lncRNA-ATB functions as a competing endogenous RNA to promote YAP1 by sponging miR-590-5p in malignant melanoma

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Abstract. The critical long non-coding RNAs (lncRNAs) involved in the carcinogenesis and progression of malignant melanoma (MM) have not been fully investigated. In the present study, it was identified that lncRNA activated by transforming growth factor-β (lncRNA-ATB) was upregulated in MM tissues and cells compared with benign nevus cells and human melanocytes, via comparative lncRNA screening from Gene Expression Omnibus datasets and reverse transcription-quantitative polymerase chain reaction analysis. Furthermore, lncRNA-ATB promoted the cell proliferation, cell migration, and cell invasion of MM cells *in vitro*, and tumor growth *in vivo*. It was additionally identified that lncRNA-ATB attenuated cell cycle arrest and inhibited cellular apoptosis in MM cells. Finally, it was demonstrated that lncRNA-ATB functions as a competing endogenous RNA (ceRNA) to enhance Yes associated protein 1 expression by competitively sponging microRNA miR-590-5p in MM cells. In conclusion, the present study revealed the expression and roles of lncRNA-ATB in MM, and indicated that lncRNA-ATB functions as a ceRNA to promote MM proliferation and invasion by sponging miR-590-5p.

Introduction

Malignant melanoma (MM) is one of the most aggressive forms of cutaneous neoplasm, the incidence of which has increased markedly in recent decades (1). An estimated 87,110 newly diagnosed MM cases and 9,730 MM-associated mortalities were predicted for 2017 in the United States (1). Despite the notable improvements in diagnosis and treatment made in recent years, the prognosis remains poor for patients diagnosed at the metastatic stage, with a median survival time of 6-9 months and a 5-year survival rate of only 10% (2-4). Thus, it is necessary to establish effective biomarkers for the early diagnosis and efficient evaluation of prognosis following surgery.

Long non-coding RNAs (lncRNAs) are a class of transcripts >200 nucleotides in length with limited protein coding potential (5-7). Recently, studies have demonstrated that lncRNAs have multiple functions in a wide range of biological process, including proliferation (8), apoptosis (9), cell migration (10) and cell invasion (11). A novel regulatory mechanism whereby lncRNAs function as competing endogenous (ce)RNAs to sponge microRNAs (miRNAs) and regulate their downstream signaling pathways has been proposed and confirmed preliminarily (12-14). Although it has been reported that a number of lncRNA transcripts are involved in the carcinogenesis and development of MM (15-17), the miRNA sponge roles of lncRNAs in the modulation of cell proliferation and migration in MM remain unclear.

The present study screened for candidate lncRNAs responsible for the malignant phenotype of MM from the Gene Expression Omnibus (GEO) datasets. It was identified that long non-coding RNA activated by transforming growth factor (TGF)-β (lncRNA-ATB) was the most markedly upregulated lncRNA in MM tissues and A375 cells compared with benign nevus cells and human melanocytes. The roles and underlying mechanism of lncRNA-ATB in the proliferation, apoptosis and invasion-metastasis cascade of MM cells were investigated and identified *in vivo* and *in vitro*.

Materials and methods

Cell culture. Highly invasive human MM A2058 cells, moderately invasive human MM A375 cells, normal epidermal melanocyte HEMa-LP cells, 293 and B16/F10 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), where they were characterized by mycoplasma detection, DNA-fingerprinting, isozyme detection and

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Abbreviations: MM, malignant melanoma; lncRNA, long noncoding RNA; lncRNA-ATB, long noncoding RNA activated by transforming growth factor-β; YAP1, Yes associated protein 1

Key words: malignant melanoma, lncRNA-ATB, microRNA-590-5p, YAP1, competing endogenous RNA

cell vitality detection. A2058, A375, 293 and B16/F10 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. HEMa-LP cells were cultured in Medium 254 (Invitrogen; Thermo Fisher Scientific, Inc.) suplemented with human melanocyte growth supplement (Hyclone; GE Healthcare Life Sciences) at 37˚C in a humidified atmosphere of 95% air and 5% CO_2.

Oligonucleotide transfection. pGMLV-lncRNA-ATB negative control (NC), pGMLV-lncRNA-ATB short hairpin (sh)RNA, miRNA (miR)-590-5p NC, miR-590-5p mimics and miR-590-5p inhibitors were purchased from GenePharma Co. Ltd. (Shanghai, China). The sequence for miR-590-5p was GAGCUUAUUCA UAAAAUGCAG; miR-NC was GUCCAGUGAAUUCCCAG; and miR-590-5p inhibitors was GACGUAAAAUACUUAUUC GAG. The lncRNA-ATB overexpressing plasmid was constructed by Shanghai GeneChem Co., Ltd. (Shanghai, China) using pCDNA3.1. An empty vector was used as the control (Shanghai GeneChem Co., Ltd.). When the cell confluence of A375 and A2058 cells reached 50-60%, oligonucleotide transfections were performed using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Oligonucleotides (50 nmol) were mixed with 5 μl Lipofectamine® 2000 in 500 μl medium. The transfection solutions were added to each well containing 500 μl medium. Following transfection, cell samples were collected at 48 h for further analyses.

RNA/miRNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. For lncRNA-ATB quantification, total RNA was extracted from the cells or tissues using TRI reagent (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), according to the manufacturer's protocol. RNA samples were reverse transcribed into cDNA using RT regeants (Takara Bio, Inc., Otsu, Japan) with lncRNA specific primers, and β-actin was used as internal loading controls. RT was performed at 45˚C for 60 min and 70˚C for 10 min. SYBR Mix (Takara Bio, Inc.) was used to detect and quantify lncRNA-ATB and β-actin expression. For miR-590-5p quantification, total miRNA was extracted from the cells or tissues using the miRNeasy RNA isolation kit (Qiagen, Inc., Valencia, CA, USA), according to the manufacturer's protocol. Total miRNA samples (1 μl; 1 μg/μl) were reverse transcribed into cDNA using the miScript RT kit (Qiagen GmbH, Hilden, Germany) with miR-590-5p specific primers, and universal small nuclear U6 RNA was used as an internal loading control. An Applied Biosystems™ SYBR™ Green dye miRNA RT-qPCR kit (Thermo Fisher Scientific, Inc.) was used to detect and quantify miR-590-5p and U6 expression. Data were analyzed with 7500 software v.2.0.1 (Applied Biosystems; Thermo Fisher Scientific, Inc.), with the automatic Cq setting for adapting baseline and threshold for Cq determination (18). Each sample was examined in triplicate. The primer sequences used in the present study are listed in Table I. RT-qPCR assays were performed under the following thermocycling conditions: 95˚C for 5 min, followed by 45 cycles of 95˚C for 15 sec, 60˚C for 30 sec and 72˚C for 30 sec.

Western blotting. Cells were washed in PBS three times prior to the proteins being extracted. Cells were lysed using radioimmunoprecipitation assay buffer (JingCai Technologies, Inc., Xi'an, China) and the protein concentration was determined using the bicinchoninic acid method. Each protein sample (30 μg) was denatured in SDS sample buffer and separated via 10% SDS-PAGE. Separated proteins were transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA), blocked with 5% bovine serum albumin (BSA; Beyotime Institute of Biotechnology, Haimen, China) for 2 h at room temperature, and incubated overnight with primary antibodies at 4˚C. Blotting was performed with primary antibodies against Yes associated protein 1 (YAP1; 1:300; cat. no. 14074; Cell Signaling Technology, Inc., Danvers, MA, USA). Goat anti-rabbit immunoglobulin horseradish peroxidase-linked F(ab)2 fragments (1:5,000; cat. no. TA130071; OriGene Technologies, Inc., Beijing, China) were used as secondary antibodies for 2 h at room temperature. β-actin (1:4,000; cat. no. ab8226; Abcam, Cambridge, UK) was used as a loading control. Blots were then washed three times (10 min/wash) in TBS-Tween-20 and developed using an enhanced chemiluminescence system (JingCai Technologies, Inc.). ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to analyze the gray values of each blot.

Cell counting kit-8 (CCK-8) assay. A2058 cells transfected with lncRNA-ATB NC or lncRNA-ATB shRNA 24 h previously, and A375 cells transfected with empty vector or lncRNA-ATB overexpressing vector 24 h previously, were seeded in a 96-well culture plate at 2,000 cells/well. Each group was established in nine wells. CCK-8 reagents (cat. no. 40203ES60; Yeasen Biological Technology, Inc., Shanghai, China) were added into each well at 24, 48, 72, 96 and 120 h post-seeding, and each group was cultured for a further 50 min at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. The optical density values was measured at 490 nm using a microplate reader.

Flow cytometry. MM cells in a 6-well culture plate were harvested by trypsinization, and washed three times with PBS. For cellular apoptosis analysis, cells were suspended in 500 μl binding buffer at a density of $2x10^6$ cells/ml, and incubated with Annexin V-fluoroscein isothiocyanate and propidium iodide (PI; BD Biosciences, San Jose, CA, USA) for 15 min in the dark at room temperature. For cell cycle analysis, PI was added into A2058 cells tranfected with lncRNA-ATB NC or lncRNA-ATB, and A375 cells transfected with empty vector or lncRNA-ATB overexpressing vector, and incubated for 20 min in the dark at room temperature. Cellular apoptosis and the cell cycle were analyzed using a flow cytometer and Kaluza analysis software version 2.0 (both Beckman Coulter, Inc., Brea, CA, USA).

Cell migration and invasion. Cell migration and invasion capacity were measured *in vitro* using Transwell migration assays (EMD Millipore). A2058 cells transfected with lncRNA-ATB NC or lncRNA-ATB, and A375 cells transfected with empty vector or lncRNA-ATB overexpressing vector were suspended in DMEM with 10 g/l BSA at a density of $5x10^5$ cells/ml. Cell suspensions (200 μl) were seeded in the

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction analysis.

Gene name	Forward primer (5'→3')	Reverse primer (5'→3')
lncRNA-ATB	CTTCACCAGCACCCAGAGA	AAGACAGAAAAACAGTTCCGAGTC
β-actin	CGTCTTCCCCTCCATCGT	GAAGGTGTGGTGCCAGATTT
miR-590-5p	GGAATTCTTCAGTTGTAACCCAG	CGGGATCCTTGAGATGTCACCAA
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

lncRNA-ATB, long non-coding RNA-activated by transforming growth factor-β; miR, microRNA.

upper chamber with membrane coated with (for the Transwell invasion assay) or without (for the migration assay) Matrigel (BD Biosciences). To attract the cells, 600 μl DMEM with 10% serum was added to the bottom chamber. When the cells had been allowed to migrate for 24 h or to invade for 48 h, the penetrated cells on the filters were fixed in dried methanol for 1 min and stained with 4 g/l crystal violet for 10 min at room temperature. The numbers of migrated or invasive cells were determined from five random fields using a light microscope (Olympus Corporation, Tokyo, Japan) at x10 magnification.

Ethics statement. All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals, 1996, by the National Research Council (US) Institute for Laboratory Animal Research (19). Animal experiments were approved by and carried out according to the guidelines of the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China).

RNA pull-down assay. The full length lncRNA-ATB sequence or NC sequence was amplified by PCR kit (Takara Bio, Inc.) using a T7-containing primer. The following primer sequences were used: lncRNA-ATB forward, 5'-TCCCTGACTCCTCTA TGGCATCTGTGG-3'; lncRNA-ATB reverse, 5'-CCTTTGCT TCCTCTTTTCTCATCTACTC-3'. The thermocycling conditions were as stated above. The DNA fragments were cloned into pcDNA3.1. The restriction enzyme *Xho*I was used to linearize the plasmids. T7 RNA polymerase (Takara Bio, Inc.) and Biotin RNA Labeling Mix (Roche Diagnostics, Indianapolis, IN, USA) was used to make biotin-labeled RNAs that were reverse transcribed. The products were treated with RNase-free DNase I (Roche Diagnostics) and purified with an RNeasy Mini kit (Qiagen, Inc.), and subsequently mixed with RNA extract from A2058 cells and incubated at room temperature for 1 h. The pull-down complexes were analyzed by RT-qPCR as previously stated following subsequent washes.

Tumor xenograft assays. All mice were housed and maintained under specific pathogen-free conditions at 18-22˚C, with 20% humidity, a 12-h light and 12-h dark cycle, with feeding *ad libitum*. All experiments were approved by the Animal Care and Use Committee of Xi'an Jiaotong University and performed in accordance with institutional guidelines. For the tumorigenesis assays, xenograft tumors were generated via subcutaneous injection of A2058 cells ($5x10^6$), including A2058 lncRNA-ATB NC and A2058 lncRNA-ATB shRNA, into the hind limbs of 18-20-g 4-6-week-old Balb/C female athymic nude mice (nu/nu; Animal Center of Xi'an Jiaotong University, Xi'an, China; total number, 10 mice; n=5 mice/group). Tumor size was measured using a vernier caliper. Tumor volume was determined by the following formula: 0.5x Ax B^2, where A represents the diameter of the base of the tumor and B represents the corresponding perpendicular value. After 35 days, the mice were sacrificed and the tumors were collected and weighed. For the lung colonization assay, B16/F10 cells ($5x10^6$), which included lncRNA-ATB NC-luciferase and lncRNA-ATB shRNA-luciferase were intravenously injected into 18-20-g 6-week-old female C57/B6 mice (total number, 10 mice; n=5 mice/group). Lung colonization was measured using Xenogen IVIS Kinetic imaging systems (PerkinElmer, Inc., Waltham, MA, USA) on days 0, 7 and 14 post-injection.

Luciferase assays. The putative binding sites of miR-590-5p for lncRNA-ATB were predicted using RNAhybrid (20) and miRDB (http://www.mirdb.org/). Two hundreds and ninety-three cells were seeded in a 96-well plate at 70% confluence. The lncRNA-ATB was cloned into pMir-Report (Ambion; Thermo Fisher Scientific, Inc.), yielding pMir-Report-lncRNA-ATB. Mutations were introduced in potential miR-590-5p binding sites using the QuikChange site-directed mutagenesis kit (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA). miR-590-5p NC or mimics were transfected into 293 cells with 30 ng wild-type (WT) or mutant (Mut) 3'-untranslated region (UTR) of lncRNA-ATB using Lipofectamine® 2000, as previously stated. Cells were collected 48 h post-transfection, and luciferase assays were performed using a *Photinus pyralis-Renilla reniformis* dual luciferase reporter assay system, according to the manufacturer's protocol (Promega Corporation, Madison, WI, USA). The luciferase activity of each lysate was normalized to *Renilla* luciferase activity.

Statistical analysis. Differentially expressed lncRNAs were identified from the GEO database GSE3189 with false discovery rate (FDR) <0.01 and |logFC| >1 using the R package (21). The raw P-value was corrected using the Benjamini and Hochberg method (22) to circumvent the multi-test bias. A fold-change value >2 or <0.25 and FDR <0.01 were selected as cutoff criteria for differentially expressed lncRNAs. Statistical analysis was performed using SPSS statistical software (version 21.0; IBM Corp., Armonk, NY, USA). The differences in characteristics between two groups were examined by the Student's t-test. The differences in characteristics between three groups were examined by analysis of variance, and the least significant difference test was applied to detect the differences between

each pair of groups. The correlation between miR-590-5p expression and YAP1 expression was analyzed by Pearson correlation analysis. All P-values were determined from two-sided tests, and P<0.05 was considered to indicate a statistically significant difference. All experiments were repeated three times and the data are presented as the mean ± standard deviation from three independent experiments.

Results

lncRNA-ATB is upregulated in MM cell lines. To elucidate the critical lncRNAs involved in the carcinogenesis and progression of MM, comparative lncRNA profiling was performed in 45 MM, 18 benign skin nevus cell (BN), and seven normal skin tissue specimens from the GEO dataset GSE3189 (23). Vectorgram analysis further identified that lncRNA-ATB was upregulated in MM specimens compared with BN (Fig. 1A and Table II). The volcano plot illustrates that the lncRNA-ATB expression level was >4-fold increased between cases and controls (P<0.001; Fig. 1B). The significant upregulation of lncRNA-ATB was further confirmed using RT-qPCR in the human epidermal melanocyte cell line HEMa-LP and in MM cell lines (Fig. 1C).

Effects of lncRNA-ATB on the cell proliferation and apoptosis of MM cells. To investigate the functional role of lncRNA-ATB in MM cells, gain- and loss-of function experiments were performed by transfecting an shRNA, shLncRNA-ATB, into the highly invasive MM cell line A2058 to knock down lncRNA-ATB expression (Fig. 1D; P<0.05). An lncRNA-ATB overexpressing plasmid was also transfected into the moderately invasive MM cell line A375 to stably overexpress lncRNA-ATB. CCK-8 assays indicated that the proliferation of A2058 cells transfected with shLncRNA-ATB was inhibited compared with that in the control (Fig. 2A). However, the proliferation of A375 cells transfected with the lncRNA-ATB overexpressing plasmid was enhanced compared with that in the control (Fig. 2B). Cellular apoptosis assays demonstrated that the percentage of early apoptotic cells increased in A2058 cells transfected with shLncRNA-ATB. Whereas, the percentage of early apoptotic cells decreased in A375 cells transfected with the lncRNA-ATB overexpressing plasmid (Fig. 2C and D).

Effects of lncRNA-ATB on the cell cycle distribution of MM cells. Cell cycle assays (Fig. 3) demonstrated that A2058 cells transfected with shLncRNA-ATB displayed a significant increase in the percentage of cells in the G1 phase and a decrease in the percentage of cells in the S phase (Fig. 3A and C). By contrast, the percentage of cells in the G1 phase decreased and the percentage of cells in the S phase increased in A375 cells transfected with the lncRNA-ATB overexpressing plasmid compared with that in the control (Fig. 3B and D).

Effects of lncRNA-ATB on the cell migration and invasion of MM cells. The present study further investigated the effects of lncRNA-ATB on the cell migration and invasion of MM cells using Transwell assays (Fig. 4). The cell migration assays indicated a significant decrease in the number of migratory cells among A2058 cells transfected with shLncRNA-ATB compared with the control (Fig. 4A and C). By contrast, ectopic expression of lncRNA-ATB significantly increased the number of migratory A375 cells compared with the normal control (Fig. 4A and D). As expected, cell invasion assays also confirmed a decrease in the number of invasive cells among A2058 cells transfected with shLncRNA-ATB compared with the control (Fig. 4B and C). Ectopic expression of lncRNA-ATB significantly enhanced the invasive ability of A375 cells compared with the control (Fig. 4B and D).

Effects of lncRNA-ATB on the tumorigenic ability of MM cells. The effect of lncRNA-ATB on the tumorigenic ability of MM cells was detected *in vivo*. shLncRNA-ATB and luciferase were transfected into A2058 cells and tumorigenesis assays were performed in nude mice. Cells ($5x10^6$) were injected into the flanks of the nude mice. Tumor sizes were measured using the Xenogen IVIS Kinetic imaging system and Vernier calipers every 7 days. After 35 days, the mice

Table II. Top 30 lncRNAs upregulated in malignant melanoma.

lncRNA name	Log_2 fold-change
LncRNA-ATB	4.585437915
Linc00662	3.029954694
AC144652.1	3.057751736
RP11-654D12.2	3.081628575
PVT1	3.139985752
PGM5-AS1	3.151644278
MIR24-2	3.1683935
SNHG19	3.233861413
AC112721.2	3.301811151
CTD-2066L21.3	3.34462291
MIR378D2	3.356163777
NUP50-AS1	3.420937034
SNHG9	3.427925853
AP006621.5	3.441188157
LINC00973	3.515399942
LUCAT	3.567557456
LINC01588	3.582647048
YTHDF3-AS1	3.589774049
BANCR	3.678548044
TUG1	3.78577717
SPRY4-IT1	3.826319081
H19	3.855857029
HOXD-AS1	3.879582756
FALEC	3.881343407
FTH1P3	3.915488817
ILF3-AS1	3.980602573
HOXA11-AS	4.004958111
HEIH	4.061621096
CCAT1	4.155603912
MHENCR	10.18103013

Data from the Gene Expression Omnibus dataset GSE3189. lncRNA, long non-coding RNA.

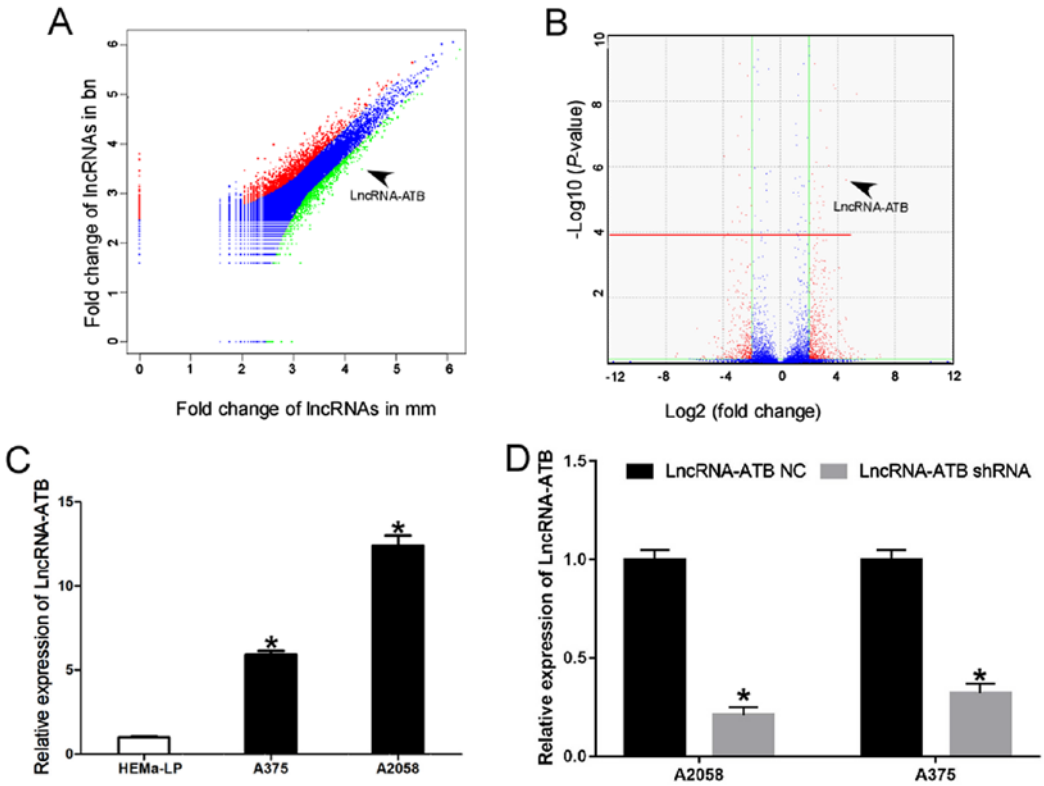

Figure 1. lncRNA-ATB is upregulated in MM cell lines of malignant melanoma. (A) Vectorgram analysis of lncRNAs in MM and BN specimens. The x-axis indicates the Log_2 fold-change in lncRNA expression in MM tissues, and the y-axis indicates the Log_2 fold-change of lncRNA expression in BN tissues. Red, lncRNAs upregulated by ≥2-fold in MM compared with BN. Green, lncRNAs upregulated by ≥2-fold in BN compared with MM. Blue, lncRNAs upregulated by <2-fold in MM compared with BN or lncRNAs upregulated by <2-fold in BN compared with MM. Data from the GEO datasets (GSE3189): Differentially expressed lncRNAs from the GEO dataset GSE3189 with FDR <0.01 and |logFC| >2 were identified using the R package. The raw P-value was corrected using the Benjamini and Hochberg method to circumvent the multi-test bias. A fold-change value >2 or <0.25 and FDR <0.01 were selected as cutoff criteria for differentially expressed lncRNAs. (B) Volcano plot of lncRNAs in MM and BN. The x-axis indicates the Log_2 fold-change in lncRNA expression between MM and BN tissues, while the y-axis indicates the Log_{10} of the adjusted P-value for each lncRNA. Values above the red line were identified to be statistically significant. (P<0.01) following application of the Benjamini and Hochberg method. lncRNA-ATB expression level was >4-fold increased between cases and controls (P<0.001 vs. BN). (C) Relative expression of lncRNA-ATB in human epidermal melanocytes and MM cells. *P<0.05 vs. HEMa-LP cells. (D) Knockdown efficiency of lncRNA-shRNA in MM cells. Data are from three experiments and are presented as the mean ± standard deviation. *P<0.05 vs. respective lncRNA-ATB NC group (Student's t-test). MM, malignant melanoma; BN, benign nevi; lncRNA, long noncoding RNA; ATB, activated by transforming growth factor-β; GEO, Gene Expression Omnibus; FDR, false discovery rate; shRNA, short hairpin RNA; NC, negative control.

were sacrificed, and the tumors were collected and weighed. It was identified that shLncRNA-ATB had a significant tumor growth-inhibitory effect, causing significant reductions in tumor size (Fig. 5A and B) and weight (Fig. 5C) in the shLncRNA-ATB group compared with the control group. B16/F10 melanoma cells transfected with shLncRNA-ATB or controls were injected intravenously into C57/B6 mice, and lung colonization was assessed using the Xenogen IVIS200 System. It was identified that lung metastasis was inhibited in mice injected with B16/F10 cells transfected with shLncRNA-ATB, in comparison with the A2058 cells transfected with a control shRNA (Fig. 5D). Taken together, these results indicated that lncRNA-ATB was upregulated in MM cells, and that knockdown of lncRNA-ATB was able to repress cell proliferation, cell migration, cell invasion and tumor growth *in vitro* and *in vivo*.

lncRNA-ATB directly sponges miR-590-5p to upregulate YAP1 expression. To examine the potential interaction between YAP1 and its targeting miRNA, miR-590-5p was identified as a potential targeting miRNA that has putative binding sites for lncRNA-ATB (Fig. 6A; data from RNA hybrid and miRDB), by searching in online bioinformatics databases. RT-qPCR assays revealed that miR-590-5p mimics and inhibitors were able to effectively upregulate and downregulate miR-590-5p expression, respectively, in MM cells (Fig. 6B; P<0.01). The biotin-labeled pulldown assay indicated that lncRNA-ATB was able to directly pull down miR-590-5p, which suggested that lncRNA-ATB may directly and specifically sponge miR-590-5p (Fig. 6C). Accordingly, miR-590-5p expression was significantly increased in A2058 cells transfected with shLncRNA-ATB, and significantly downregulated in A375 cells transfected with the lncRNA-ATB overexpressing plasmid (Fig. 6D; P<0.05). In addition, a dual-luciferase reporter assay was performed to verify whether lncRNA-ATB was the functional target of miR-590-5p. It was observed that miR-590-5p was able to reduce the relative luciferase activity of WT-LncRNA-ATB. Conversely, co-transfection of 293 cells with Mut-LncRNA-ATB and miR-590-5p resulted in a non-significant alteration in the relative luciferase activity (Fig. 6E). The RT-qPCR results showed that ectopic expression of miR-590-5p in A2058 cells inhibited lncRNA-ATB expression, while lncRNA-ATB expression was upregulated in A375 cells transfected with miR-590-5p inhibitors (Fig. 6F).

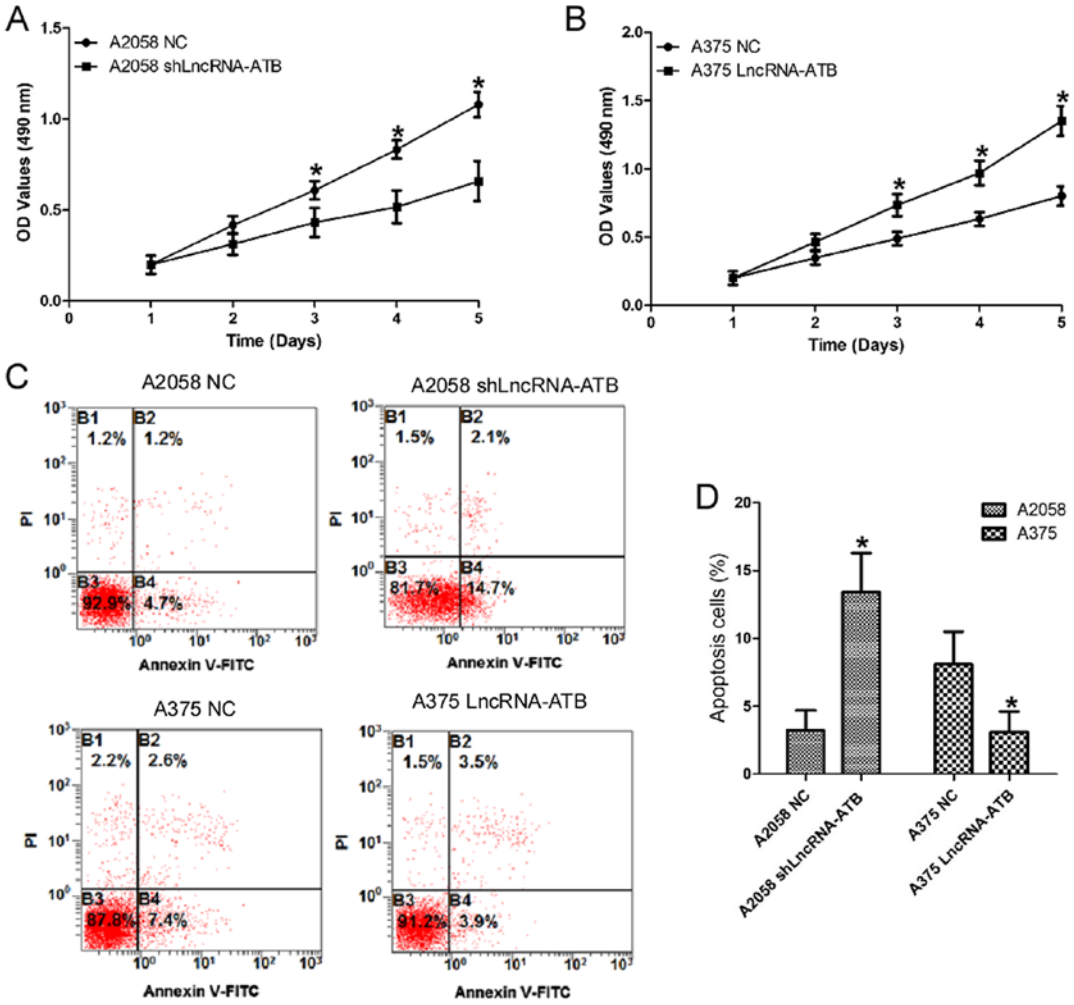

Figure 2. Effects of lncRNA-ATB on the cell proliferation and apoptosis of MM cells. (A) The effects of lncRNA-ATB shRNA on the proliferation of A2058 cells were detected by CCK-8 assays. (B) Effects of lncRNA-ATB on the proliferation of A375 cells were detected by CCK-8 assays. (C) Effects of lncRNA-ATB on the cellular apoptosis of MM cells were detected by flow cytometry using an Annexin V/PI kit, and (D) the results were quantified. Data are from three experiments and are presented as the mean ± standard deviation. *P<0.05 vs. respective NC group (Student's t-test). MM, malignant melanoma; lncRNA, long noncoding RNA; shRNA, short hairpin RNA; NC, negative control; PI, propidium iodide; FITC, fluorescein isothiocyanate; OD, optical density; CCK-8, cell counting kit-8; ATB, activated by transforming growth factor-β.

These results demonstrated that miR-590-5p was also able to directly bind to lncRNA-ATB at specific recognition sites. A previous study reported miR-590-5p may function as a tumor suppressing miRNA in A375 cells (24). In addition, it may inhibit the cell migration and invasion of A375 by directly targeting YAP1. Thus, the present study investigated the effects of lncRNA-ATB on the YAP1 protein expression level. The western blotting results demonstrated that the expression of YAP1 protein was significantly downregulated in A2058 cells transfected with shLncRNA-ATB. By contrast, ectopic expression of lncRNA-ATB in A375 cells resulted in increased YAP1 protein expression levels (Fig. 6G and H).

Discussion

In the present study, it was demonstrated that lncRNA-ATB, which had been reported to be activated by TGF-β (25), was upregulated in A375 cells compared with human melanocytes. In addition, it was observed that lncRNA-ATB was able to promote MM cell proliferation, migration and invasion *in vitro*, and MM tumor growth *in vivo*. Furthermore, it was demonstrated that lncRNA-ATB attenuated cell cycle arrest and inhibited cellular apoptosis in MM cells. Finally, the present study provided the first evidence, to the best of our knowledge, that lncRNA-ATB functions as a ceRNA to enhance YAP1 expression by competitively sponging miR-590-5p in MM cells. Taken together, these results demonstrated that lncRNA-ATB may function as a ceRNA to upregulate YAP1 expression, and promote cell proliferation and invasion, in MM cells by sponging miR-590-5p to reduce the binding of miR-590-5p to YAP1.

lncRNA-ATB has been reported to be upregulated in a number of types of cancer, including papillary thyroid cancer (26), renal cell carcinoma (27) and gastric cancer (28). lncRNA-ATB was identified as a TGF-β-activated lncRNA (25); thus, the function of lncRNA-ATB may be consistent with TGF-β pathways in different tumors, although certain lncRNAs, including HOX transcript antisense RNA, serve entirely opposite function in different types of cancer cells (29,30). It is possible that lncRNA-ATB is an example of this type of non-coding RNA. However, according to previous studies (25,31), it was noted that lncRNA-ATB serves an

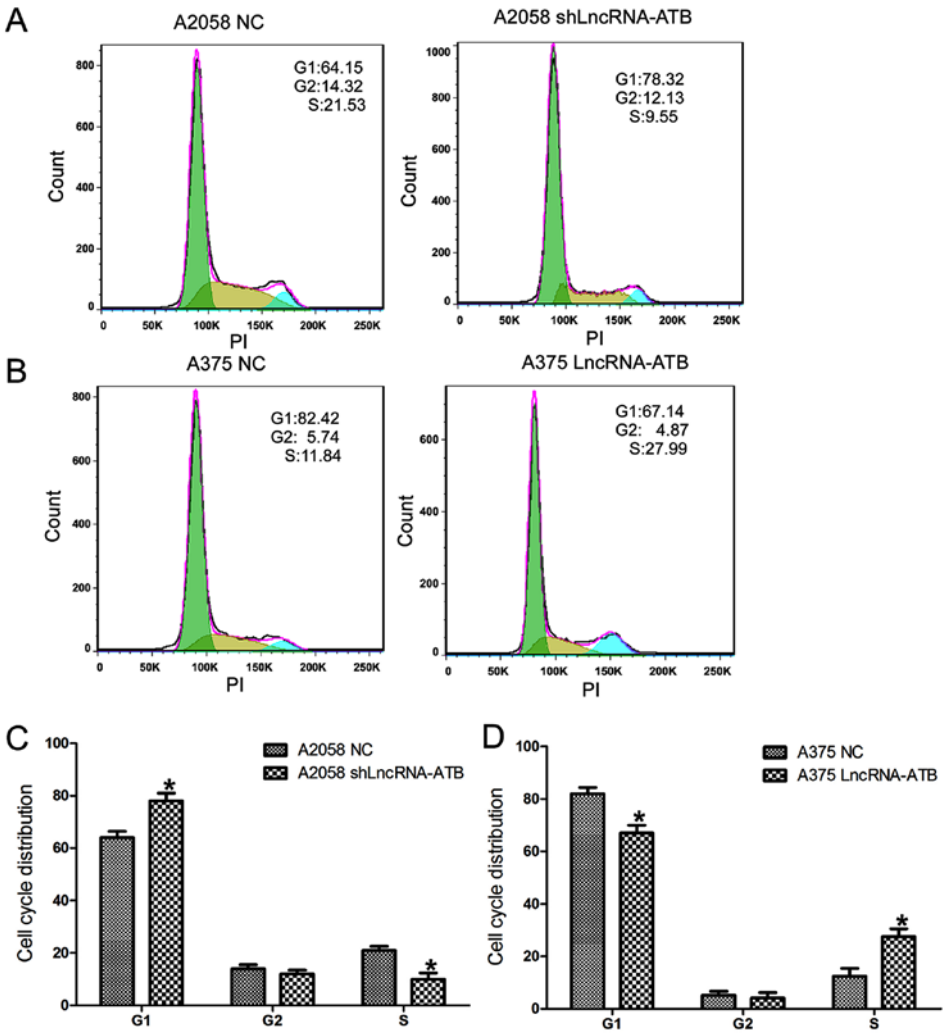

Figure 3. Effects of lncRNA-ATB on the cell cycle distribution of malignant melanoma cells. (A) Effects of lncRNA-ATB shRNA on the cell cycle distribution of A2058 cells were detected by flow cytometry using PI. (B) Effects of lncRNA-ATB on the cell cycle distribution of A375 cells were detected by flow cytometry using PI. (C) Quantification of the results in A2058 cells. (D) Quantification of the results in A375 cells. Data are from three experiments and presented as mean ± SD. *P<0.05 vs. respective NC group (Student's t-test). PI, propidium iodide; lncRNA, long noncoding RNA; shRNA, short hairpin RNA; ATB, activated by transforming growth factor-β; NC, negative control.

oncogenic role in the majority of types of cancers. In line with these previous studies, the present study identified that lncRNA-ATB was significantly upregulated in MM cell lines compared with normal human epidermal melanocyte cell lines. In non-small cell lung cancer, lncRNA-ATB promotes cellular apoptosis, and inhibits cell viability, cell migration and invasion (31). Furthermore, lncRNA-ATB was identified as a potential oncogene in osteosarcoma (32), glioma (33), prostate cancer (34) and colon cancer (35); the upregulation of lncRNA-ATB was able to promote carcinogenesis and progression in these cancer types. Notably, through searching electronic databases, a meta-analysis reported that lncRNA-ATB was associated with adverse clinical features, overall survival, progression-free survival and prognosis in human cancer (36). Studies have also confirmed that increased lncRNA-ATB expression is an independent prognostic predictor in a number of types of cancer, including non-small cell lung cancer (37), gastric cancer (38) and colorectal cancer (39).

To further determine the biological role of lncRNAs in MM, gain or loss-of- function experiments were performed. The results revealed that lncRNA-ATB promoted cell proliferation, migration and invasion, and attenuated cell cycle arrest *in vitro*. It was additionally demonstrated that knockdown of lncRNA-ATB was able to inhibit tumor growth *in vivo*. In addition to lncRNA-ATB, a number of other lncRNAs have been demonstrated to be potential oncogenes or tumor suppressors in MM, including homeobox A11-antisense (40), hepatocellular carcinoma upregulated EZH2-associated lncRNA (41) and colon cancer associated transcript 1 (42). In agreement with these studies, lncRNA-ATB was identified in the present study to be a potential oncogene in MM, and the critical roles of lncRNAs in the carcinogenesis and progression of MM were further confirmed. As it is difficult to perform gain-or-loss of function experiments using normal epidermal melanocytes, the present study did not investigate the role of lncRNA-ATB in HEMa-LP cells. Although lncRNA-ATB serves an important role in carcinogenesis, it was considered likely that the proliferation of HEMa-LP may not exhibit a marked alteration, due to the number of tumor suppressor genes in HEMa-LP cells to suppress the function of lncRNA-ATB. It was also observed that the basal

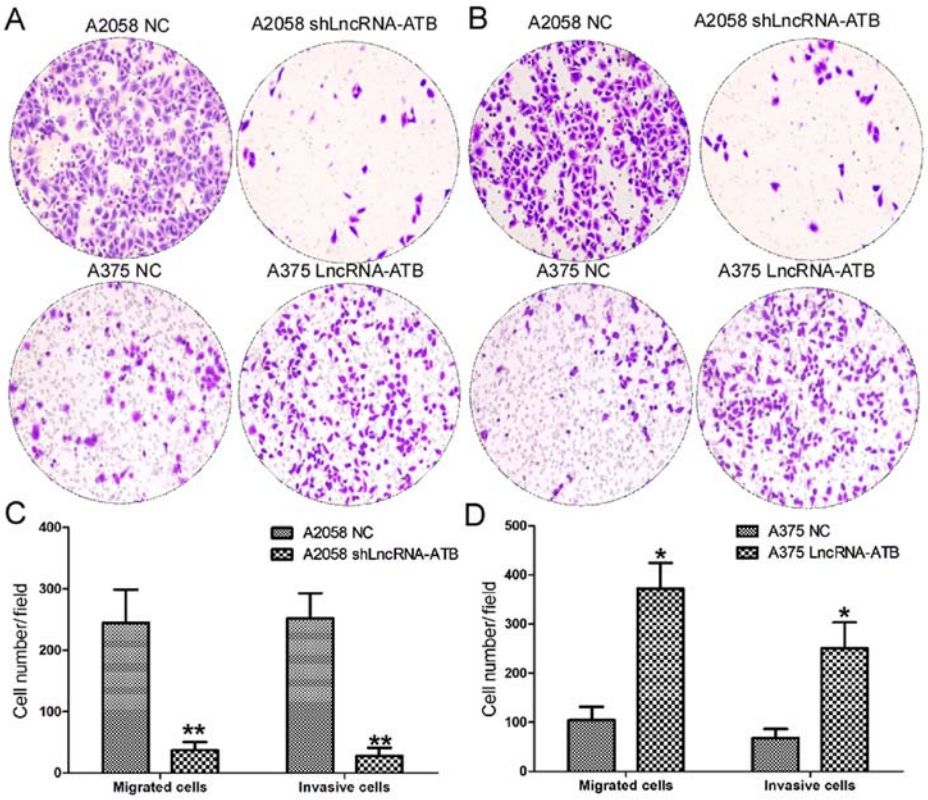

Figure 4. Effects of lncRNA-ATB on the cell migration and invasion of MM cells. (A) Effects of lncRNA-ATB shRNA on the cell migration of A2058 and A375 cells were detected by Transwell assays (magnification, x200). (B) Effects of lncRNA-ATB shRNA on the cell invasion of A2058 and A375 cells were detected by Transwell assays (magnification, x200). (C) Analysis of the migration and invasion data from A2058 cells. (D) Analysis of the migration and invasion data from A375 cells. Data are from three experiments and are presented as the mean ± standard deviation. *P<0.05 and **P<0.01 vs. respective NC group (Student's t-test). NC, negative control; shRNA, short hairpin RNA; lncRNA, long noncoding RNA; ATB, activated by transforming growth factor-β.

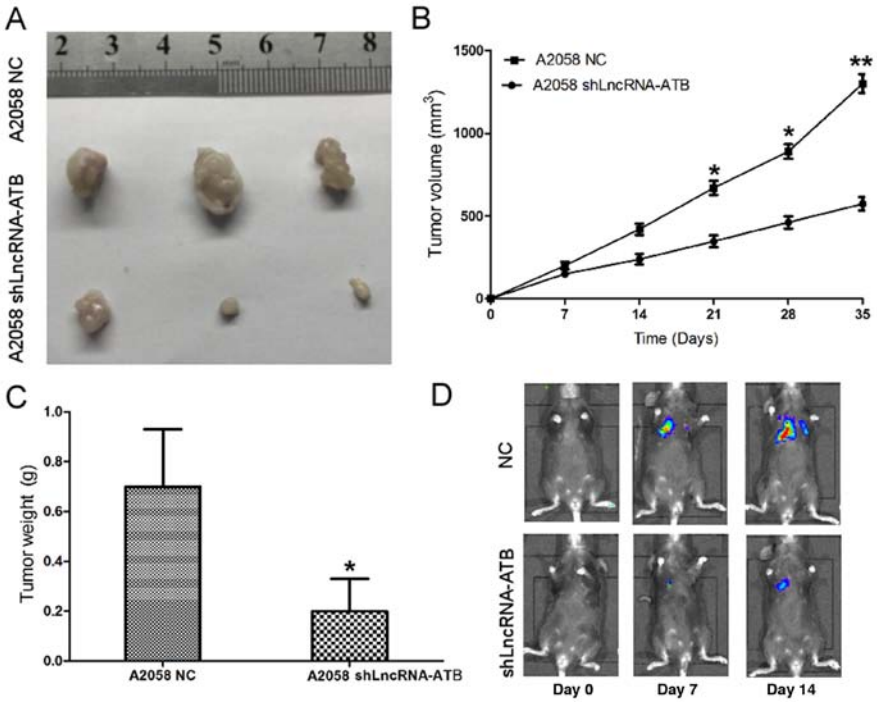

Figure 5. Effects of lncRNA-ATB on the tumorigenic ability and lung colonization of MM cells. (A) Tumors collected from the lncRNA-ATB shRNA-luciferase groups and A2058 lncRNA-ATB NC-luciferase groups. (B) Tumor growth curve of A2058 lncRNA-ATB shRNA-luciferase cells and A2058 lncRNA-ATB NC-luciferase cells in nude mice. (C) Tumor weights of lncRNA-ATB shRNA-luciferase and A2058 lncRNA-ATB NC-luciferase groups at day 35. (D) Effects of lncRNA-ATB shRNA on lung metastasis were measured using a Xenogene IVIS Kinetic imaging system every 7 days subsequent to injecting B16/F10 lncRNA-ATB shRNA-luciferase cells and B16/F10 lncRNA-ATB NC-luciferase cells intravenously into C57/B6 mice. Data are from three experiments and are presented as the mean ± standard deviation. *P<0.05 vs. respective NC group (Student's t-test). NC, negative control; shRNA, short hairpin RNA; lncRNA, long noncoding RNA; ATB, activated by transforming growth factor-β.

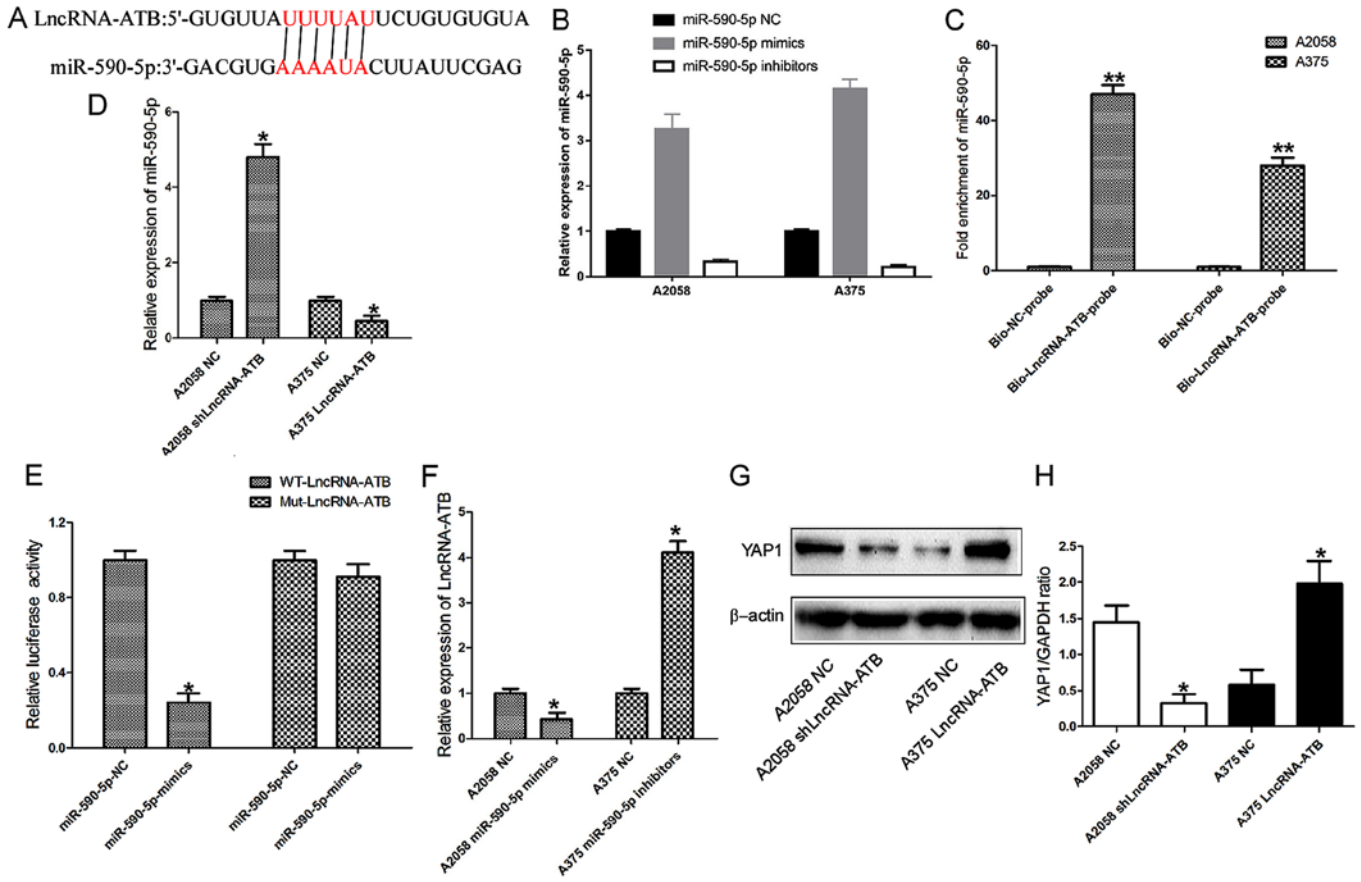

Figure 6. lncRNA-ATB sponges miR-590-5p to promote YAP1 expression in MM cells. (A) lncRNA-ATB and its putative binding sequence in miR-50-5p. (B) Efficacy of miR-590-5p mimics or inhibitors in MM cells. (C) Relative expression of miR-590-5p in the same sample pulled down by biotinylated lncRNA-ATB and NC probe. (D) Relative expression of miR-590-5p in MM cells transfected with lncRNA-ATB shRNA and lncRNA-ATB overexpressing plasmid. (E) Relative luciferase activity in 293 cells transfected with WT or Mut lncRNA-ATB plasmid. (F) Relative expression of lncRNA-ATB in MM cells transfected with miR-590-5p mimics or inhibitors. (G) Immunoblotting analysis and (H) relative quantification of YAP1 expression levels in MM cells transfected with lncRNA-ATB shRNA or overexpressing plasmid. Data are from three experiments and are presented as the mean ± standard deviation. *P<0.05 and **P<0.01 vs. respective NC group (Student's t-test). MM, malignant melanoma; NC, negative control; shRNA, short hairpin RNA; lncRNA, long noncoding RNA; ATB, activated by transforming growth factor-β; WT, wild-type; Mut, mutant; YAP1, Yes associated protein 1; miR, microRNA; Bio, biotinylated.

measurements of A2058 cells and A375 cells with respect to the cell cycle, cell migration and cell invasion were different. There may be a number of reasons to account for this discrepancy. Firstly, the source of A2058 and A375 is different. Compared with A375, A2058 is a more invasive cell line. Thus, the number of migrated and invasive A2058 cells transfected with lncRNA-ATB NC was greater compared with the A375 cells transfected with lncRNA-ATB NC. Secondly, the different levels of lncRNA-ATB may be one of the factors accounting for the increased invasive ability of A2058 cells.

Recently, studies identified lncRNAs that were able to act as ceRNAs to interfere with miRNAs and their downstream pathways, which affect post-transcriptional regulation (43,44). ceRNA regulatory networks are implicated in numerous biological processes in cancer, including tumorigenesis (45), epithelial-mesenchymal transition (46) and the invasion-metastasis cascade (47). For example, lncRNA metastasis associated lung adenocarcinoma transcript 1 is overexpressed in MM and upregulates the expression levels of matrix metallopeptidase 14 and snail family transcriptional repressor 1 by competitively sponging miR-22, thus promoting tumor growth and metastasis (48). Similarly, the lncRNA melanoma highly expressed competing endogenous lncRNA for miR-425 and miR-489 also functions as a ceRNA to upregulate insulin like growth factor 1 and spindling 1 expression by sponging miR-425 and miR-489, thereby promoting tumor growth and metastasis in MM (49). lncRNA-ATB was also demonstrated to be a ceRNA in hepatocellular carcinoma, upregulating zinc finger E-box binding homeobox 1 and 2 expression, and promoting the invasion-metastasis cascade by competitively binding miR-200 (50). The present study further increased understanding of the ceRNA function of lncRNA-ATB. The potential miR-590-5p binding sites were identified in lncRNA-ATB transcripts, and it was demonstrated that lncRNA-ATB expression was negatively regulated by miR-590-5p. Notably, it was confirmed that lncRNA-ATB may function as a ceRNA by sponging miR-590-5p to upregulate YAP1 expression in MM cells.

In conclusion, the present study demonstrated that lncRNA-ATB is a potential oncogene in MM. lncRNA-ATB was able to promote the tumor growth and metastasis of MM cells *in vivo* and *in vitro* by sponging miR-590-5p, functionally releasing YAP1 mRNA transcripts that are normally targeted by miR-590-5p. The present study helped to reveal the regulatory mechanism of lncRNA-ATB in MM and may lead to novel therapeutic strategies for MM.

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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

LW conceived the experiments and drafted the manuscript. KM and BL conducted the experiments and helped to analyze the data. MD performed data analysis. XM, YZ and DH performed data analysis and revised the manuscript. All authors contributed to revise the manuscript and approved the final version for publication.

Ethics approval and consent to participate

Animal experiments were approved by and carried out according to the guidelines of the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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