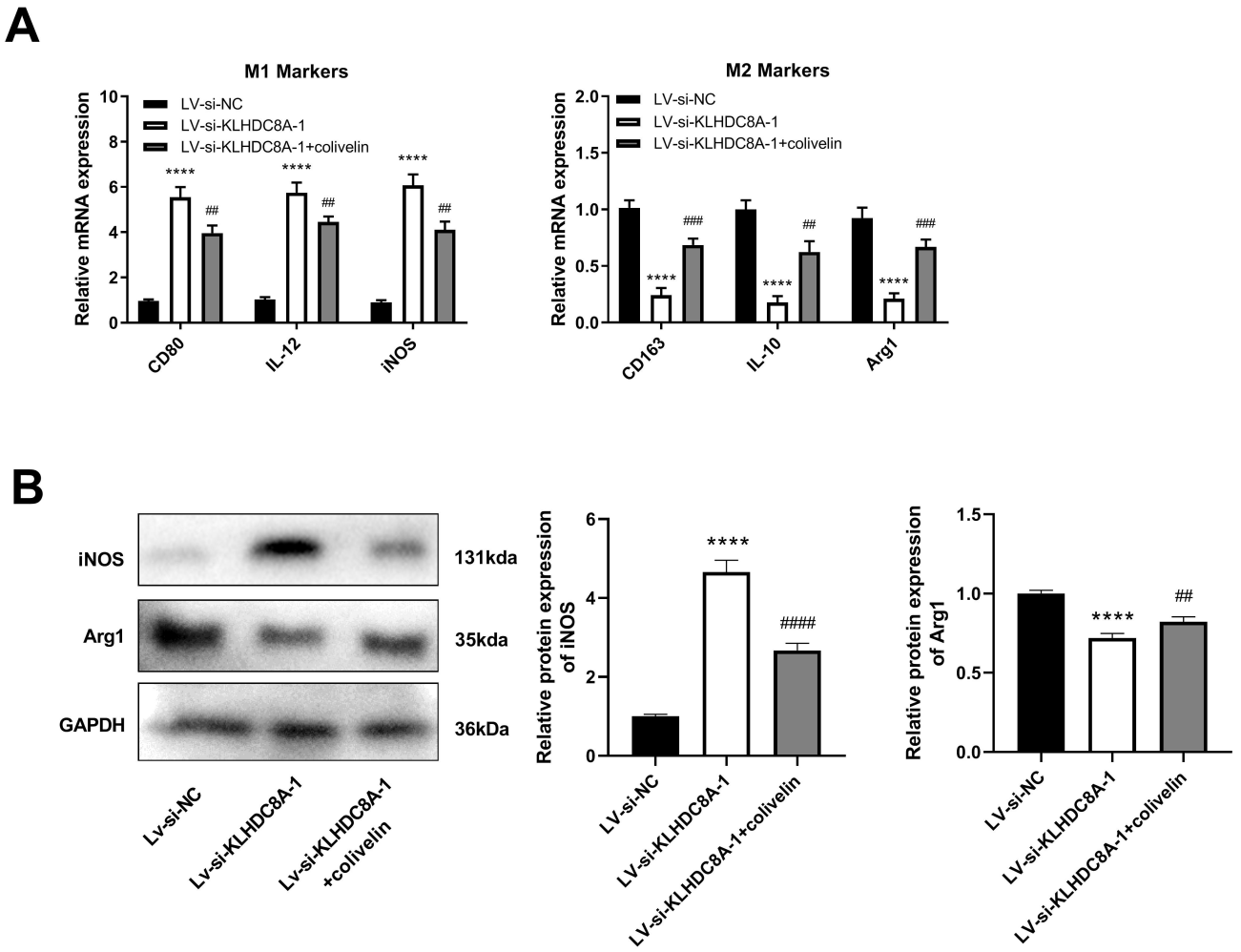

Fig. 7. Colivelin weakens the influence of KLHDC8A knockdown on macrophage polarization-related markers. (A) The levels of M1 markers (*CD80*, *IL-12*, and *iNOS*) and the M2 markers (*CD163*, *IL-10*, and *Arg1*) were determined by qRT-PCR. (B) The expression of iNOS and Arg1 was detected by western blot. $^{****}p < 0.0001$ *vs.* LV-si-NC; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ *vs.* LV-si-KLHDC8A-1 (n = 6).

tic strategy for PTC by regulating macrophage polarization. Macrophages are mainly affected by various microenvironmental stimuli, which are functionally heterogeneous cell populations [29,30]. Previous reports have illustrated that CEBPD contributes to M2 macrophage polarization via IL-4/IL-13, which is involved in the PTC progression [13]. Similarly, in this study, the knockdown of KLHDC8A inhibited PTC cell-induced M2 macrophage polarization, possibly by affecting the secretion of IL-4 or IL-13, which can be investigated further in the future.

Researchers have reported that PD-1 and PD-L1 are connected to TME, which are pivotal in cancer. PD-1 acts as an immune checkpoint that promotes tumor development by binding to PD-L1 to drive immune escape [31]. Macrophage phagocytosis is increased *in vivo* by inhibiting PD-1/PD-L1, thus hindering tumor growth and elongating the survival of mice in cancer mouse models in a macrophage-dependent fashion [32]. M2 to M1 macrophage reprogramming can enhance the expression of PD-L1, which can be transcriptionally activated by STAT3 [33]. Rab37/IL-6/STAT3 transcription axis promotes M2 polarization of macrophages and further up-regulates PD-1 in T cells in lung cancer [34]. IGF binding protein 2 activates the EGFR/STAT3 pathway to regulate PD-L1 levels in malignant melanoma [31]. Moreover, Liang *et al.* [35] have suggested that STAT3 expression was decreased by curcumin contributes to inhibiting TPC-1 cell viability, migration, and invasion. In this research, we found a significant reduction in PD-1 and phosphorylation of STAT3 after the knockdown of KLHDC8A. Furthermore, our data showed that the STAT3 activator, colivelin, attenuated the inhibitory roles of KLHDC8A knockdown on cell viability, invasion, migration, and tumor growth. Meanwhile, the impacts of KLHDC8A knockdown on macrophage polarization were reversed by colivelin. Therefore, these findings suggested that KLHDC8A silencing inhibited PTC malignant development and polarization of M2 macrophages via down-regulating PD-1/STAT3 axis. Similar to our results,

Chi *et al*. [36] have suggested that the upregulation of B7H4 reduces the PD-1/STAT3 pathway *in vitro* and *in vivo*, and colivelin reverses the inhibitory impacts of B7H4 silencing on tumor growth and macrophage polarization.

However, there are still some shortcomings in the study. *In vivo*, assays on the effect of KLHDC8A on PTC are still superficial, and other experiments can be performed in future studies. Furthermore, this study elucidated that silencing of KLHDC8A inhibited M2 polarization by regulating the PD-1/STAT3 pathways to alleviate PTC. Still, the potential key factors of PTC cells affecting M2 macrophage polarization have not been explored, which can be used as a future research direction.

Conclusions

In summary, KLHDC8A was highly expressed in PTC. KLHDC8A silencing exerted inhibitory roles on cell viability, invasion, and migration *in vitro* and suppressed tumor growth *in vivo*. Additionally, the silencing of KLHDC8A regulated the M1/M2 macrophage polarization by suppressing the PD-1/STAT3 pathway. These findings offer potential therapeutic strategies for the clinical therapy of PTC.

Availability of Data and Materials

All data in the manuscript is available through the responsible corresponding author.

Author Contributions

YP and MW: conception, design and analysis of data, performed the data analyses and wrote the manuscript; JiaY, CH and JicY: contributed to the conception of the study; wrote the manuscript. All authors have read and approved the manuscript, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics Approval and Consent to Participate

Animal study has been approved by the Animal Ethics Committee of Ganzhou Hospital Affiliated to Nanchang University (20220305). This study was conducted in accordance with the ethics committee and in compliance with China Animal Welfare Legislation. We confirm that the study is reported in accordance with ARRIVE guidelines.

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202436188.172.

References

[1] Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, *et al*. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA: A Cancer Journal for Clinicians. 2021; 71: 209–249.

[2] Pizzato M, Li M, Vignat J, Laversanne M, Singh D, La Vecchia C, *et al*. The epidemiological landscape of thyroid cancer worldwide: GLOBOCAN estimates for incidence and mortality rates in 2020. The Lancet. Diabetes & Endocrinology. 2022; 10: 264–272.

[3] Park H, Park J, Park SY, Kim TH, Kim SW, Chung JH. Clinical Course from Diagnosis to Death in Patients with Well-Differentiated Thyroid Cancer. Cancers. 2020; 12: 2323.

[4] Haddad RI, Bischoff L, Ball D, Bernet V, Blomain E, Busaidy NL, *et al*. Thyroid Carcinoma, Version 2.2022, NCCN Clinical Practice Guidelines in Oncology. Journal of the National Comprehensive Cancer Network: JNCCN. 2022; 20: 925–951.

[5] Chen DW, Lang BHH, McLeod DSA, Newbold K, Haymart MR. Thyroid cancer. The Lancet. 2023; 401: 1531–1544.

[6] Coca-Pelaz A, Shah JP, Hernandez-Prera JC, Ghossein RA, Rodrigo JP, Hartl DM, *et al*. Papillary Thyroid Cancer-Aggressive Variants and Impact on Management: A Narrative Review. Advances in Therapy. 2020; 37: 3112–3128.

[7] Chen W, Fu J, Chen Y, Li Y, Ning L, Huang D, *et al*. Circular RNA circKIF4A facilitates the malignant progression and suppresses ferroptosis by sponging miR-1231 and upregulating GPX4 in papillary thyroid cancer. Aging. 2021; 13: 16500–16512.

[8] Zhu X, Chen T, Yang H, Lv K. Lactate induced up-regulation of KLHDC8A (Kelch domain-containing 8A) contributes to the proliferation, migration and apoptosis of human glioma cells. Journal of Cellular and Molecular Medicine. 2020; 24: 11691–11702.

[9] Dhanoa BS, Cogliati T, Satish AG, Bruford EA, Friedman JS. Update on the Kelch-like (KLHL) gene family. Human Genomics. 2013; 7: 13.

[10] Sekine Y, Hatanaka R, Watanabe T, Sono N, Iemura S, Natsume T, *et al*. The Kelch repeat protein KLHDC10 regulates oxidative stress-induced ASK1 activation by suppressing PP5. Molecular Cell. 2012; 48: 692–704.

[11] Mukasa A, Wykosky J, Ligon KL, Chin L, Cavenee WK, Furnari F. Mutant EGFR is required for maintenance of glioma growth in vivo, and its ablation leads to escape from receptor dependence. Proceedings of the National Academy of Sciences of the United States of America. 2010; 107: 2616–2621.

[12] Hui L, Chen Y. Tumor microenvironment: Sanctuary of the devil. Cancer Letters. 2015; 368: 7–13.

[13] Yang Y, Xia S, Zhang L, Wang W, Chen L, Zhan W. MiR-324-5p/PTPRD/CEBPD axis promotes papillary thyroid carcinoma progression via microenvironment alteration. Cancer Biology & Therapy. 2020; 21: 522–532.

[14] Qing W, Fang WY, Ye L, Shen LY, Zhang XF, Fei XC, *et al*.

Density of tumor-associated macrophages correlates with lymph node metastasis in papillary thyroid carcinoma. Thyroid: Official Journal of the American Thyroid Association. 2012; 22: 905–910.

[15] Xiong H, Mittman S, Rodriguez R, Moskalenko M, Pacheco-Sanchez P, Yang Y, *et al*. Anti-PD-L1 Treatment Results in Functional Remodeling of the Macrophage Compartment. Cancer Research. 2019; 79: 1493–1506.

[16] Yuan L, Ye J, Fan D. The B7-H4 gene induces immune escape partly via upregulating the PD-1/Stat3 pathway in non-small cell lung cancer. Human Immunology. 2020; 81: 254–261.

[17] Huo N, Cong R, Sun ZJ, Li WC, Zhu X, Xue CY, *et al*. STAT3/LINC00671 axis regulates papillary thyroid tumor growth and metastasis via LDHA-mediated glycolysis. Cell Death & Disease 2021; 12: 799.

[18] Fu S, Zhao N, Jing G, Yang X, Liu J, Zhen D, *et al*. Matrine induces papillary thyroid cancer cell apoptosis in vitro and suppresses tumor growth in vivo by downregulating miR-182-5p. Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie. 2020; 128: 110327.

[19] Roman BR, Morris LG, Davies L. The thyroid cancer epidemic, 2017 perspective. Current Opinion in Endocrinology, Diabetes, and Obesity. 2017; 24: 332–336.

[20] Viola D, Valerio L, Molinaro E, Agate L, Bottici V, Biagini A, *et al*. Treatment of advanced thyroid cancer with targeted therapies: ten years of experience. Endocrine-related Cancer. 2016; 23: R185–R205.

[21] Lian YF, Yuan J, Cui Q, Feng QS, Xu M, Bei JX, *et al*. Upregulation of KLHDC4 Predicts a Poor Prognosis in Human Nasopharyngeal Carcinoma. PloS One. 2016; 11: e0152820.

[22] He K, Jia S, Lou Y, Liu P, Xu LX. Cryo-thermal therapy induces macrophage polarization for durable anti-tumor immunity. Cell Death & Disease. 2019; 10: 216.

[23] Belgiovine C, Digifico E, Anfray C, Ummarino A, Torres Andón F. Targeting Tumor-Associated Macrophages in Anti-Cancer Therapies: Convincing the Traitors to Do the Right Thing. Journal of Clinical Medicine. 2020; 9: 3226.

[24] Wang T, Shi J, Li L, Zhou X, Zhang H, Zhang X, *et al*. Single-Cell Transcriptome Analysis Reveals Inter-Tumor Heterogeneity in Bilateral Papillary Thyroid Carcinoma. Frontiers in Immunology. 2022; 13: 840811.

[25] Ryder M, Gild M, Hohl TM, Pamer E, Knauf J, Ghossein R, *et al*. Genetic and pharmacological targeting of CSF-1/CSF-1R inhibits tumor-associated macrophages and impairs BRAF-induced thyroid cancer progression. PloS One. 2013; 8: e54302.

[26] Liu H, Dong H, Jiang L, Li Z, Ma X. Bleomycin inhibits proliferation and induces apoptosis in TPC-1 cells through reversing M2-macrophages polarization. Oncology Letters. 2018; 16: 3858–3866.

[27] Xie Z, Li X, He Y, Wu S, Wang S, Sun J, *et al*. Immune Cell Confrontation in the Papillary Thyroid Carcinoma Microenvironment. Frontiers in Endocrinology. 2020; 11: 570604.

[28] Mazzoni M, Mauro G, Erreni M, Romeo P, Minna E, Vizioli MG, *et al*. Senescent thyrocytes and thyroid tumor cells induce M2-like macrophage polarization of human monocytes via a PGE2-dependent mechanism. Journal of Experimental & Clinical Cancer Research: CR. 2019; 38: 208.

[29] Orecchioni M, Ghosheh Y, Pramod AB, Ley K. Macrophage Polarization: Different Gene Signatures in M1(LPS+) vs. Classically and M2(LPS-) vs. Alternatively Activated Macrophages. Frontiers in Immunology. 2019; 10: 1084.

[30] Zhu L, Zhao Q, Yang T, Ding W, Zhao Y. Cellular metabolism and macrophage functional polarization. International Reviews of Immunology. 2015; 34: 82–100.

[31] Li T, Zhang C, Zhao G, Zhang X, Hao M, Hassan S, *et al*. IGFBP2 regulates PD-L1 expression by activating the EGFR-STAT3 signaling pathway in malignant melanoma. Cancer Letters. 2020; 477: 19–30.

[32] Gordon SR, Maute RL, Dulken BW, Hutter G, George BM, McCracken MN, *et al*. PD-1 expression by tumour-associated macrophages inhibits phagocytosis and tumour immunity. Nature. 2017; 545: 495–499.

[33] Cai H, Zhang Y, Wang J, Gu J. Defects in Macrophage Reprogramming in Cancer Therapy: The Negative Impact of PD-L1/PD-1. Frontiers in Immunology. 2021; 12: 690869.

[34] Kuo IY, Yang YE, Yang PS, Tsai YJ, Tzeng HT, Cheng HC, *et al*. Converged Rab37/IL-6 trafficking and STAT3/PD-1 transcription axes elicit an immunosuppressive lung tumor microenvironment. Theranostics. 2021; 11: 7029–7044.

[35] Liang Y, Kong D, Zhang Y, Li S, Li Y, Dong L, *et al*. Curcumin inhibits the viability, migration and invasion of papillary thyroid cancer cells by regulating the miR-301a-3p/STAT3 axis. Experimental and Therapeutic Medicine. 2021; 22: 875.

[36] Chi J, Liu Y, Yang L, Yang J. Silencing of B7H4 Represses the Development of Oral Squamous Cell Carcinoma Through Promotion of M1 Macrophage Polarization. Journal of Oral and Maxillofacial Surgery: Official Journal of the American Association of Oral and Maxillofacial Surgeons. 2022; 80: 1408–1423.