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ORIGINAL RESEARCH

Long Noncoding RNA *LINC00173* Promotes the Malignancy of Melanoma by Promoting the Expression of IRS4 Through Competitive Binding to microRNA-493

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Fan Yang[1]
Pengzhen Lei[2]
Weihui Zeng[3]
Jianwu Gao[1]
Na Wu[1]

[1]Department of Dermatology, Shaanxi Provincial People's Hospital, Xi'an, Shaanxi 710068, People's Republic of China; [2]Department of Orthopedics, Shaanxi Provincial People's Hospital, Xi'an, Shaanxi 710068, People's Republic of China; [3]Department of Dermatology, The Second Affiliated Hospital, School of Medicine, Xi'an Jiaotong University, Xi'an, Shaanxi 710048, People's Republic of China

Purpose: Long intergenic non-protein-coding RNA 173 (*LINC00173*) plays crucial roles in lung cancer. However, the expression and biological functions of *LINC00173* in melanoma have not yet been investigated. In this study, we aimed to characterize the involvement of *LINC00173* in melanoma and elucidate its mechanisms of action.

Materials and Methods: Reverse-transcription quantitative PCR was performed to measure *LINC00173* expression in melanoma. A CCK-8 assay, flow cytometry, and migration and invasion assays were applied to examine melanoma cell proliferation, apoptosis, migration, and invasion, respectively. A xenograft tumor experiment was performed to determine the tumorous growth of melanoma cells in vivo.

Results: We found that *LINC00173* was upregulated in melanoma tissues and cell lines. High *LINC00173* expression was closely associated with TNM stage, lymph node metastasis, and shorter overall survival of patients with melanoma. Functional assays revealed that *LINC00173* upregulation inhibited melanoma cell proliferation, migration, and invasion and induced apoptosis, suggesting that *LINC00173* acts as an oncogenic RNA. *LINC00173* knockdown retarded the tumorous growth of melanoma cells in vivo. Mechanistically, *LINC00173* increased insulin receptor substrate 4 (IRS4) expression by sponging microRNA-493 (miR-493), thereby acting as a competing endogenous RNA. The effects of *LINC00173* knockdown on the malignant phenotype of melanoma cells were reversed by overexpression of IRS4 or knockdown of miR-493.

Conclusion: The *LINC00173*–miR-493–IRS4 pathway regulates melanoma characteristics by increasing the expression of IRS4 via competitive binding of *LINC00173* to miR-493, suggesting that this pathway is a potential target for the diagnosis, prognosis, and/or treatment of melanoma.

Keywords: *LINC00173*, miR-493, melanoma, therapeutic target

Introduction

Melanoma, resulting from malignant transformation of melanocytes located at the basement of the epidermis, is the most frequent and aggressive type of skin cancer.[1,2] The global morbidity rate of melanoma has been increasing gradually in recent years.[3] It is estimated that there would be over 150,000 novel cases and 50,000 deaths due to melanoma yearly worldwide.[4,5] Even though some sophisticated therapeutic regimens have evolved from surgery, chemotherapy, targeted therapy, and immunotherapy,[6–10]

Correspondence: Fan Yang
Department of Dermatology, Shaanxi Provincial People's Hospital, No. 256 Youyi West Road, Xi'an, Shaanxi 710068, People's Republic of China
Email fanfanniu1985@126.com

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the prognosis of patients with melanoma has not improved significantly.[11] The 5-year survival rate of these patients is lower than 15%, which is a consequence of the high metastatic potential and uncontrollable growth.[12] Melanoma cases represent only 4% of patients with skin tumors but are responsible for ~74% of skin tumor–related deaths.[13] UV damage has been validated as a major risk factor of melanoma; genetic and epigenetic changes also exert important actions during melanoma initiation and progression.[14] Nonetheless, the exact events underlying the pathogenesis of melanoma remain largely unknown. Therefore, comprehensive elucidation of melanoma pathogenesis is urgently needed to identify novel and promising therapeutic techniques.

Long noncoding RNAs (lncRNAs) are a group of endogenous RNA transcripts (with a length of >200 bp) that cannot be translated into proteins.[15] LncRNAs are implicated in nearly all physiological and pathological activities, especially in carcinogenesis and cancer progression.[16] The actions of lncRNAs are mediated by diverse molecular mechanisms, such as transcriptional modulation, chromatin remodeling, histone modification, regulation of mRNA splicing, and the competing endogenous RNA (ceRNA) mechanism.[17,18] Existing studies have identified a close relation between lncRNAs and human cancers.[19–21] Much evidence suggests that numerous lncRNAs are differentially expressed in melanoma, and their dysregulation is involved in melanoma progression because lncRNAs can act as oncogenic factors or tumor suppressors.[22–24] Accordingly, in-depth exploration of lncRNAs' functions in the formation and progression of melanoma is essential for identifying new targets for the diagnosis and management of this fatal disease.

MicroRNAs (miRNAs), a family of single-stranded, highly conserved and noncoding RNAs, are identified as gene regulators by directly binding to the 3′-untranslated region (3′-UTR) of their target mRNAs, and thereby resulting in mRNA degradation and/or translation suppression.[25,26] Interestingly, accumulating studies demonstrated the enrollment of miRNAs in the oncogenesis and progression of melanoma.[27–29] MiRNAs may perform tumor-suppressing or tumor-promoting activities in melanoma, and are implicated in the regulation of multiple aggressive processes.[30,31] Hence, studying the expression and roles of miRNAs in melanoma my highlight potential targets for managing melanoma.

LINC00173 is a crucial modulator of the malignancy of lung cancer.[32,33] Nevertheless, the expression and biological roles of *LINC00173* in melanoma have not yet been explored. Here, we attempted to analyze the expression of *LINC00173* in melanoma and its clinical significance. Effects of *LINC00173* on the malignancy characteristics of melanoma cells in vitro and in vivo were tested. The next step was investigation of the mechanism of *LINC00173*-mediated promotion of melanoma progression.

Materials and Methods

Clinical Tissue Sample Collection

This study was performed with the approval of the Research Ethics Committee of Shaanxi Provincial People's Hospital. In addition, written informed consent forms were signed by all the patients who participated in this research. Melanoma tissue samples and adjacent normal tissues were obtained from 45 patients with melanoma undergoing a surgical procedure at the Shaanxi Provincial People's Hospital. Patients who had received chemotherapy, radiotherapy, targeted therapy, or immunotherapy were excluded from this study. All tissue samples were immediately frozen and stored in liquid nitrogen.

Cell Culture

Human epidermal melanocytes (HEMs) were bought from ScienCell Research Laboratories, Inc. (San Diego, CA, USA) and grown in a melanocyte medium (ScienCell Research Laboratories, Inc.). Four human melanoma cell lines, ie, A375, A2058, SKMEL1, and HT144, were purchased from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific), 100 U/mL penicillin, and 100 µg/mL streptomycin was utilized for culturing melanoma cells. All cell lines were maintained at 37°C in a humidified atmosphere supplied with 5% CO_2.

Cell Transfection

An miR-493 mimic, negative control (NC) miRNA mimic (miR-NC), miR-493 inhibitor, and NC inhibitor were acquired from GenePharma Technology (Shanghai, China). Small interfering RNAs (siRNA) specific to *LINC00173* (si-LINC00173) and NC siRNA (si-NC) were synthesized by RiboBio (Guangzhou, China). IRS4-overexpressing plasmid pcDNA3.1-IRS4 was bought from Sangon Biotech (Shanghai, China). Cells were grown up to 60% confluence and transfected with the miRNA mimic (100 pmol), miRNA inhibitor (100 pmol), siRNA (100 pmol) or plasmid (4 µg) using Lipofectamine 2000® (Invitrogen; Thermo Fisher Scientific).

Reverse-Transcription Quantitative PCR (RT-qPCR)

The TRIzol reagent (Invitrogen; Thermo Fisher Scientific) was applied for total-RNA isolation. An absorbance ratio (A_{260}/A_{280}), which was determined using Nanodrop 2000 (Invitrogen; Thermo Fisher Scientific) was used to analyze the quality of the isolated total RNA. To quantitate the expression of miR-493, first-strand cDNAs were produced from the total RNA using the miScript Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany). qPCR was performed using an miScript SYBR Green PCR Kit (Qiagen GmbH). The expression of miR-493 was normalized to that of *U6* small nuclear RNA. To quantify *IRS4* mRNA and *LINC00173* expression, the total RNA was reverse-transcribed into cDNA with a PrimeScript RT Reagent Kit (Takara Bio, Dalian, China). The synthesized cDNA was analyzed by qPCR with the SYBR Premix Ex Taq™ Kit (Takara Bio). *GAPDH* was regarded as an endogenous control for *IRS4* mRNA and *LINC00173* normalization. All reactions were performed on a 7500 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific). All data were analyzed by the $2^{-\Delta\Delta Cq}$ method.

The primers were designed as follows: miR-4[illegible]; 5′-TGTGATTGGAATGGAAATTTAATTT-3′ (forward) and 5′-ACTATCCTACACTCCCCTACCCTAC-3′ (reverse); [illegible] 5′-CTCGCTTCGGCAGCACA-3′ (forward) and [illegible] AAC[illegible] CTTCACGAATTTGCGT-3′ (reverse); LINC00[illegible] 5′-GC[illegible] AATGTTGCGATCCTCTGG-3′ (forward) and 5′-CAGC CATGTCTCAGAGGTGA-3′ (reverse); IRS4, [illegible] CCGACA CCTCATTGCTCTTTTC-3′ (forward) and 5′-TTTCCTGC TCCGACTCGTTCTC[illegible] (reverse); and GAPDH, 5′-CAGC CTCAAGATCATC[illegible]GCA-3′ (forward) and 5′- TGTGGT CATGAGTCCTTC[illegible]A-3′ (reverse).

Cell Counting Kit-8 (CCK-8) Assay

Suspensions of transfected cells were diluted to a certain concentration and then seeded in 96-well plates at an initial density of 3000 cells/well. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for four periods after inoculation: 0, 24, 48, or 72 h. A total of 10 μL of the CCK-8 solution (Beyotime Institute of Biotechnology, Shanghai, China) was added into each well at each time point. Subsequent to additional 2 h incubation, the absorbance value of every well at a wavelength of 450 nm was measured on a microplate reader.

Apoptosis and Cell Cycle Assessment via Flow-Cytometric Analysis

Apoptotic cells were quantified using an Annexin V-Fluorescein Isothiocyanate (FITC) Apoptosis Detection Kit (BioLegend, San Diego, CA, USA). After 48 h culture, transfected cells were collected via treatment with trypsin without EDTA, and precooled phosphate-buffered saline was utilized to wash the transfected cells thrice. The transfected cells were centrifuged and resuspended in 100 μL of flow cytometry binding buffer, after which the cells were labeled with 5 μL of Annexin-V-FITC and [illegible] propidium iodide (PI) solution at room temperature in the dark for 15 min. The apoptotic cells were quantified on a flow cytometer (FACScan™, BD Biosciences, Franklin Lakes, NJ, USA).

Transfected cells were fixed in 70% ethanol at 4°C overnight, followed by centrifugation at 4°C for 5 min. The supernate was discarded and the transfected cells were probed with 5 μL RNase ([illegible] μg/mL) at 37°C for 20 min. Following incubation at room temperature with 25 μL PI (BioLegend) diluted in 500 μL cell-staining buffer.Finally, flow cytometry was utilized to analyze the cell cycle status.

Migration and Invasion Assays

The transfected cells were resuspended in FBS-free DMEM. For the invasion assay, 200 μL of a cell suspension containing 5×10^4 transfected cells was seeded on the upper insert of a 24-well Transwell plate (8 μm pore size; Corning Inc., Corning, NY, USA) that was precoated with Matrigel (BD Biosciences). The basolateral inserts were covered with 600 μL DMEM that was supplemented with 10% FBS functioning as a chemoattractant. After 24 h incubation, noninvasive cells (remaining on the top layer of the membrane) were gently wiped away with a cotton swab, and the invasive cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. After extensive washing and air drying, the invasive cells were counted under an inverted microscope (Olympus Corporation, Tokyo, Japan) at 200× magnification to evaluate the invasiveness of cancer cell lines. A migration assay was performed following the same experimental steps as in the invasion assay except that the membranes were not coated with Matrigel.

Xenograft Tumor Experiment and Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

Short hairpin RNA (shRNA) specific to *LINC00173* (sh-LINC00173) and NC shRNA (sh-NC) was acquired from

GenePharma Technology and subsequently inserted into the lentiviral pLKO vector, thus yielding lentiviruses pLKO-sh-*LINC00173* and pLKO-sh-NC, respectively. To stably silence *LINC00173* in A375 cells, either the pLKO-sh-LINC00173 or pLKO-sh-NC lentivirus was transduced into A375 cells. A375 cells stably expressing sh-LINC00173 were selected with puromycin.

All animal experiments were performed with the approval of the animal ethics committee of Shaanxi Provincial People's Hospital and in conformity with the Animal Protection Law of the People's Republic of China-2009 for experimental animals. Male, BALB/c nude mice at 4 weeks of age were bought from Huafukang Bioscience Co., Inc. (Beijing, China). A375 cells stably expressing either sh-LINC00173 or sh-NC were collected, resuspended in phosphate-buffered saline, and subcutaneously injected into the flank of the mice. Each group contained three mice. Starting at 7 days postinjection, the length (L) and width (W) of the tumor xenografts were measured every 2 days; the tumor volume was computed as $L \times W^2 \times 0.5$. All mice were euthanized through cervical dislocation at 4 weeks after the tumor xenografting. Tumor xenografts were collected, weighed, and analyzed with RT-qPCR and Western blotting.

After fixation in 4% formalin, the tumor xenografts were embedded in paraffin and subjected to A[illegible] in situ terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit (Roche Applied [illegible]ence) for the detection of cell apoptosis. The numb[illegible] of apoptotic cells was counted in both sh-LINC0017[illegible]nd sh-NC groups.

Nuclear-and-Cytoplasmic Separation Assay

RNA located in the cytoplasm or nucleus of melanoma cells was separated using a Cytoplasmic and Nuclear RNA Purification Kit (Norgen, Thorold, ON, Canada). Cytoplasmic RNA and nuclear RNA samples were analyzed by RT-qPCR to determine *LINC00173* expression distribution within the melanoma cell.

Bioinformatic Analysis and the Luciferase Reporter Assay

LINC00173–miRNA interactions were predicted using starBase 3.0 (http://starbase.sysu.edu.cn/). Fragments of *LINC00173* carrying either the putative wild-type (WT) or mutant (MUT) miR-493–binding sequence were generated by GenePharma Technology, followed by insertion into the psi-CHECK2 luciferase reporter vector (Promega Corporation, Madison, WI, USA). The luciferase reporter plasmids were named as LINC00173-WT and LINC00173-MUT, respectively. Lipofectamine 2000® was employed to transfect melanoma cells with either the miR-493 mimic or miR-NC plus either LINC00173-WT or LINC00173-MUT. The cells were harvested at 48 h post-transfection and subjected to the measurement of luciferase activity via a Dual Luciferase Reporter Assay System (Promega Corporation). The activity of firefly luciferase was normalized to that of *Renilla* luci[illegible]

RNA Immunoprecipitation (RIP) Assay

This assay was performed for testing the interaction between *LINC00173* and miR-[illegible]3 in melanoma cells. We used the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Bur[illegible]ton, MA, USA). In short, cells were lysed in RIP lysis buffer. A[illegible] anti-Argonaute 2 (AGO2) antibody or control IgG (both from Millipore) was conjugated to magnetic beads and was incubated with the cell extract. After overnight incubation at 4°C, the magnetic beads were collected, washed, and digested with Proteinase K. The immunoprecipi[illegible]RNA was extracted and analyzed by RT-qPCR to assess the enrichment of *LINC00173* and miR-493 in the AGO2-containing beads.

Western Blotting

Total protein was extracted using radioimmunoprecipitation assay buffer (Beyotime, Shanghai, China). A BCA Protein Assay Kit (Beyotime) was employed to quantity the total-protein samples. Equivalent amounts of protein were separated by SDS 10% polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. After 2 h blocking with 5% fat-free milk, primary antibodies were incubated with the membranes overnight at 4°C. The membranes were probed with a horseradish peroxidase–conjugated antibody (ab6721; 1:5,000 dilution; Abcam) at room temperature for 2 h. The protein signals were developed using the ECL Detection Kit (GE Healthcare Life Sciences, Chalfont, UK). The primary antibodies included IRS4 (cat. # ab52622; 1:500; Abcam, Cambridge, MA, USA), Bcl-2 (cat. # ab182858; 1:500; Abcam), BAX (cat. # ab32503; 1:500; Abcam), Bcl-XL (cat. # ab178844; 1:500; Abcam), CDK-2 (cat. # ab32147; 1:500; Abcam), CDK-4 (cat. # ab199728; 1:500; Abcam), cyclin D1 (cat. # ab16663; 1:500; Abcam), and GAPDH (ab128915; 1:500; Abcam).

Statistical Analysis

All experiments were performed at least in triplicate, and repeated three times. All measurement data are presented as mean ± standard deviations. Comparisons between two groups were evaluated with Student's *t* test. One-way analysis of variance (ANOVA) together with Tukey's *post hoc* test was performed to compare the data among multiple groups. The chi-square test was performed to determine the association between *LINC00173* expression and clinical features of the patients with melanoma. Overall-survival curves were constructed by the Kaplan–Meier method, and the logrank test was performed to analyze the differences. Spearman correlation analysis was performed on some parameters. All statistical analyses were performed using SPSS 20.0 software (IBM Corp., Armonk, NY, USA), and P less than 0.05 was considered indicative of a statistically significant difference.

Results

LINC00173 Is Upregulated in Melanoma Tissues and Cell Lines

To analyze the expression status of *LINC00173* in melanoma, RT-qPCR analysis was performed to measure the amounts of *LINC00173* in the 45 pairs of melanoma tissue samples and adjacent normal tissues. *LINC00173* was overexpressed in the melanoma tissues compared to the adjacent normal tissues (Figure 1A). *LINC00173* expression in HEMs and four human melanoma cell lines (A375, A2058, SKMEL1, and HT144) was determined by RT-qPCR. *LINC00173* level was higher in the four tested melanoma cell lines than in HEMs (Figure 1B).

Evaluation of the relation between *LINC00173* expression and clinical features indicated that higher *LINC00173* expression was significantly associated with TNM stage (P = 0.007) and lymph node metastasis (P = 0.002) in the 45 patients with melanoma (Table 1). Patients with melanoma featuring high *LINC00173* expression showed shorter overall survival than did the patients with low *LINC00173* expression (Figure 1C; P = 0.026). Therefore, *LINC00173* was upregulated in melanoma, and this upregulation negatively correlated with the patients' overall survival, implying that *LINC00173* may perform crucial functions in melanoma progression.

Downregulation of LINC00173 Restrains Melanoma Cell Proliferation, Migration, and Invasion and Induces Apoptosis in vitro

Given that *LINC00173* was more highly expressed in melanoma cell lines A375 and HT144 compared to the other two melanoma cell lines, these two cell lines were chosen for further experiments. To investigate whether dysregulation of *LINC00173* is functionally implicated in melanoma tumorigenesis, knockdown experiments were performed on A375 and HT144 cells using the siRNA specific to *LINC00173* (si-LINC00173). RT-qPCR analysis confirmed good knockdown efficiency of si-LINC00173. Transfection with si-LINC00173 resulted in a marked decrease of

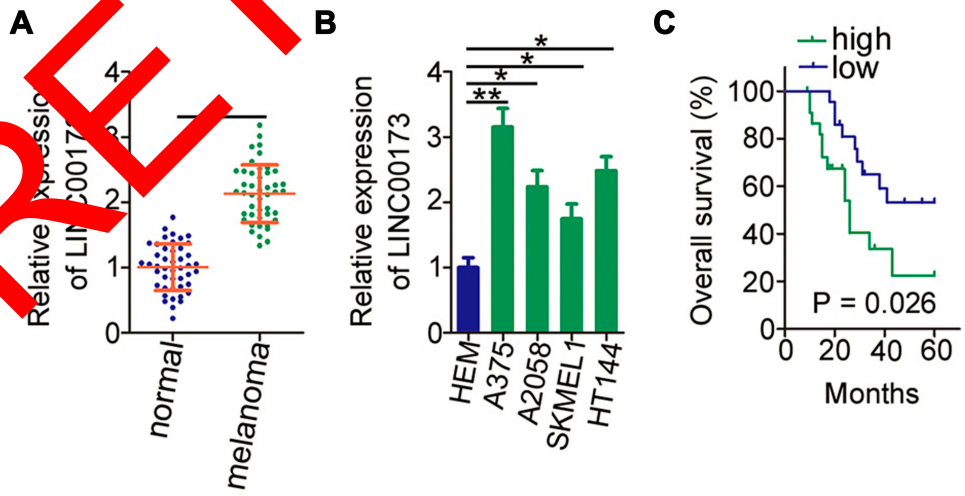

Figure 1 *LINC00173* is upregulated in melanoma and negatively correlates with patients' clinical outcomes. (**A**) Relative expression of *LINC00173* in 45 pairs of melanoma tissues and adjacent normal tissues was determined by RT-qPCR. (**B**) RT-qPCR analysis was performed to assess *LINC00173* expression in human epidermal melanocytes (HEMs) and four human melanoma cell lines (A375, A2058, SKMEL1, and HT144). (**C**) Kaplan–Meier analysis uncovered a correlation between *LINC00173* expression and overall survival of the 45 patients with melanoma (P = 0.026). *P < 0.05 and **P < 0.01.

Table 1 Correlation Between *LINC00173* Expression and Clinical Characteristics of Patients with Melanoma (n = 45 Patients)

Clinicopathological Characteristic	*LINC00173* Expression (Number of Patients)		P value
	High	Low	
Age			0.550
<50 years	8	10	
≥50 years	15	12	
Gender			0.542
Males	13	15	
Females	10	7	
Family history of cancer			0.284
Yes	7	3	
No	16	19	
TNM stage			0.007
I–II	6	15	
III	17	7	
Lymph node metastasis			0.002
Absent	8	18	
Present	15	4	

LINC00173 expression in both A375 and HT144 cells (Figure 2A). *LINC00173* knockdown obviously promoted the proliferation of A375 and HT144 cells (Figure 2B), as determined by the CCK-8 assay. The effect of *LINC00173* silencing on melanoma cell apoptosis and cell cycle was tested by flow cytometry. *LINC00173* depletion notably promoted the apoptosis (Figure 2C) and induced the G0/G1 cycle arrest (Figure 2D) of A375 and HT144 cells.

In addition, expression of apoptotic-associated proteins (Bcl-2, BAX and Bcl-XL) and cell cycle-associated proteins (CDK-2, CDK-4 and cyclin D1) in the LINC00173-deficiency A375 and HT144 cells was measured via Western blotting. The results (Figure 2E) were in accord with the respective cell apoptotic rate and cell cycle progression data.

To test whether *LINC00173* could affect the migration and invasiveness of melanoma cells, the migratory and invasive abilities of *LINC00173*-deficient A375 and HT144 cells were determined in the migration and invasion assays. Treatment with si-LINC00173 drastically impaired the migration (Figure 2F) and invasiveness (Figure 2G) of A375 and HT144 cells. Thus, *LINC00173* enhanced the malignancy of melanoma cells.

LINC00173 Serves as a ceRNA in Melanoma Cells by Sponging miR-493

Mechanistically, lncRNAs can function as ceRNAs that sponge miRNAs and thereby increase the expression of miRNAs' target mRNAs.[34] To explore the mechanisms behind the strong involvement of *LINC00173* in melanoma, we first evaluated *LINC00173* expression distribution in the melanoma cell. According to the nuclear-and-cytoplasmic separation assay, *LINC00173* was enriched in the cytoplasm of A375 and HT144 cells (Figure 3A), suggesting that *LINC00173* may act as a ceRNA in melanoma. In online database starBase, version 3.0, *LINC00173* was predicted to harbor a potential binding site for miR-493 (Figure 3B).

A luciferase reporter assay was performed to validate the direct binding between *LINC00173* and miR-493 in melanoma cells. The miR-493 mimic–mediated upregulation of miR-493 (Figure 3C) effectively reduced the luciferase activity of plasmid LINC00173-WT in A375 and HT144 cells. In contrast, the luciferase activity of LINC00173-MUT was unaffected by the miR-493 mimic introduction (Figure 3D). The RIP assay revealed that *LINC00173* and miR-493 were both enriched on the anti-AGO2 antibody containing magnetic beads (Figure 3E). These observations confirmed the interaction between *LINC00173* and miR-493 in melanoma cells.

To test whether miR-493 can be sponged by *LINC00173* in melanoma cells, RT-qPCR was performed to measure miR-493 expression in A375 and HT144 cells after transfection with either si-LINC00173 or si-NC. The results showed that miR-493 was obviously induced in si-LINC00173–transfected A375 and HT144 cells relative to cells transfected with si-NC (Figure 3F). Clinical tissue test results indicated that miR-493 was downregulated in melanoma tissues (Figure 3G), consistently with the results of another study.[35] An inverse association between *LINC00173* and miR-493 expression levels in melanoma tissue samples was identified by Spearman correlation analysis (Figure 3H; $r = -0.6006$, $P < 0.0001$). Therefore, *LINC00173* as a ceRNA is capable of sponging miR-493 in melanoma cells.

LINC00173 Positively Modulates IRS4 Expression in Melanoma Cells

IRS4 mRNA is a direct target of miR-493 in melanoma cells.[35] To examine the regulatory relation between *LINC00173* and IRS4 in melanoma, the expression of IRS4 was evaluated in A375 and HT144 cells after knockdown of *LINC00173*. RT-qPCR and Western blotting

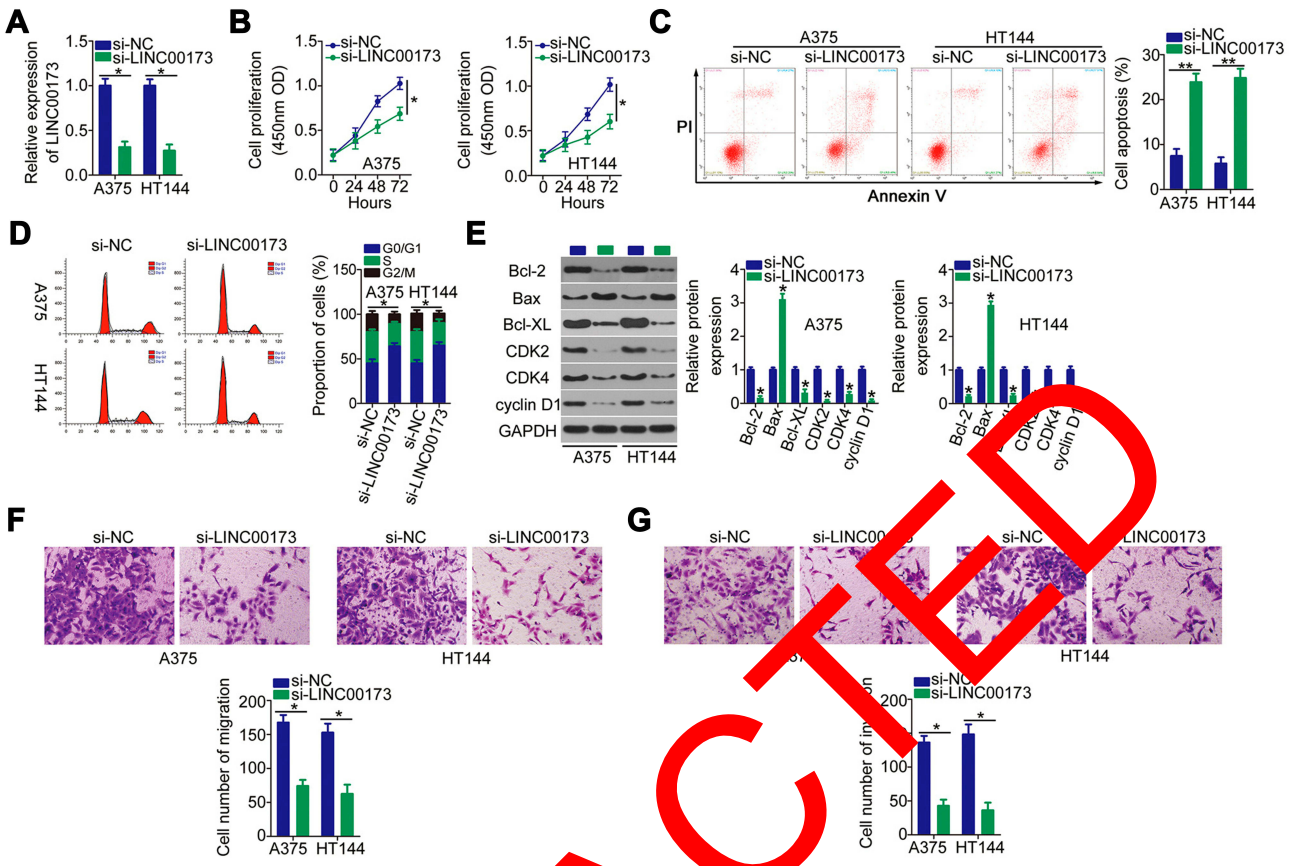

Figure 2 *LINC00173* knockdown restricts the proliferation, migration, and invasiveness and induces the apoptosis of A375 and HT144 cells. (**A**) *LINC00173* expression was evaluated in A375 and HT144 cells through RT-qPCR analysis following transfection of si-LINC00173 or si-NC. (**B**) CCK-8 assay was performed to analyze the proliferation of A375 and HT144 cells after *LINC00173* silencing. (**C**) The percentage of apoptotic A375 and HT144 cells transfected with either si-LINC00173 or si-NC was determined by flow cytometry. (**D**) The cell cycle status of *LINC00173*-deficient A375 and HT144 cells was determined by flow cytometry. (**E**) Western blotting was conducted to detect the expression levels of apoptotic associated proteins (Bcl-2, BAX and Bcl-XL) and cell cycle-associated proteins (CDK-2, CDK-4 and cyclin D1) in A375 and HT144 cells after *LINC00173* knockdown. (**F** and **G**) Migratory and invasive abilities were determined in *LINC00173*-deficient A375 and HT144 cells by migration and invasion assays. *P < 0.05 and **P < 0.01.

results showed that mRNA (Figure 4A) and protein (Figure 4B) levels of IRS4 in A375 and HT144 cells were obviously downregulated by si-*LINC00173* transfection. We found that *IRS4* mRNA was overexpressed in the melanoma tissue samples (Figure 4C), showing a positive correlation with *LINC00173* expression (Figure 4D; r = 0.527, P = 0.0004). Rescue assays were then performed to determine whether the positive influence of *LINC00173* on IRS4 expression is dependent on the sponging of miR-493. For this purpose, the miR-493 inhibitor was cotransfected with either si-LINC00173 or si-NC into A375 and HT144 cells. The transfection efficiency of the miR-493 inhibitor was validated by RT-qPCR (Figure 4E). Silencing of *LINC00173* expression increased the expression of miR-493 in A375 and HT144 cells, and this phenomenon was reversed by cotransfection with the miR-493 inhibitor (Figure 4F). Downregulation of IRS4 mRNA (Figure 4G) and protein (Figure 4H) by si-LINC00173 in A375 and HT144 cells was attenuated by miR-493 inhibitor cotransfection. Consequently, *LINC00173* acts as a ceRNA of miR-493 and thereby positively modulates IRS4 expression in melanoma cells.

Cancer-Promoting Activities of LINC00173 in Melanoma Cells are Dependent on Upregulation of miR-493–IRS4 Axis Output

Two other rescue assays were performed to test whether *LINC00173* exerts its oncogenic actions via regulation of the miR-493–IRS4 axis. First, *LINC00173*-deficient A375 and HT144 cells were transfected with the miR-493 inhibitor. The downregulation of *LINC00173* significantly inhibited proliferation (Figure 5A), promoted the apoptosis (Figure 5B) and induced the G0/G1 cycle arrest (Figure 5C) of A375 and HT144 cells, and these effects were abrogated

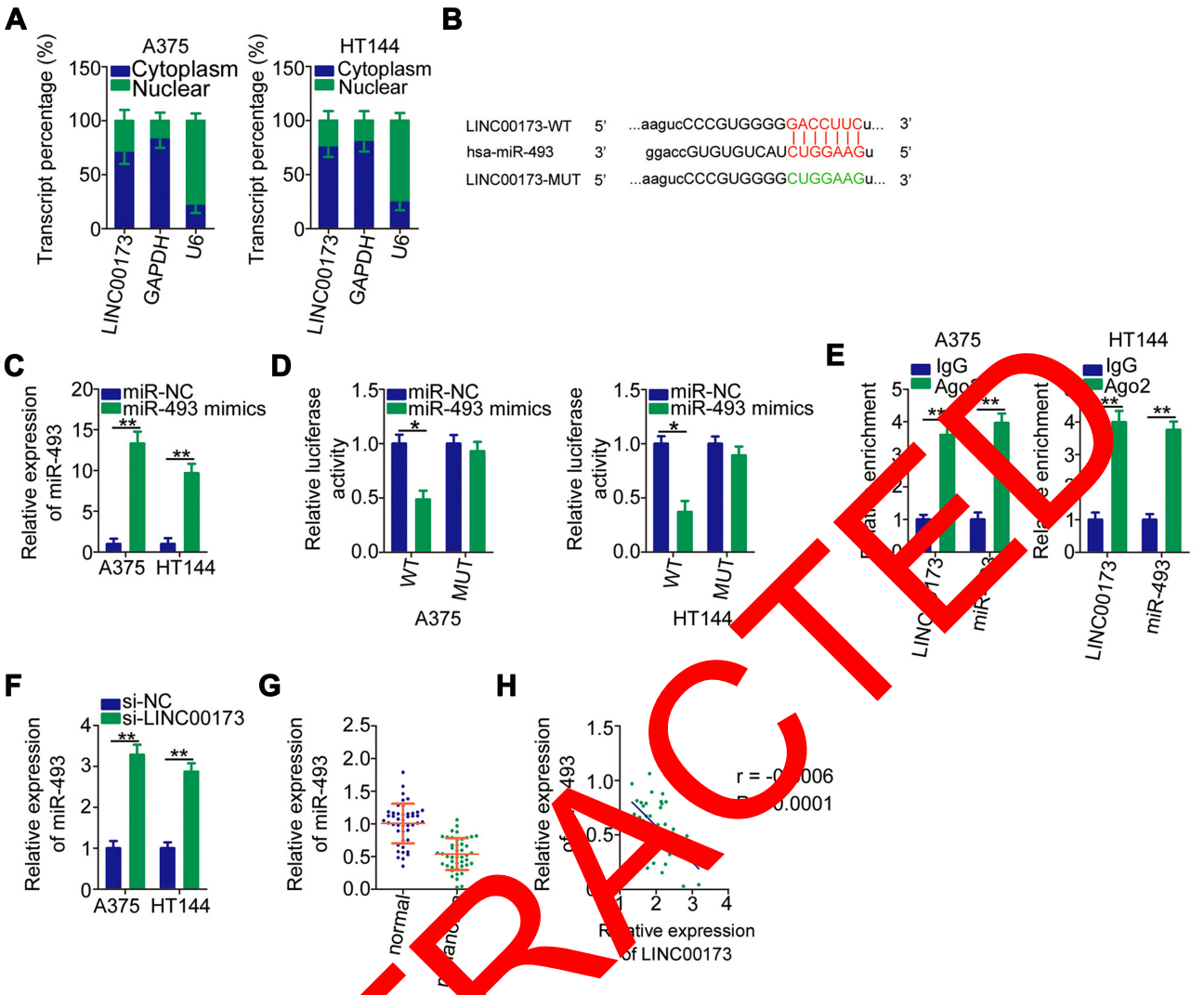

Figure 3 *LINC00173* serves as a ceRNA in melanoma cells and sponges miR-493. (**A**) Expression localization of *LINC00173* in A375 and HT144 cells was identified by a nuclear and cytoplasmic separation assay with RT-qPCR analysis. *GAPDH* and *U6* RNA served as the cytoplasmic and nuclear control transcripts, respectively. (**B**) The potential miR-493–binding site in *LINC00173*. Mutant binding sequences are shown too. (**C**) MiR-493 expression was examined by RT-qPCR analysis in A375 and HT144 cells following transfection with either the miR-493 mimic or miR-NC. (**D**) Either the miR-493 mimic or miR-NC along with either LINC00173-WT or LINC00173-MUT was introduced into A375 and HT144 cells. The luciferase reporter assay was applied to determine the binding of miR-493 to *LINC00173* in melanoma cells. (**E**) RIP assays were performed to analyze the interaction between miR-493 and *LINC00173* in melanoma cells. The enrichment of miR-493 and *LINC00173* in A375 and HT144 cells was validated by RT-qPCR. (**F**) The efficacy of the transfected si-LINC00173 or si-NC on miR-493 expression are shown in A375 and HT144 cells. (**G**) Total RNA was isolated from the 45 pairs of melanoma tissue samples and adjacent normal tissues and then was subjected to RT-qPCR analysis to evaluate miR-493 expression status. (**H**) Correlation between miR-493 and *LINC00173* expression levels in the 45 melanoma tissue samples was analyzed through Spearman correlation analysis ($r = -0.6006$, $P < 0.0001$). *$P < 0.05$ and **$P < 0.01$.

by the miR-493 inhibitor cotransfection. Similarly, the effect of the *LINC00173* knockdown on the migration (Figure 5D) and invasiveness (Figure 5E) of A375 and HT144 cells was reversed by miR-493 inhibition.

A rescue assay was performed on A375 and HT144 cells after cotransfection with si-*LINC00173* and either IRS4-overexpressing plasmid pcDNA3.1-IRS4 or the empty pcDNA3.1 vector. Transfection with pcDNA3.1-IRS4 notably raised IRS4 protein (Figure 6A) levels in A375 and HT144 cells, as evidenced Western blotting. Functional experiments suggested that overexpression of IRS4 attenuated the *LINC00173* depletion–induced effects on cell proliferation (Figure 6B), apoptosis (Figure 6C), cell cycle (Figure 6D), migration (Figure 6E), and invasiveness (Figure 6F) of A375 and HT144 cells. In brief, the oncogenic roles of *LINC00173* in melanoma cells were found to be dependent on upregulation of miR-493–IRS4 axis output.

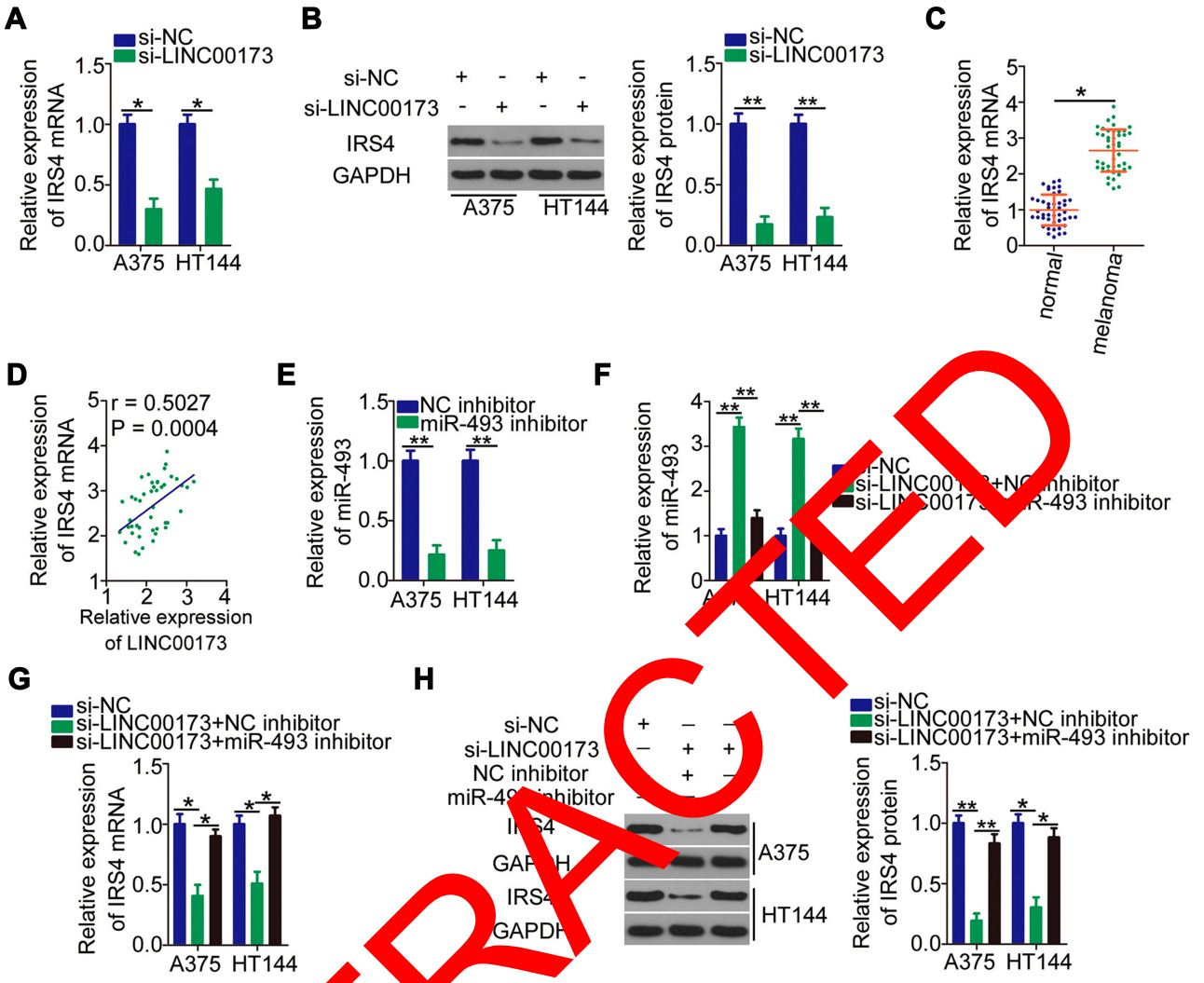

Figure 4 *LINC00173* sponges miR-493 and consequently increases IRS4 expression in melanoma cells. (**A** and **B**) IRS4 mRNA and protein expression was examined using RT-qPCR and Western blotting analysis, respectively, of A375 and HT144 cells transfected with either si-LINC00173 or si-NC. (**C**) RT-qPCR was performed to measure *IRS4* mRNA expression in the 45 pairs of melanoma tissue samples and adjacent normal tissues. (**D**) Spearman correlation analysis of the association between *IRS4* mRNA and *LINC00173* levels in the 45 melanoma tissues (r = 0.5027, P = 0.0004). (**E**) The miR-493 inhibitor was transfected into A375 and HT144 cells to silence endogenous miR-493 expression. (**F**) A375 and HT144 cells were cotransfected with si-LINC00173 and either the miR-493 inhibitor or NC inhibitor. MiR-493 expression was determined via RT-qPCR. (**G** and **H**) The mRNA and protein expression of IRS4 in the aforementioned cells was respectively measured by RT-qPCR and Western blotting. *P < 0.05 and **P < 0.01.

Knockdown of LINC00173 Inhibits the Tumorous Growth of Melanoma Cells in vivo by Enhancing miR-493 Expression and Reducing IRS4 Expression

A xenograft tumor experiment was performed to examine the effect of *LINC00173* on the growth of melanoma cells in vivo. A375 cells stably transfected with either sh-LINC00173 or sh-NC were subcutaneously inoculated into the flank of nude mice. The tumorous growth of the transplanted tumor cells was dramatically slower in the sh-LINC00173 group than in the sh-NC group (Figure 7A and B). At 4 weeks after tumor xenografting, all the mice were euthanized, and the tumor xenografts were resected. The weight of tumor xenografts was significantly lower in the sh-LINC00173 group than in the sh-NC group (Figure 7C). TUNEL assay manifested that cell apoptosis was obviously promoted by low expression of LINC00173 in the nude mice (Figure 7D). RT-qPCR and Western blotting analyses of these tumor xenografts indicated that miR-493 expression was higher (Figure 7E) but the IRS4 protein amount was lower (Figure 7F) in the tumor xenografts derived from stably sh-LINC00173–transfected A375 cells. Thus, *LINC00173* knockdown impeded the tumorous growth of melanoma cells in vivo by decreasing the output of the miR-493–IRS4 axis.

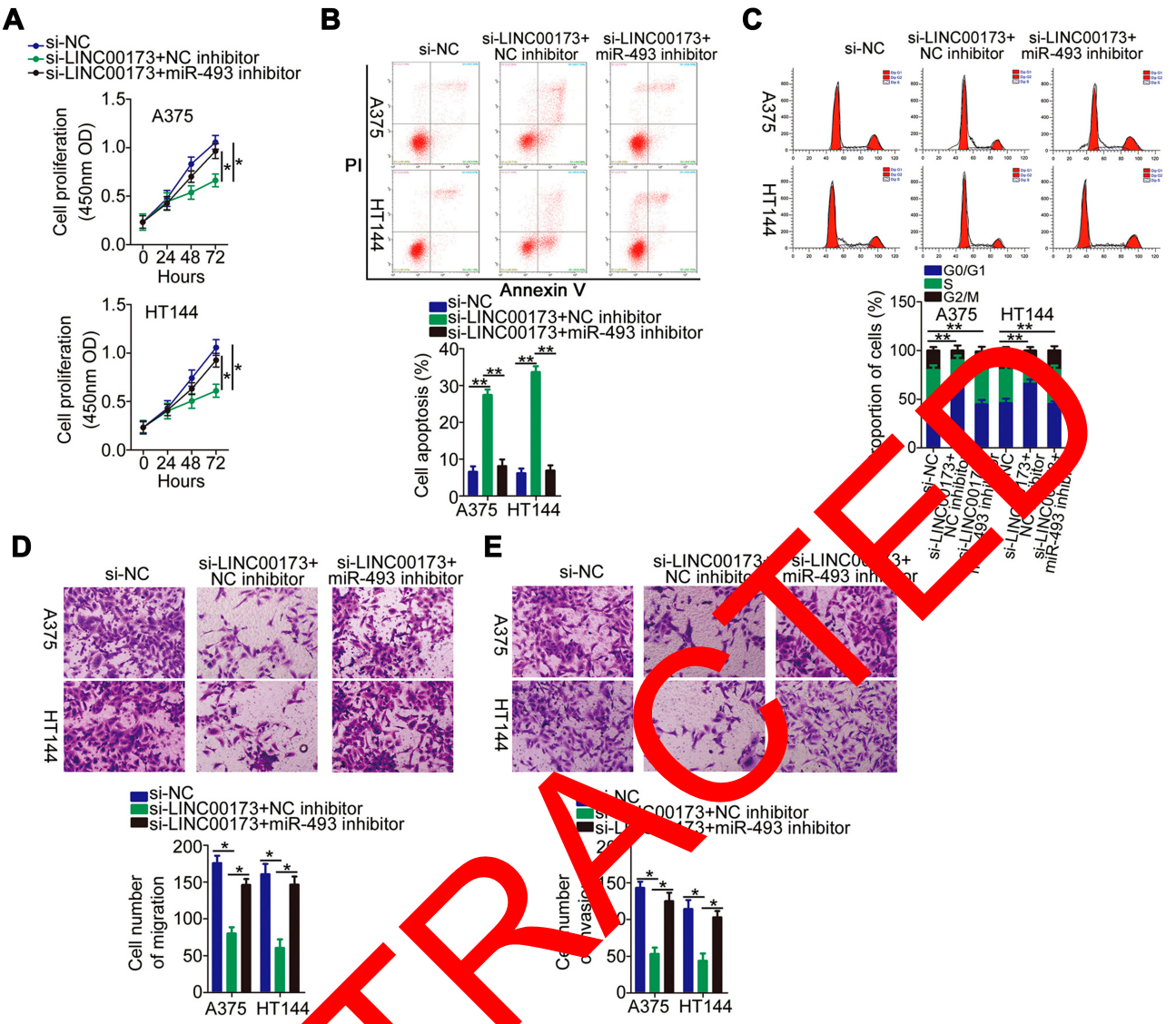

Figure 5 Inhibition of miR-493 can reverse the inhibitory impact of the *LINC00173* knockdown on the malignant behavior of melanoma cells. (**A**–**C**) The proliferation, apoptosis and cell cycle distribution of A375 and HT144 cells transfected with si-LINC00173 and either the miR-493 inhibitor or NC inhibitor were measured via the CCK-8 assay and flow cytometry, respectively. (**D** and **E**) Migration and invasion assays were performed to determine the migratory and invasive abilities of A375 and HT144 cells treated as described above. *P < 0.05, **P < 0.01.

Discussion

In recent years, lncRNAs were identified as novel regulators of tumorigenesis and tumor progression.[36,37] Approximately one fifth of lncRNAs are predicted to control the aggressive phenotype of human cancers.[38] Regarding melanoma, a variety of lncRNAs are abnormally expressed and are closely related to the patients' prognosis.[39–41] They play an important part in the malignant characteristics of melanoma in vitro and in vivo because these lncRNAs exert oncogenic or tumor-suppressive effects.[42–44] Therefore, studying the specific roles of lncRNAs in melanoma may uncover effective therapeutic targets in this disease. Here, we first assessed the expression of *LINC00173* in melanoma tissues and cell lines. Second, we applied siRNA to silence endogenous *LINC00173* expression in melanoma cells in order to evaluate the influence of the *LINC00173* knockdown on the malignant phenotype of melanoma cells in vitro and in vivo. Third, we explored the events behind the oncogenic activities of *LINC00173* in melanoma cells.

LINC00173 is upregulated in non-small cell lung cancer[32] and small cell lung cancer.[33] Upregulation of *LINC00173* is closely related to chemoresistance and a more advanced stage in patients with small cell lung cancer.[33] Patients with small cell lung cancer overexpressing *LINC00173* manifest shorter

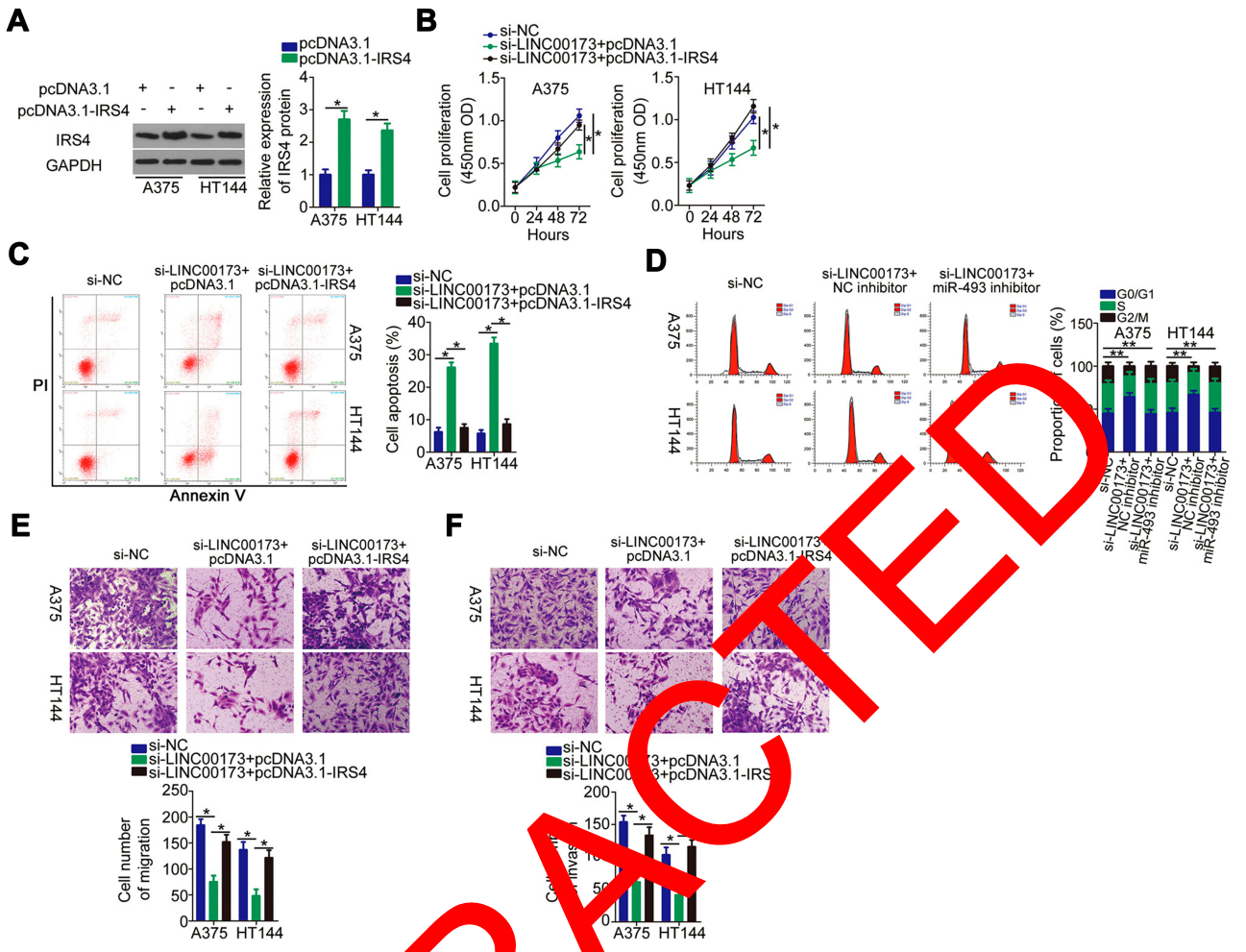

Figure 6 Restoration of IRS4 can reverse the inhibitory impact of *LINC00173* knockdown on the malignant behavior of melanoma cells. (**A**) The protein level of IRS4 in A375 and HT144 cells transfected with either pcDNA3.1-IRS4 or the empty pcDNA3.1 vector was tested by Western blotting. (**B**–**F**) *LINC00173*-deficient A375 and HT144 cells were next transfected with either pcDNA3.1-IRS4 or pcDNA3.1. After cotransfection, proliferation, apoptosis, cell cycle distribution, migration, and invasion were evaluated via the CCK-8 assay, flow-cytometric analysis, and migration and invasion assays, respectively. *P < 0.05 and **P < 0.01.

overall survival compared with patients with low *LINC00173* expression.[33] Functionally, *LINC00173* has been confirmed as an oncogenic lncRNA in small cell lung cancer and is known to promote cancer cell chemoresistance, proliferation, and metastasis in vitro and tumor chemoresistance and growth in vivo.[33] However, the expression pattern, clinical value, and detailed participation of *LINC00173* in melanoma are poorly understood. In this work, our results indicate that *LINC00173* expression is high in melanoma tissues and cell lines. High *LINC00173* expression was associated with adverse clinical features and shorter overall survival of patients with melanoma. *LINC00173* knockdown suppressed melanoma cell proliferation, migration, and invasion in vitro; increased apoptosis in vitro; and restricted tumorous growth in vivo.

Subcellular distribution of lncRNAs determines the functions of lncRNAs. The ceRNA theory indicates that when an lncRNA is mainly enriched in the cytoplasm, this RNA acts as a molecular sponge sequestering target miRNAs consequently de-repressing the miRNAs' targets at the post-transcriptional level.[34,45] Here, *LINC00173* was demonstrated to be predominantly localized in the cytoplasm of melanoma cells, suggesting that *LINC00173* may work as a ceRNA. Bioinformatic analysis then revealed that *LINC00173* contains a binding site for miR-493. This prediction was validated by luciferase reporter and RIP assays. Knockdown of *LINC00173* was found to decrease the expression of IRS4 (the target of miR-493) in melanoma cells, whereas this regulatory impact was abrogated by inhibition of miR-493 expression. A positive correlation between *LINC00173* and

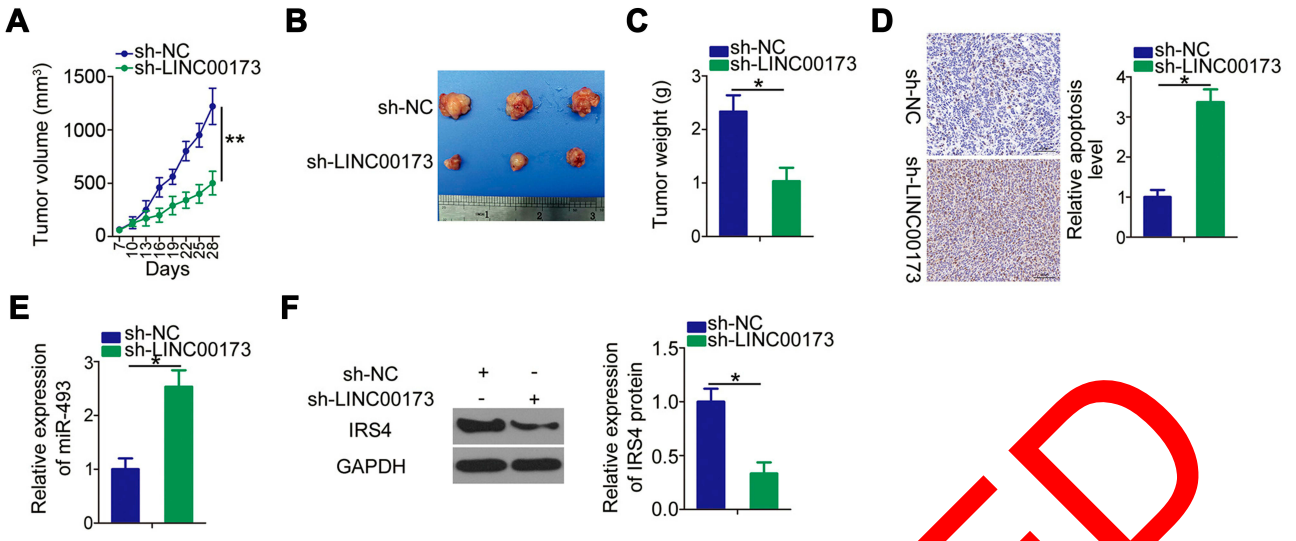

Figure 7 Downregulation of *LINC00173* restrains melanoma cell growth in vivo. (**A**) The volume of tumor xenografts was measured every 2 days. The growth curves were plotted accordingly. (**B**) After 4 weeks, all the mice were euthanized, and the tumor xenografts were excised and photographed. (**C**) The weight of the subcutaneous tumor xenografts was measured at 28 days after cell inoculation. (**D**) TUNEL assay was utilized to measure cell apoptosis of tumor xenografts obtained from sh-LINC00173 group and sh-NC group. (**E**) RT-qPCR was applied to assess miR-493 expression in the tumor xenografts obtained from the sh-LINC00173 group and sh-NC group. (**F**) The IRS4 protein in tumor xenografts was quantified by Western blotting. *P < 0.05 and **P < 0.01.

IRS4 levels was confirmed in the melanoma tissues. Our rescue assays indicate that the oncogenic actions of *LINC00173* on the progression of melanoma are dependent on enhancement of miR-493–IRS4 axis output. Therefore, the influence of *LINC00173* on the aggressiveness of melanoma can be partly explained by the ceRNA mechanism involving *LINC00173*, miR-493, and *IRS4* mRNA.

MiR-493 is dysregulated in multiple types of cancer,[46–51] including melanoma.[35] Functionally, exogenous miR-493 expression attenuates cell proliferation and induces cell cycle arrest in melanoma.[35] Mechanistically, *IRS4* is a direct target gene of miR-493 in melanoma cells.[35] IRS4 is a part of a family of cytoplasmic docking proteins mediating signals from cell surface receptors to downstream effectors.[52] In humans, IRS4 is increasingly implicated in cancer initiation and progression. Overexpression of IRS4 is seen in hepatocellular carcinoma,[53] breast cancer,[54] colorectal cancer,[55] and lung cancer.[56] Our results indicate that IRS4 is upregulated by the *LINC00173*–miR-493 axis in melanoma. *LINC00173* can interact with miR-493 directly to raise the expression of IRS4. Hence, a new *LINC00173*–miR-493–IRS4 pathway was identified here in melanoma cells and seems to control tumorigenesis and tumor progression. This study may offer novel ideas for the discovery of antimelanoma therapies.

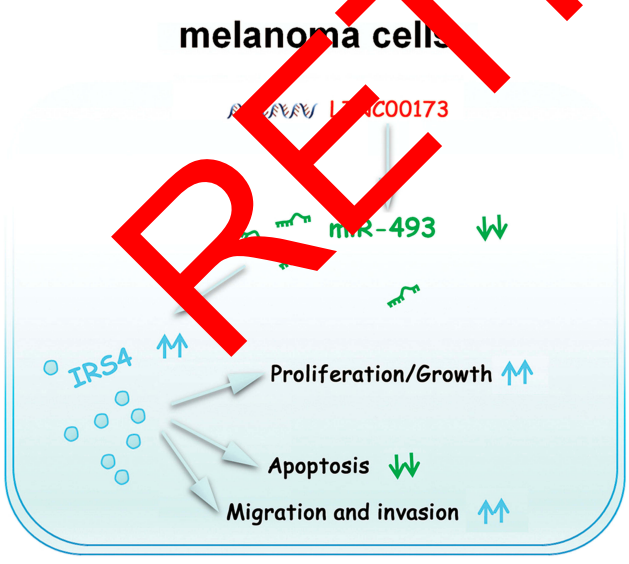

Figure 8 Schematic diagram of proposed mechanism. *LINC00173* promotes the malignant characteristics of melanoma by increasing IRS4 expression via competitive binding to miR-493.

Conclusions

Our study identified an oncogenic lncRNA, *LINC00173*, involved in melanoma. *LINC00173* promotes the malignant characteristics of melanoma by increasing IRS4 expression via competitive binding to miR-493. Validating the participation of the *LINC00173*–miR-493–IRS4 pathway (Figure 8) in melanoma pathogenesis may be useful in the identification of novel therapeutic targets.

Abbreviations

3′-UTR, 3′-untranslated region; CCK-8, Cell Counting Kit-8; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal

bovine serum; FITC, fluorescein isothiocyanate; lncRNA, long noncoding RNA; miRNA, miR, microRNA; MUT, mutant; NC, negative control; RIP, RNA immunoprecipitation; RT-qPCR, reverse-transcription quantitative PCR; shRNA, short hairpin RNA; siRNA, small interfering RNA; WT, wild-type.

Ethics and Consent Statement

This study was performed with the approval of the Research Ethics Committee of Shaanxi Provincial People's Hospital. In addition, written informed consent forms were signed by all the patients who participated in this research. All animal experiments were performed with the approval of the animal ethics committee of Shaanxi Provincial People's Hospital and in conformity with the Animal Protection Law of the People's Republic of China-2009 for experimental animals.

Disclosure

The authors declare that they have no competing interests.

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