Regulation of the TUG1/miR-145-5p/SOX2 axis on the migratory and invasive capabilities of melanoma cells

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Abstract. Melanoma is the most prevalent malignancy of cutaneous carcinomas. Taurine-upregulated gene 1 (TUG1), a lncRNA, is a pivotal regulator of cutaneous malignancies. The present study aimed to investigate the impact and possible mechanisms of action of TUG1 behind the progression of melanomas. Reverse transcription-quantitative PCR was conducted to detect the expression levels of TUG1, microRNA (miR)-145-5p and SOX2 in melanoma tissues and cell lines. Cell Counting Kit-8 (CCK-8) assays were performed to measure the proliferative ability of melanoma cells and transwell assays were used to examine the migration and invasion of melanoma cells. Dual luciferase reporter and RNA immunoprecipitation (RIP) assays were utilized to identify the interactions among TUG1, miR-145-5p and SOX2. Western blotting and immunohistochemical assays were performed to determine the expression profile of SOX2. The impact of TUG1 on melanoma tumorigenesis was assessed using tumorigenicity assays. TUG1 expression levels were elevated in melanoma tumor tissues and cell lines. Reduced TUG1 expression levels significantly inhibited the proliferative, migratory and invasive abilities of melanoma cells. The expression levels of miR-145-5p were decreased in melanoma tumor tissues and cell lines. TUG1 directly targeted miR-145-5p and downregulated miR-145-5p. Upregulation of TUG1 counteracted the promotion of the proliferative, migratory and invasive abilities of melanoma cells induced by the overexpression of miR-145-5p. SOX2 was a target of miR-145-5p and its expression was negatively regulated by miR-145-5p, while positively regulated by TUG1. TUG1 regulated SOX2 expression through sponging miR-145-5p. Silencing of TUG1 also inhibited melanoma tumorigenesis in mice. In conclusion, the TUG1/miR-145-5p/SOX2 axis regulated the migration and invasion of melanoma cells.

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Introduction

Melanoma, brought on by the malignant transformation of melanocytes, has been reported as the deadliest skin cancer (1). Melanoma easily metastasizes and its 5-year survival rate is only 18% (2). The incidence of melanoma worldwide has been rising year by year (3). Its high mortality is related to its unresectability and easy movability (3). Though present therapeutic approaches are capable of curing early-stage melanoma by surgical removal, immune-based methods and chemoprevention, the prognosis of patients with advanced melanoma remains poor (4).

Long non-coding RNAs (lncRNAs), >200 nucleotides in length, play key roles in several types of human cancer (5). LncRNAs have been found to be dysregulated in various types of cancer, including lung cancer, breast cancer, liver cancer, colorectal cancer and melanoma (6). There are growing numbers of studies that have investigated melanoma-related lncRNAs, such like lncRNA cancer susceptibility candidate 2 (7), as a melanoma-suppressing lncRNA; and homeobox A11-antisense RNA (8), ZNFX1 antisense RNA 1 (9) and H19 Imprinted Maternally Expressed Transcript (10), as melanoma-accelerating lncRNAs. Taurine-upregulated gene 1 (TUG1) is a lncRNA that consists of 6.7-kb nucleotides and TUG1 has been shown to play important roles in tumorigenesis (11).

MicroRNAs (miRNAs) are a large group of endogenous RNAs consisting of 18-22 nucleotides, which are regarded as key regulators of development and progression of various types of human tumors (12). miRNAs perform regulatory roles by directly binding to the 3' untranslated region (3'UTR) of their target mRNAs to silence translation, which can affect the proliferation, metastasis and apoptosis of tumor cells (13). Previous studies have indicated that miRNA (miR)-145-5p expression levels are decreased in breast cancer (14), bladder cancer (15) and melanoma (16) cell lines, which suggests that miR-145-5p may function as a tumor-suppressor.

SOX2, a member of the SOX gene family, has been shown to be associated with the regulation of certain biological processes during tumor development (17). A previous study found that SOX2 contributes to oxidative metabolism, as

well as elevates drug resistance and metastatic capacity in melanoma cells (18). SOX2 is dispensable for primary melanoma metastasis and formation (19). Nevertheless, whether miR-145-5p and SOX2 are required for the mechanisms of action behind how TUG1 functions during the development of melanomas remains unclear.

In the present study, the expression levels of TUG1 were found to be elevated in melanoma tumor tissues and cell lines. In addition, the effects of TUG1 on the proliferative, migratory and invasive abilities of melanoma cells were investigated. Downstream target and regulatory mechanism of TUG1 in melanoma cells were also explored.

Materials and methods

Clinical specimens and cell culture. The present study was approved by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University (Hefei, China). Tumor tissues and adjacent noncancerous tissues (normal tissues) were collected from 27 patients with melanoma (aged 29-71 years; 15 male and 12 female), who were diagnosed at the First Affiliated Hospital of Anhui Medical University between March 2014 and January 2018. No patients received chemotherapy or any other types of therapy before surgery. Inclusion criteria included histologically confirmed melanoma. Exclusion criteria included: i) Current or previous history of any other severe or uncontrolled diseases, including previous history of cancer, bleeding dyscrasia and immune system disease; ii) currently on medications that could interfere with assessment of biological outcomes, including estrogens, progestogens, androgens, prednisone or psychoactive drugs; and iii) aged <16 years. All patients signed a written informed consent. Fresh tissues were immediately frozen and stored in liquid nitrogen.

Human melanoma cells (M14 and A375) and human primary normal epidermal melanocytes HEMa-LP were purchased from the American Type Culture Collection and cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.) in a humidified atmosphere with 5% CO_2 at 37˚C, unless otherwise stated.

Transfection with oligonucleotides and plasmids. Specific small interfering (si)-RNAs against TUG1 (si-TUG1#1, si-TUG1#2 and si-TUG1#3; final concentration, 20 nM), the scrambled negative control (NC; si-NC; final concentration, 20 nM), miR-145-5p mimic (final concentration, 15 nM), miR-NC (final concentration, 15 nM), pcDNA-TUG1 (final concentration, 1.5 μg/ml), si-SOX2 (final concentration, 20 nM), miR-145-5p inhibitor (in-miR-145-5p; final concentration, 25 nM), its negative control (in-miR-NC; final concentration, 25 nM), pcDNA-SOX2 (final concentration, 1 μg/ml), specific short hairpin (sh)RNA against TUG1 (sh-TUG1) and its negative control sh-NC were all synthesized by Shanghai GenePharma Co., Ltd. The pcDNA plasmid was purchased from Thermo Fisher Scientific, Inc. The sequences for the siRNAs, miRNA mimics and inhibitors used in the present study were shown in Table I.

The aforementioned oligonucleotides or plasmids were transfected into M14 and A375 cells using Lipofectamine™ 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. Briefly, M14 and A375 cells were cultured for 24 h, before the constructs were transfected into the cells using 500 μl Opti-MEM containing 20 μl Lipofectamine™ 2000 (1 mg/ml) in a humidified atmosphere with 5% CO_2 at 37˚C. After incubating for 6 h, culture medium was replaced by fresh medium. Subsequently, 24, 48 or 72 h after transfection, the cells were collected for further analysis.

Reverse transcription-quantitative (RT-qPCR). For the detection of mRNA expression levels, RNA was isolated from melanoma tissues or cells using TRIzol® Reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA was reverse-transcribed into cDNA using M-MLV Reverse Transcriptase (cat. no. 28025021; Invitrogen; Thermo Fisher Scientific, Inc.) with RNaseOUT™ Recombinant Ribonuclease Inhibitor (cat. no. 10777; Thermo Fisher Scientific, Inc.), dNTPs (cat. no. 10297117; Thermo Fisher Scientific, Inc.) and the oligo (dT) 12-18 primer (cat. no. 18418012; Thermo Fisher Scientific, Inc.) following the standard protocols: 65˚C for 5 min; 50 min at 37˚C; and 70˚C for 15 min. Subsequent qPCR was conducted using iQ™ SYBR® Green Supermix (cat. no. 1708882; Bio-Rad Laboratories, Inc.). For the detection of miR-145-5p expression levels, miRNA was extracted using a miRNeasy Mini Kit (Qiagen, GmbH). Reverse transcription and RT-qPCR were conducted using a TaqMan™ Reverse Transcription kit and TaqMan™ MicroRNA Assays (Applied Biosystems; Thermo Fisher Scientific, Inc.). β-actin was used as the internal control for TUG1 and SOX2, whereas U6 was used as the internal control for miR-145-5p. The primers for TUG1, SOX2, β-actin, miR-145-5p and U6 are as follows: TUG1 forward, 5'-GGACCTGGAACCCGAAAGAG-3' and reverse, 5'-TGGTGGTAGTGCTTGCTCAG-3'; SOX2 forward, 5'-CAGCGCATGGACAGTTACG-3' and reverse, 5'-TTCATGTAGGTCTGCGAGCTG-3'; β-actin forward, 5'-TGGACTTCGAGCAAGAGATGG-3' and reverse, 5'-ACGTCACACTTCATGATGGAG-3'; miR-145-5p forward, 5'-CAGTCTTGTCCAGTTTTCCCAG-3' and reverse, 5'-TATGCTTGTTCTCGTCTCTGTGTC-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. RT-qPCR was performed on a StepOnePlus™ Real-time PCR Systems (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following thermocycling conditions: Initial denaturation for 10 min at 95˚C; 40 cycles of 95˚C for 15 sec and 60˚C for 1 min; followed by final elongation at 72˚C for 10 min. The expression levels of TUG1, SOX2 and miR-145-5p were analyzed using the $2^{-\Delta\Delta Cq}$ method (20).

Cell counting kit-8 (CCK-8) assay. For the detection of cell proliferative ability, cells were seeded in 96-well plates containing DMEM with 10% FBS (1,000 cells per well) for 0, 24, 48 and 72 h. The CCK-8 reagent (MedChemExpress) was used for this assay. After collection, 10 μl CCK-8 reagent was added into each well. In total, after incubation at 37˚C for 2 h, the proliferative ability was determined through measuring the absorbance at 450 nm of each well using a spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.).

Transwell assay. Transwell migration and invasion assay was performed using a transwell chamber (8 μm pore size; EMD

Table I. Sequences for the siRNAs, miRNA mimics and inhibitors used in the present study.

Name	Sequence (5'-3')
si-TUG1#1	CAGUCCUGGUGAUUUAGACAGUCUU
si-TUG1#2	CCCAGAAGUUGUAAGUUCACCUUGA
si-TUG1#3	CAGCUGUUACCAUUCAACUUCUUAA
si-NC	UUCUCCGAACGUGUCACGUTT
miR-145-5p mimic	GUCCAGUUUUCCCAGGAAUCCCU
miR-145-5p inhibitor	AGGGAUUCCUGGGAAAACUGGAC
miR-NC mimic	ACUCUAUCUGCACGCUGACUU
miR-NC inhibitor	CAGUACUUUUGUGUAGUACAA
sh-TUG1	CCGGCTGTTGACCTTGCTGTGAGAACTCGA GTTCTCACAGCAAGGTCAACAGTTTTTTG
sh-NC	CCTAAGGTTAAGTCGCCCTCG
si-SOX2 sense	UGAUGGAGACGGAGCUGAAUU
si-SOX2 antisense	UUCAGCUCCGUCUCCAUCAUU

Si, small interfering; miR, microRNA; TUG1, taurine-upregulated gene 1; SOX2, SRY (sex determining region Y)-box 2.

Millipore). For migration assays, $3x10^4$ transiently transfected M14 and A375 cells in serum-free DMEM were seeded into the upper chamber, whilst DMEM supplemented with 10% FBS was added to the lower chamber. Subsequently, the chamber was incubated at 37˚C. In total, 24 h later, the migrated cells were fixed with 4% paraformaldehyde for 15 min at room temperature, before being stained with 0.1% crystal violet for 30 mins at room temperature and counted under an inverted light microscope (Nikon Eclipse TS100; Nikon Corporation) at x20 magnification. For invasion assays, the protocol used was identical, but the upper chamber was precoated with 50 μl Matrigel (8 mg/ml; 1:8 dilution; Corning, Inc.) at 4˚C overnight.

Dual-luciferase reporter assay. The downstream target miRNAs of TUG1 were predicted using the online website LncBase Predicted v.2 (http://carolina.imis.athenainnova-tion.gr/diana_tools/web/index.php?r=lncbasev2%2Findex-predicted). Direct targets of miR-145-5p were predicted by online databse miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/php/detail.php?mirtid=MIRT000307). The wild-type (WT) or mutant (MUT) sequences of TUG1 and SOX2 were amplified and cloned into pGL3 luciferase promoter vector (Promega Corporation) to synthesize TUG1 WT, TUG1 MUT, SOX2 WT and SOX2 MUT. *Renilla* luciferase reporter plasmids were used as the control for normalization. In total, $1x10^6$ M14 and A375 cells were co-transfected with 8 μg recombinant luciferase reporter plasmids, 0.16 μg pRL-TK plasmids and miR-145-5p mimics (final concentration, 15 nM) or miR-NC (final concentration, 15 nM) using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instruction. The luciferase activity was evaluated using the Dual-Luciferase® reporter system (Beyotime Institute of Biotechnology) according to the protocols supplied by the manufacturer.

RNA immunoprecipitation (RIP) assay. The RIP assay was performed using the extracts obtained from the melanoma M14 and A375 cells using the EZ-Magna RIP kit (cat. no. 17-701; EMD Millipore) according to the manufacturer's instructions, as previously described (21). In brief, the extracted supernatant (150 μg protein) was incubated with 1 mg magnetic beads that had been pre-coated with human anti-argonaute2 (Ago2) antibodies (cat. no. ab186733, 1:50, Abcam) or IgG (cat. no. PP64B, 1:20, EMD Millipore) at 4˚C overnight, which was used as a negative control. After overnight incubation at 4˚C, the RNA complex were incubate with proteinase K (RIP wash buffer, 10% SDS and 10 mg/ml proteinase K; EMD Millipore) for 30 min at 55˚C to digest the protein remaining on the beads. The RNA was then purified from the samples using phenol: chloroform: isoamyl alcohol separation. RNA detection was conducted using RT-qPCR as aforementioned.

Western blot assay. Cell lysates of melanoma cells (M14 and A375) were prepared using the RIPA Reagent (Cell Signaling Technology, Inc.). The protein concentration was quantified with bicinchoninic acid reagent (Sigma-Aldrich; Merck KGaA). A total of 30 μg protein samples were fractionated on 15% SDS-polyacrylamide gels and then transferred onto PVDF membranes. Thereafter, the membranes were blocked with 5% skimmed milk at room temperature for 2 h and probed with primary antibodies targeting SOX2 (1:1,000, cat. no. cat. no. ab171380; Abcam) or β-actin (1:1,000; cat. no. ab8227; Abcam) at 4˚C overnight. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:1,000; cat. no. ab205718, Abcam) at room temperature for 2 h. The bands were visualized using an ECL detection kit (Thermo Fisher Scientific, Inc.) and quantified using the ImageJ 1.8.0 software (National Institute of Health). The aforementioned antibodies used for this investigation were purchased from Abcam (Cambridge, MA, USA).

Xenograft mouse model. The animal experiments, which lasted 28 days, were approved by the Animal Care and Scientific

Committee of the First Affiliated Hospital of Anhui Medical University (Hefei, China). A total of 12 male BALB/c nude mice (5-week-old; 16-20 g weight range) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (grouped into a sh-TUG1 group and sh-NC group, n=6 per group) and maintained under temperature (20-22˚C) and humidity-controlled (55±10%) pathogen-free specific conditions with a 12-h light/dark cycle and given water and food *ad libitum*. The stable A375 cells expressing sh-NC or sh-TUG1 were produced and maintained in medium containing 3 μg/ml puromycin (Sigma-Aldrich; Merck KGaA). Prior to injection, the stable A375 cells were resuscitated and grown for two passages. A total of $4x10^6$ stable A375 cells expressing sh-NC or sh-TUG1 were collected and suspended in 100 μl sterile PBS solution and then subcutaneously inoculated into the right flank of the nude mice. Tumor volume was measured every 7 days for 28 days and calculated according to the following equation: Volume=(length x width^2)/2. The health and behavior of nude mice involved in the animal experiment were also monitored every 7 days after inoculation. All mice were sacrificed at day 28 following inoculation, for a pre-set 28-day test cycle. The 12 nude mice were anesthetized using 2% methoxyflurane (an inhalant anesthetic) (22). Subsequently, the animals were sacrificed by cervical dislocation. The xenograft tumor tissues were then collected for measurement of weight and used for RT-qPCR, western blot analyses and immunohistochemical assays.

Immunohistochemical (IHC) assay. The IHC assays were conducted to detect the expression profile of SOX2 in resected tumors from the sh-NC group and sh-TUG1 group, following the protocols from a previous report (23). In brief, resected tumors from the xenograft models were fixed in 3% formaldehyde overnight at 4˚C and embedded in paraffin and then cut into 5-μm sections. The slices were then incubated with 0.3% hydrogen peroxide (H_2O_2) solution in methanol for 20 min at room temperature to block the activity of endogenous peroxidases. Sections were blocked in 10% horse serum at room temperature for 1 h and then incubated with goat anti-SOX2 antibodies (1:100; cat. no. GT15098; Neuromics) at 4˚C overnight, followed by incubation with peroxidase-conjugated horse anti-goat IgG (cat. no. PI-9500-1; 1:2,000; Vector Laboratories, Inc.) at room temperature for 30 min. Immunoreactivity was measured using NovaRed peroxidase substrate (Vector Laboratories, Inc.) under an Eclipse TS100 fluorescence microscope (Nikon Corporation) at x20 magnification.

Statistical analysis. All data analyses were conducted using SPSS 22.0 software (IBM Corp.). Data from three independent experiments are presented as the mean ± SD. Difference between means was analyzed using paired Student's t-tests (for 2 groups) and one-way ANOVAs (for ≥3 groups) followed by Tukey's post hoc tests. Pearson correlation coefficient was used to analyze the correlation among TUG1, miR-145-5p and SOX2. Differences were considered significant at $P<0.05$.

Results

TUG1 expression is upregulated in melanoma tissues and cells. To investigate the role of TUG1 in melanoma, the expression levels of TUG1 were analyzed using RT-qPCR. As shown in Fig. 1A, TUG1 expression was significantly upregulated in melanoma tissues compared with that in normal tissues. In addition, the expression levels of TUG1 were significantly raised in the melanoma M14 and A375 cell lines compared with that in normal melanocyte cell line, HEMa-LP cells (Fig. 1B).

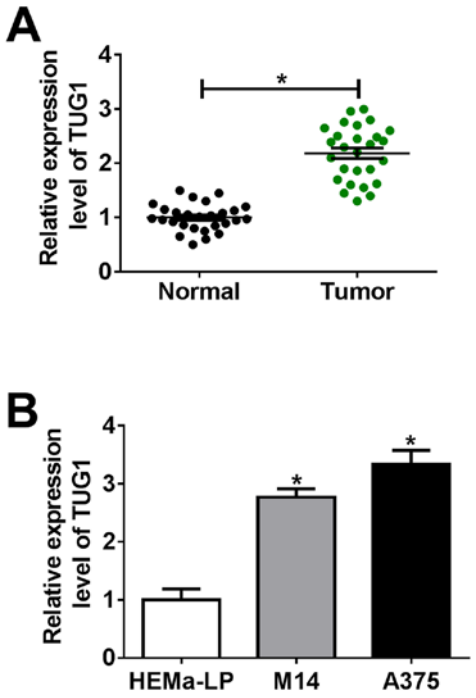

Figure 1. TUG1 is upregulated in melanoma tissues and cells. (A) RT-qPCR was used to detect the expression levels of TUG1 in melanoma tissues and normal tissues. (B) RT-qPCR was also performed to analyze the expression levels of TUG1 in melanoma M14 and A375 cells, as well as the normal melanocyte cell line, HEMa-LP. *P<0.05 compared with Normal or HEMa-LP. RT-qPCR, reverse transcription-quantitative PCR; TUG1, taurine-upregulated gene 1.

Silencing of TUG1 expression inhibits the proliferative, migratory and invasive abilities of melanoma cells. siRNAs against TUG1 (si-TUG1#1, si-TUG1#2 and si-TUG1#3) were transfected into M14 and A375 cells to construct TUG1-silenced melanoma cells to explore the effect of TUG1 on the proliferative, migratory and invasive abilities of melanoma cells. RT-qPCR assays were conducted to detect the silencing efficiency of the 3 siRNAs (si-TUG1#1, si-TUG1#2 and si-TUG1#3) on the TUG1 expression in the transfected M14 and A375 cells. The mRNA expression levels of TUG1 in M14 and A375 cells were decreased after the indicated transfections, with the silencing efficiency of si-TUG1#3 being the most significant (Fig. 2A and B). As such, si-TUG1#3 was used for subsequent functional experiments. CCK-8 assays were employed to detect the proliferative ability of transfected M14 and A375 cells, which indicated that a reduced TUG1 expression inhibited the proliferative ability of the two cell lines (Fig. 2C and D). Transwell assay indicated that down-regulation of TUG1 also repressed the migration and invasion of M14 and A375 cells (Fig. 2E and F).

miR-145-5p is a direct target of TUG1 and is negatively regulated by TUG1. LncBase Predicted v.2 was applied to seek

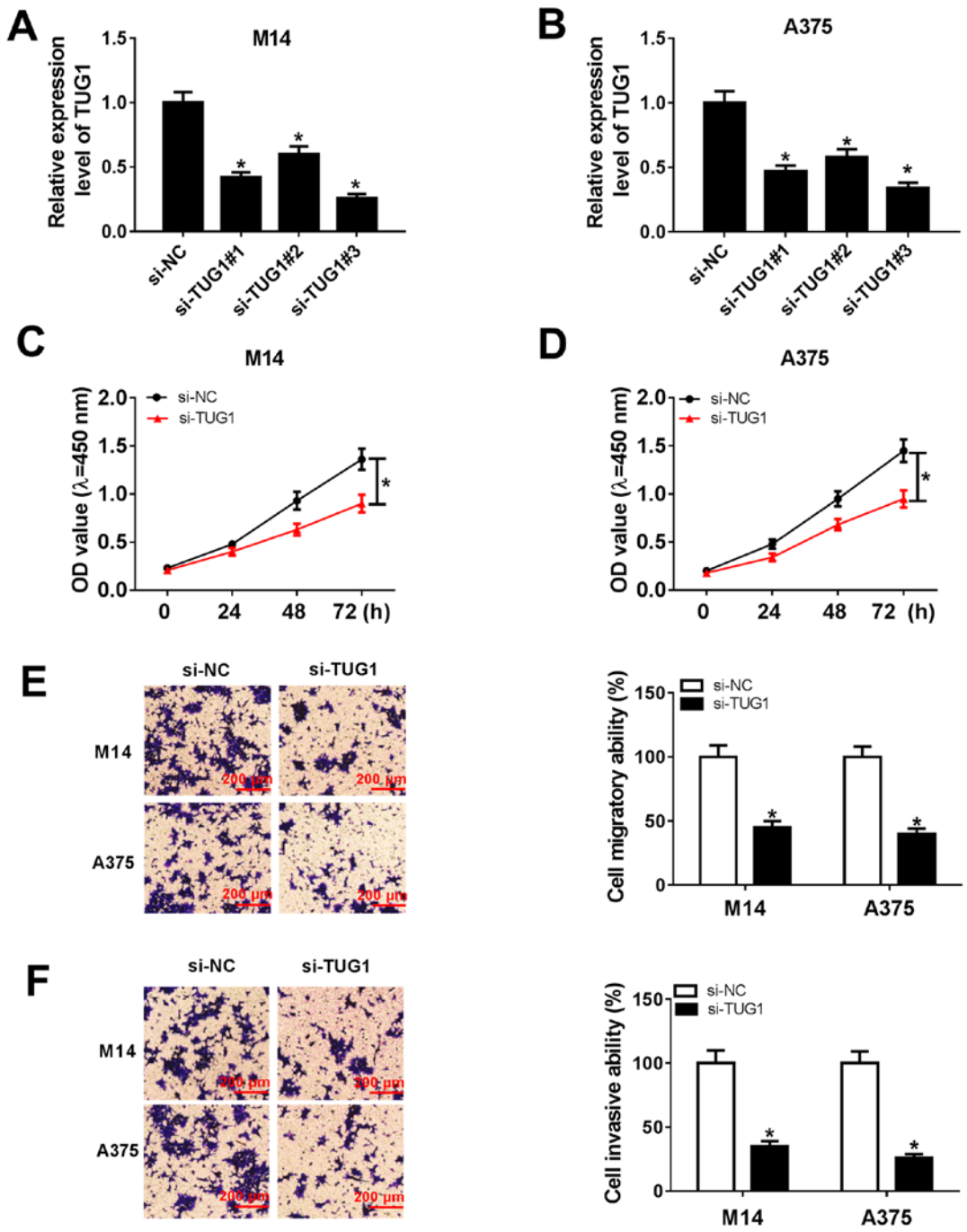

Figure 2. Silencing TUG1 expression inhibits the proliferative, migratory and invasive abilities of melanoma cells. (A) M14 and (B) A375 cells were transfected with si-NC, si-TUG1#1, si-TUG1#2 or si-TUG1#3. The relative expression levels of TUG1 in M14 or A375 cells were assessed using RT-qPCR assay. M14 and A375 cells were then transfected with si-TUG1. The proliferative ability of transfected (C) M14 and (D) A375 cells after 24, 48 and 72 h was tested using Cell Counting Kit-8 assays, measuring the OD_{450}. Transwell assays were performed to detect cell (E) migratory and (F) invasive abilities of M14 and A375 cells. Scale bars, 200 μm. *P<0.05 vs. si-NC group. NC, negative control; OD, optical density; OD_{450}, OD at 450 nm; si, small interfering; TUG1, taurine-upregulated gene 1.

the downstream target of TUG1 and it was hypothesized that miR-145-5p could bind to TUG1 (Fig. 3A). Dual-luciferase assays were performed to validate the relationship between TUG1 and miR-145-5p. RT-qPCR assay indicated that the expression of miR-145-5p was significantly upregulated by transfection of miR-145-5p mimic (Fig. S1A), but was significantly suppressed more by transfection with the miR-145-5p inhibitor (Fig. S1B). In addition, both transfection of TUG1 wt or TUG1 mut significantly elevated TUG1 expression (Fig. S1C). As depicted in Fig. 3B and C, the luciferase activity of M14 and A375 cells co-transfected with TUG1 WT and miR-145-5p mimics was obviously lower than that of cells co-transfected with TUG1 WT and miR-NC mimics. By contrast, the luciferase activity of M14 and A375 cells co-transfected with TUG1 MUT and miR-145-5p mimics or miR-NC mimics showed no significant difference. A RIP assay was performed to confirm the interaction between TUG1 and miR-145-5p, which showed that TUG1 mRNA

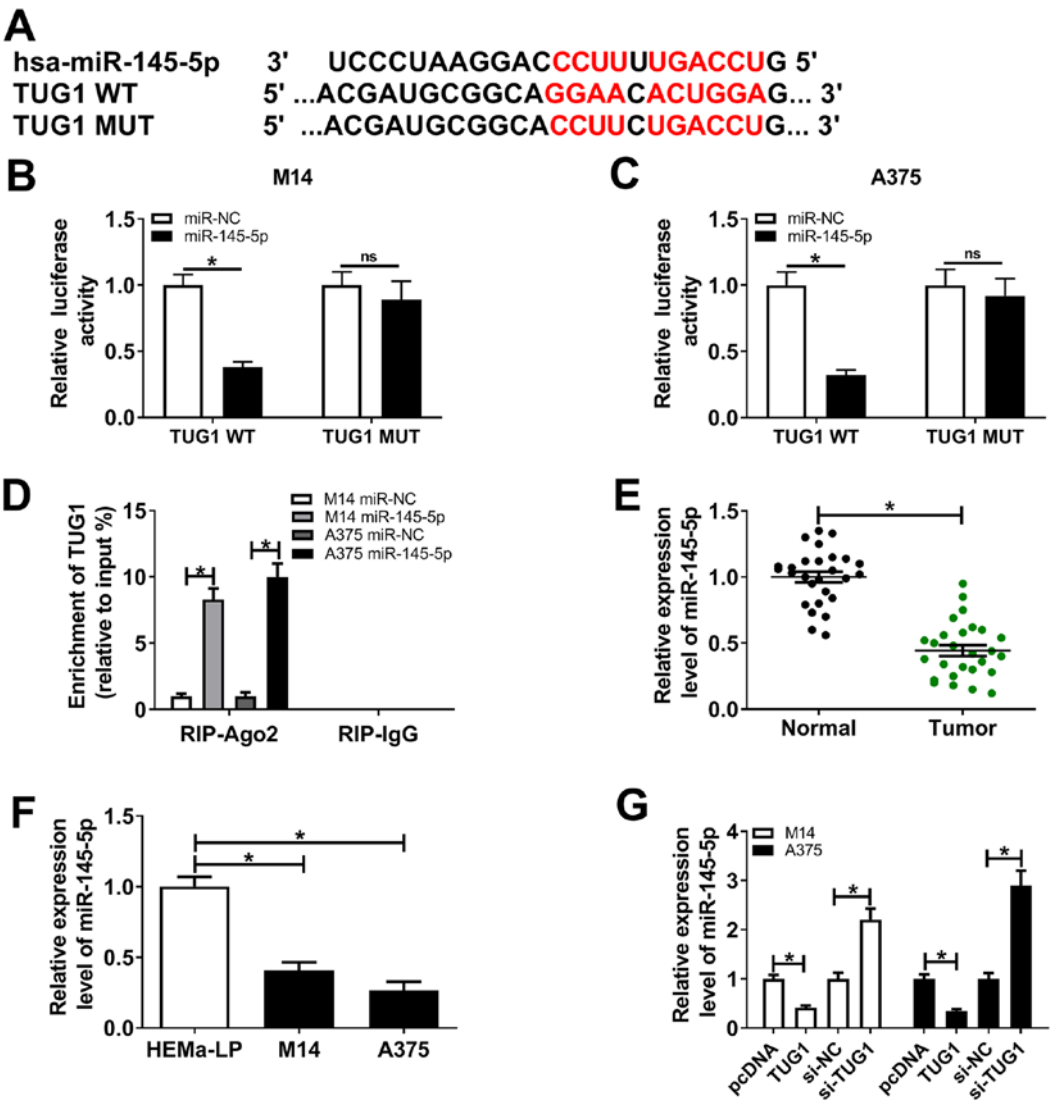

Figure 3. TUG1 targets miR-145-5p and negatively regulates miR-145-5p. (A) The predicted binding site between miR-145-5p and TUG1 and its MUT were predicted by LncBase Predicted v.2. Dual-luciferase assays were conducted to measure the luciferase activity of (B) M14 and (C) A375 cells that were co-transfected with TUG1 WT + miR-NC mimics, TUG1 WT + miR-145-5p mimics, TUG1 MUT + miR-NC mimics or TUG1 MUT + miR-145-5p mimics. (D) RIP assays were conducted and the enrichment of TUG1 was determined by the samples which had bound to the Ago2 antibody or IgG in M14 and A375 cells. (E) RT-qPCR was used to measure TUG1 expression levels in melanoma tissues. (F) The expression levels of TUG1 in melanoma cells were evaluated using RT-qPCR. (G) M14 and A375 cells were transfected with pcDNA, pcDNA-TUG1, si-NC or si-TUG1. RT-qPCR was used to measure miR-145-5p levels in transfected melanoma cells. *P<0.05. Ago2, argonaute2; miR, microRNA; MUT, mutant; NC, negative control; ns, no significance; RT-qPCR, reverse transcription-quantitative PCR; si, small interfering; TUG1, taurine-upregulated gene 1; WT, wild-type.

could be specifically recruited to the miRNA complexes isolated using anti-Ago2 antibody following miR-145-5p transfection, suggesting that TUG1 could interact with miR-145-5p (Fig. 3D). RT-qPCR was performed to analyze the expression level of miR-145-5p in melanoma tissues and cells. miR-145-5p was found to be downregulated in melanoma tissues compared with normal tissues (Fig. 3E). In addition, downregulation of miR-145-5p was also detected in melanoma M14 and A375 cells compared with HEMa-LP cells (Fig. 3F). Introduction of TUG1 repressed the expression levels of miR-145-5p in both M14 and A375 cells relative to cells transfected with pcDNA; while silencing of TUG1 promoted miR-145-5p expression in the two cell lines compared with cells transfected with si-NC, indicating that TUG1 inversely regulated miR-145-5p expression levels (Fig. 3G).

TUG1 regulates the proliferative, migratory and invasive abilities of melanoma cells by targeting miR-145-5p. To clarify whether TUG1 regulates the proliferative, migratory and invasive abilities of melanoma cells by targeting miR-145-5p, recovery experiments were performed. RT-qPCR assays showed that miR-145-5p mimics significantly elevated miR-145-5p expression levels in both M14 and A375 cells, while introduction of TUG1 counteracted the promoting impact of miR-145-5p mimics on miR-145-5p expression (Fig. 4A and B). CCK-8 assays also indicated that upregulation of miR-145-5p inhibited the proliferative ability of M14 and A375 cells, whereas elevated TUG1 almost reversed the repressive effect of miR-145-5p mimics on the proliferative ability of the two cell lines (Fig. 4C and D). Subsequently, the impact of miR-145-5p alone or miR-145-5p combined with TUG1, on the migratory and invasive abilities of melanoma

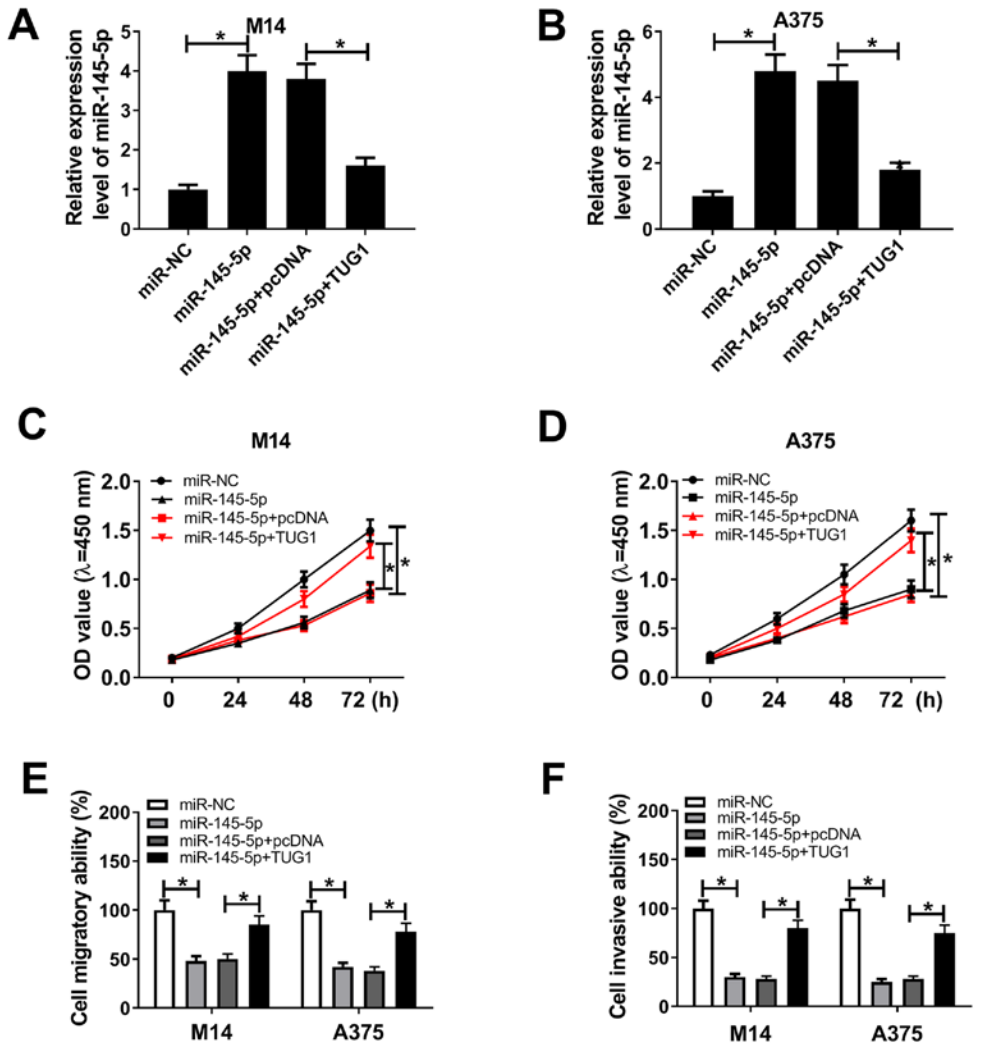

Figure 4. TUG1 regulates the proliferative, migratory and invasive abilities of melanoma cells by targeting miR-145-5p. M14 and A375 cells were transfected with miR-NC, miR-145-5p, miR-145-5p + pcDNA or miR-145-5p + TUG1. RT-qPCR was used to investigate miR-145-5p levels in (A) M14 and (B) A375 cells after transfections. Cell Counting Kit-8 assays were performed to investigate the proliferative ability of (C) M14 and (D) A375 cells after transfections, measuring the OD_{450}. Transwell assays investigating the (E) migratory and (F) invasive abilities of M14 and A375 cells after transfection. *P<0.05. miR, microRNA; NC, negative control; OD, optical density; OD_{450}, OD at 450 nm; TUG1, taurine-upregulated gene 1.

cells was assessed using Transwell assays. As displayed in Fig. 4E and F, overexpression of miR-145-5p significantly inhibited the migratory and invasive abilities of M14 and A375 cells, while raised expression levels of TUG1 significantly weakened the repressive impact of miR-145-5p on the migratory and invasive abilities of M14 and A375 cells.

miR-145-5p targets SOX2 and TUG1 regulates SOX2 expression by sponging miR-145-5p. The online software mirTarBase identified SOX2 as a direct target of miR-145-5p. The putative binding site and its MUT for miR-145-5p on the 3'-UTR of SOX2 mRNA are displayed in Fig. 5A. RT-qPCR assay indicated that SOX2 expression was significantly elevated by SOX2 wt or SOX2 mut transfection (Fig. S1E). Subsequently, the targeted relationship between miR-145-5p and SOX2 was validated using dual-luciferase reporter assays. The assay indicated that upregulation of miR-145-5p significantly constrained the luciferase activity of SOX2 WT reporter in co-transfected M14 and A375 cells, compared to the miR-NC transfected cells. However, neither the miR-145-5p nor the miR-NC had little impact on the luciferase activity of the SOX2 MUT reporter in co-transfected M14 and A375 cells (Fig. 5B and C). Subsequently, RIP assays were also implemented to confirm the direct interaction between miR-145-5p and SOX2, and it was suggested that transfection with miR-145-5p triggered SOX2 enrichment in the RIP-Ago2 group in melanoma M14 and A375 cells compared with that in the RIP-IgG group, supporting the notion that SOX2 was a direct target of miR-145-5p (Fig. 5D). RT-qPCR assays indicated that SOX2 was significantly upregulated in melanoma tissues in comparison to normal tissues (Fig. 5E). Additionally, western blot assays suggested that the protein expression levels of SOX2 were higher in melanoma M14 and A375 cells than that in HEMa-LP cells (Fig. 5F). Western blot assays that the revealed introduction of miR-145-5p reduced the protein expression levels of SOX2 and that silencing of TUG1 also reduced the SOX2 protein expression levels (Fig. 5G and H). Pearson analysis illuminated a negative correlation between miR-145-5p and SOX2 expression levels (Fig. 5I), as well as between TUG1 and miR-145-5p expression levels (Fig. 5J),

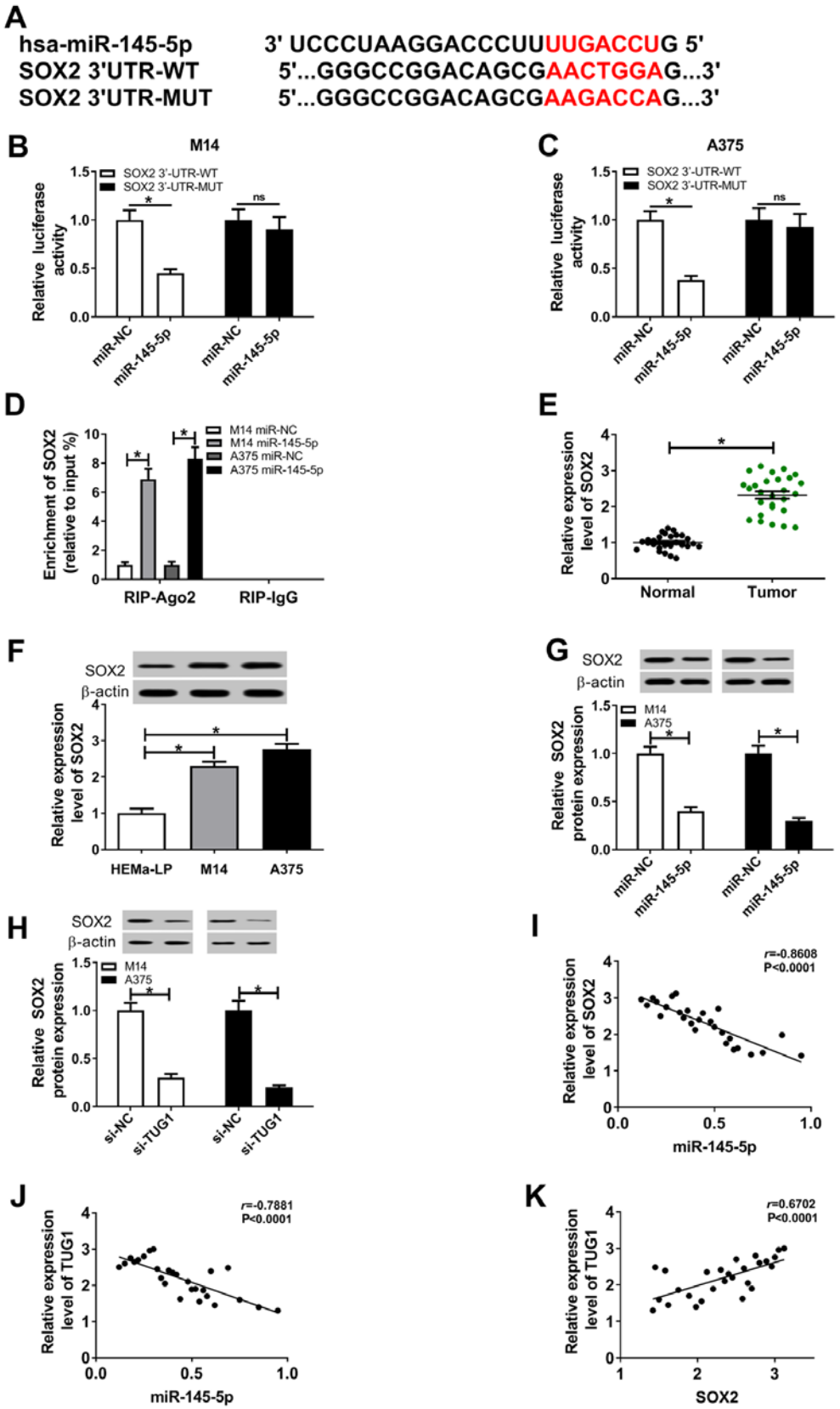

Figure 5. miR-145-5p targets SOX2 and TUG1 regulates SOX2 expression by sponging miR-145-5p. (A) Bioinformatics analysis showed the potential binding sites of miR-145-5p on SOX2, using mirTarBase. Dual-luciferase assays were performed for the analysis of luciferase activity of the (B) M14 and (C) A375 cells co-transfected with SOX2 WT + miR-NC mimics, SOX2 WT + miR-145-5p mimics, SOX2 MUT + miR-NC mimics or SOX2 MUT + miR-145-5p mimics. (D) RIP assays were performed and SOX2 enrichment was detected in the samples bound to the Ago2 antibody in M14 and A375 cells. (E) Reverse transcription-quantitative PCR for SOX2 expression levels in melanoma tissues. (F) Western blot assays for SOX2 expression levels in melanoma cells. (G) Western blot assays for SOX2 expression levels in transfected M14 and A375 cells. (H) The protein expression levels of SOX2 in transfected M14 and A375 cells were analyzed using western blot assays. Correlation analyses between (I) miR-145-5p and SOX2; (J) TUG1 and miR-145-5p2 and (K) TUG1 and SOX2. *P<0.05. Ago2, argonaute2; miR, microRNA; MUT, mutant; NC, negative control; ns, no significance; TUG1, taurine-upregulated gene 1; UTR, untranslated region; WT, wild-type.

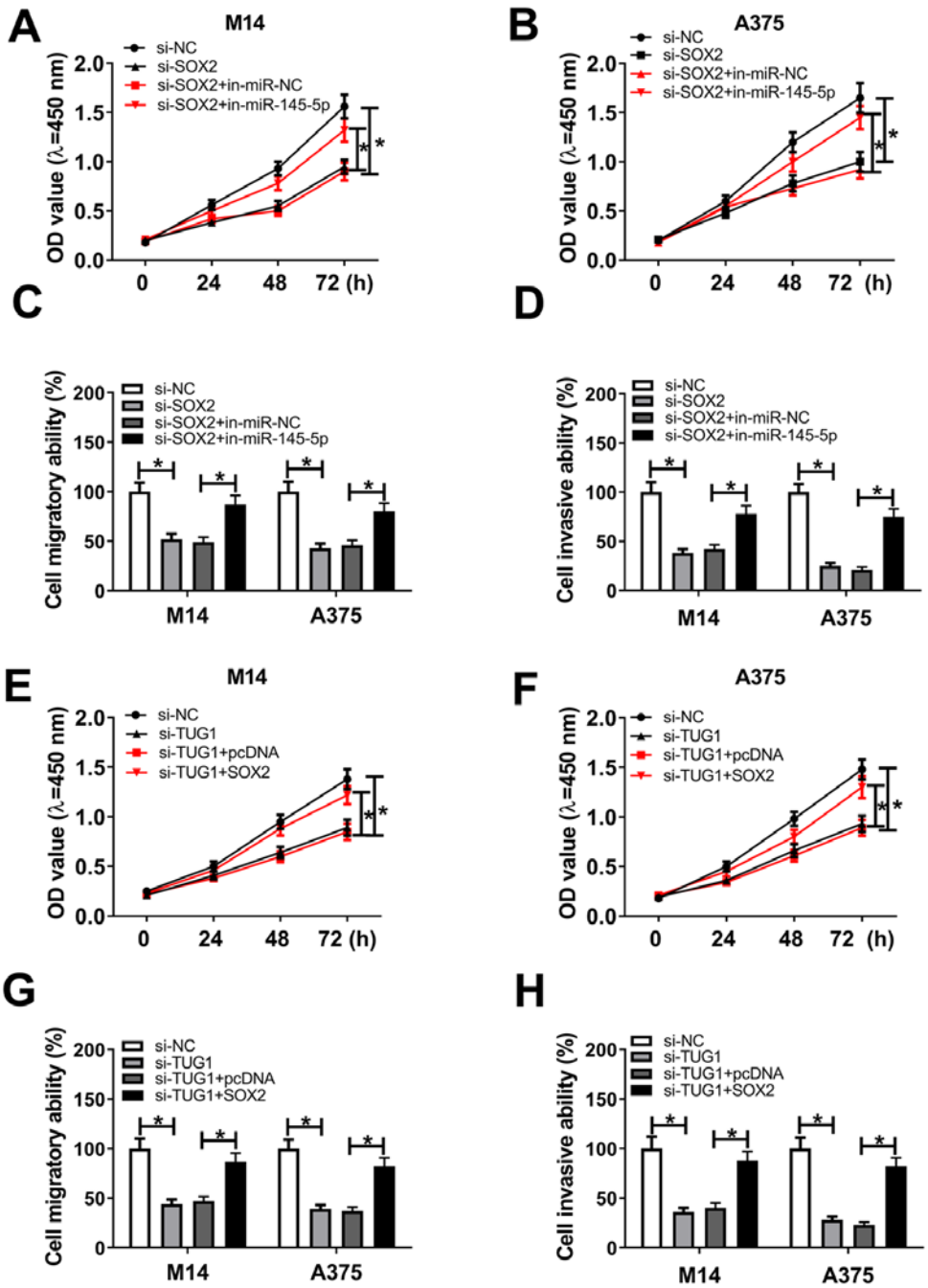

Figure 6. Silencing SOX2 restrains the proliferative, migratory and invasive abilities of melanoma cells, with TUG1 regulating the function of SOX2 by sponging miR-145-5p. M14 and A375 cells were transfected with si-NC, si-SOX2, si-SOX2 + in-miR-NC or si-SOX2 + in-miR-145-5p. (A) The proliferative abilities of transfected (A) M14 and (B) A375 cells, were determined using CCK-8 assays, through the measurement of OD450. The (C) migratory and (D) invasive abilities of M14 and A375 cells were measured using transwell assays. M14 and A375 cells were transfected with si-NC, si-TUG1, si-TUG1 + pcDNA or si-TUG1 + pcDNA-SOX2. CCK-8 assays were performed on the transfected (E) M14 and (F) A375 cells. The (G) migratory and (H) invasive abilities of the transfected melanoma cells were measured using transwell assays. *P<0.05. CCK, Cell Counting Kit; in-miR, miR inhibitor; miR, microRNA; NC, negative control; OD, optical density; OD_{450}, OD at 450 nm; si, small interfering; TUG1, taurine-upregulated gene 1.

while a positive correlation was found between TUG1 and SOX2 expression levels (Fig. 5K) in melanoma tissues.

Silencing of SOX2 restrains the cell proliferative, migratory and invasive abilities of melanoma cells, and TUG1 regulates the function of SOX2 by sponging miR-145-5p. To further clarify that TUG1 mediates the proliferative, migratory and invasive abilities of melanoma cells by targeting the miR-145-5p/SOX2 axis, restoration experiments were conducted. As shown in Fig. S1D, the expression of SOX2 was significantly by transfection with si-SOX2. The effects of SOX2 on the proliferative ability of melanoma cells were subsequently evaluated. CCK-8 assays found that knockdown of SOX2 repressed the proliferative ability of M14 and A375 cells; however, coinstantaneous knockdown of miR-145-5p weakened the inhibitory impact (Fig. 6A and B). Similarly, knockdown of SOX2 blocked the migratory and invasive capacities of M14 and A375 cells, while simultaneous knockdown of miR-145-5p relieved the

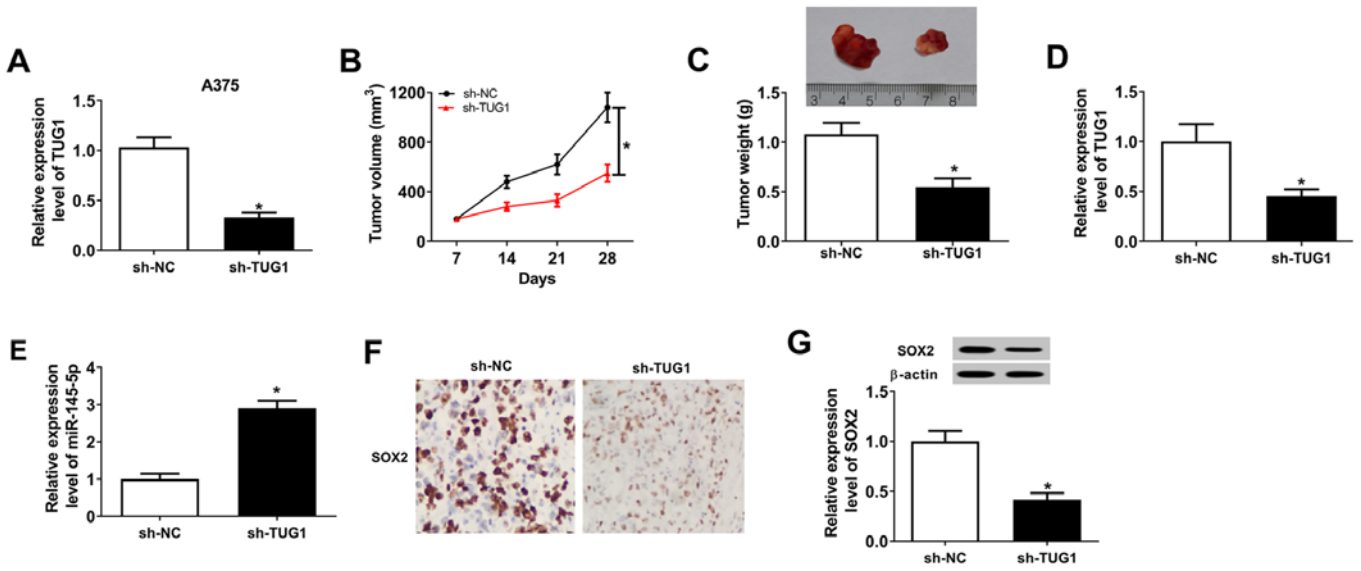

Figure 7. Downregulation of TUG1 impedes tumor growth in nude mice. (A) RT-qPCR assays for TUG1 expression levels in transfected A375 cells. (B) The tumor volumes from the nude mice. (C) The tumor weight in nude mice was measured. Images of representative isolated tumors are shown. (D) TUG1 and (E) miR-145-5p expression levels in tumors tissues derived from the nude mice were detected by RT-qPCR. (F) The expression levels of SOX2 from the resected tumors of the sh-NC group and sh-TUG1 group were detected using immunohistochemical staining. Magnification, x200. (G) Western blot assays for SOX2 protein expression levels in tumors from nude mice. *P<0.05 vs. sh-NC group. miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; sh, short hairpin; TUG1, taurine-upregulated gene 1.

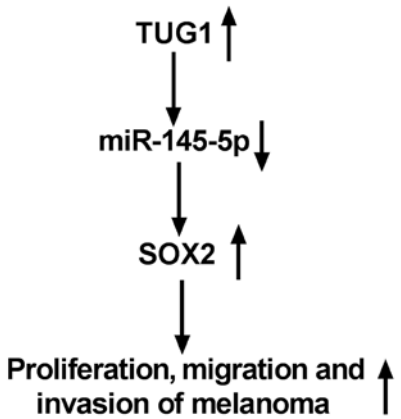

Figure 8. Schematic diagram of TUG1 regulating the proliferation, migration and invasion of melanoma cells. TUG1 regulated SOX2 expression through miR-145-5p to regulate the proliferative, migratory and invasive abilities of melanoma cells. miR, microRNA; TUG1, taurine-upregulated gene 1.

inhibitory effect (Fig. 6C and D). The CCK-8 assay also suggested that introduction of SOX2 reversed the inhibitory impact of si-TUG1 on the proliferative ability of M14 and A375 cells (Fig. 6E and F). Upregulation of SOX2 also rescued the suppressive effect of si-TUG1 on the migratory and invasive capacities of M14 and A375 cells (Fig. 6G and H).

Downregulation of TUG1 impedes tumor growth in nude mice. Finally, A375 cells stably transfected with sh-NC or sh-TUG1 were constructed, with transfections being confirmed using RT-qPCR (Fig. 7A). Downregulation of TUG1 repressed the tumor volume (Fig. 7B) and weight (Fig. 7C) in nude mice compared with the sh-NC group. In addition, the expression levels of TUG1 were detected in tumor tissues of nude mice and the results showed that TUG1 was significantly reduced in the sh-TUG1 group compared with the sh-NC group (Fig. 7D). In addition, TUG1 silencing significantly elevated miR-145-5p expression in xenograft tumor tissues (Fig. 7E). IHC staining assay for SOX2 indicated that SOX2-positive cells in sh-TUG1 group were decreased compared with that in the sh-NC group (Fig. 7F). Additionally, downregulation of TUG1 also inhibited SOX2 protein expression levels in the tumors from nude mice (Fig. 7G). In summary, TUG1 regulated the proliferation, migration and invasion of melanoma cells by modulating the miR-145-5p/SOX2 axis (Fig. 8).

Discussion

As the most common skin cancer, melanoma is extremely aggressive and metastatic (24). A large number of studies on melanoma have been conducted (2,3), but the mechanism of action behind melanoma development and progression needs to be further elucidated. The present study aimed to explore the functional roles and potential mechanisms of action of TUG1 in melanoma progression.

LncRNAs are a group of non-protein coding RNAs, which participate in the modulation of cell growth, migration and invasion, as well as other important cellular processes in various types of tumor progression, including melanoma (25,26). LncRNAs have also been shown to be linked to the development of melanoma by regulating various mechanisms and signaling pathways (27). Dysregulation of TUG1 has been demonstrated in several types of cancer, for example, TUG1 is upregulated in liver cancer, colorectal cancer, cervical cancer, ovarian cancer and gastric cancer, while it is downregulated in non-small cell lung cancer, breast cancer and glioma (11). Additionally, a former study revealed that TUG1 expression was elevated in melanoma specimens and cell lines (28), with similar conclusions being drawn in the present study. TUG1

facilitates melanoma development and metastasis through the TUG1/miR-129-5p/Astrocyte-Elevated Gene-1 axis. Wang *et al* (29) found that knockdown of TUG1 blocked melanoma cell growth and metastasis, and promoted cell apoptosis by regulating miR-29c-3p and its target gene regulator of G-protein signaling. In the present study, knockdown of TUG1 significantly inhibited the proliferative, migratory and invasive abilities of melanoma cells.

A previous study has shown that lncRNAs can function as miRNA sponges to decrease the expression levels of miRNAs available to target mRNAs (30). The bioinformatics analysis in the present study suggested that TUG1 targeted miR-145-5p; and the luciferase and RIP assays also confirmed this interaction between TUG1 and miR-145-5p. Furthermore, this targeted relationship has been reported in laryngocarcinoma, gastric cancer, hypertension and intrahepatic cholangiocarcinoma (ICC) (31-34). In laryngocarcinoma, TUG1 has been revealed to partake in laryngocarcinoma progression by regulating the miR-145-5p/ROCK1 axis and activating the RhoA/ROCK/MMPs signaling pathway (31); Ren *et al* (32) showed that TUG1 augmented cell proliferation and invasion of gastric cancer cells by directly binding to miR-145-5p and repress miR-145-5p expression; the lncRNA TUG1/miR-145-5p/FGF10 axis regulates proliferation and migration in vascular smooth muscle cells during hypertension by activation of the Wnt/β-catenin pathway (33); and as for ICC, TUG1 sponges miR-145 to contribute to cancer development and modulate glutamine metabolism via the sirtuin 3/glutamate dehydrogenase axis (34). The present study also examined the downstream genes of the TUG1/miR-145-5p axis. Moreover, the effects of the interaction between TUG1 and miR-145-5p were evaluated on proliferation, migration and invasion of melanoma cells. Upregulation of TUG1 enriched the endogenous levels of TUG1 in melanoma cells, thereby negatively regulating the expression of miR-145-5p and aggravating the inhibitory impact of TUG1 on miR-145-5p expression.

The aforementioned results suggested that miR-145-5p may play vital roles in the tumorigenesis of melanoma; therefore, the present study explored the impact and potential mechanisms of action of this miRNA. Jiang *et al* (14) claimed that miR-145-5p was downregulated in breast cancer cell lines, and that it exerts an important role in the linc01561/miR-145-5p/MMP11 regulation axis of breast cancer cell proliferation and apoptosis. In bladder cancer, it has been shown that miR-145-5p is downregulated and that miR-145-5p inhibits cell proliferation and migration in bladder cancer by directly targeting transgelin 2 (15). Additionally, miR-145-5p represses cell proliferation, invasion and migration, and induces apoptosis of melanoma cells by restraining the MAPK and PI3K/AKT pathways (16), highlighting that miR-145-5p functions as a tumor-suppressor. Reduced expression levels of miR-145-5p in melanoma tissues and cells was also detected in the present study. Moreover, it was found that TUG1 performed its inhibitory effects on the proliferative, migratory and invasive abilities of melanoma cells by targeting miR-145-5p.

To identify the regulatory mechanism of action for miR-145-5p in melanoma cells, bioinformatics analysis was also performed to identify its downstream gene. SOX2 was identified as a downstream gene of miR-145-5p. In prostate cancer, raising the expression of miR-145-5p blocks proliferation and migration in tumor cell lines, with miR-145-5p targeting SOX2 and inhibiting SOX2 expression (35). Tang *et al* (36) highlighted that miR-145-5p inhibits breast cancer cell proliferation through targeting SOX2, with both miR-145-5p and SOX2 being unfavorable prognostic factors. SOX2 serves as downstream gene of the lncRNA regulator of reprogramming/miR-145 axis, to maintain pluripotency of human amniotic epithelial stem cells and regulate their directed β islet-like cell differentiation efficiency (37). SOX2 has been validated to be connected to the development and progression of lung cancer, breast cancer, hepatocellular carcinoma, and ovarian cancer (38). SOX2 also accelerates the invasion rate of melanoma cells (39). SOX2 has also been proven to reduce the suppressive effect of miR-625 on the proliferation, migration and invasion in malignant melanoma cells (40). In the present study, the expression levels of SOX2 were upregulated, which was negatively correlated with miR-145-5p expression, but positively correlated with TUG1 expression. Silencing SOX2 hindered the proliferative, migratory and invasive abilities of melanoma cells. The present data manifested that SOX2 was involved in TUG1/miR-145-5p-mediated melanoma cell proliferation and metastasis. Additionally, TUG1 modulated SOX2 expression and exerted its role in melanoma cells by sponging miR-145-5p.

In conclusion, TUG1 and SOX2 were expressed at higher levels, while miR-145-5p expression was reduced, in melanoma tissues and cell lines, compared with those in normal tissues and cell lines. TUG1 inhibition elevated miR-145-5p expression while repressed SOX2 expression. Moreover, silencing of TUG1 inhibited the proliferative, migratory and invasive abilities of melanoma cells, which was reversed by upregulating the expression levels of miR-145-5p and downregulating the expression levels of SOX2. TUG1 regulated the expression and function of SOX2 by downregulating miR-145-5p. In addition, knockdown of TUG1 inhibited tumor growth in nude mice. Taken together, the TUG1/miR-145-5p/SOX2 axis regulated the migration and invasion of melanoma cells.

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Availability of data and materials

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

Authors' contributions

Conceptualization of the study was performed by JD and YL. JD, YL and FZ designed the investigation. JD, YL and

JS developed the method. JS and FZ performed the project administration. JD and JS collected and analyzed the data. YL provided the resources. JD and FZ supervised the study. JD and FZ wrote, reviewed and edited the manuscript. JD and FZ confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The current study was permitted by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University. All patients signed written informed consent. The current animal experiment was approved by the Animal Care and Scientific Committee of the First Affiliated Hospital of Anhui Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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