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CircRTTN upregulates EPHA2 to aggravate the malignant process of melanoma via sponging miR-890

Yaqin Wang[1]*, Junzuo Gong[2]*, Xiaojie Ding[3] and Shu Luo[2]

[1]Department of Pathology, [2]Departement of Emergency and [3]Department of Dermatology, Affiliated Hospital of North Sichuan Medical College, Nanchong City, Sichuan Province, China

*These authors contributed equally to this paper

Summary. Background. Malignant melanoma is a kind of tumor derived from melanocytes, which has the characteristics of drug resistance and distant metastasis. Accumulating evidence has demonstrated that circular RNAs (circRNAs) are involved in the pathogenesis of melanoma. Our current study aimed to investigate the role and mechanism of circRTTN in melanoma progression.

Methods. The levels of circRTTN, microRNA-890 (miR-890) and EPH receptor A2 (EPHA2) were examined via quantitative real-time PCR (qRT-PCR) and Western blot. Cell Counting Kit-8 (CCK-8), colony formation, 5-Ethynyl-2'-deoxyuridine (EdU) staining, flow cytometry, transwell and tube formation assays were conducted to estimate the effects of circRTTN on growth, apoptosis, migration, invasion and angiogenesis of melanoma cells. Western blot was used to measure related marker protein levels. The interaction between miR-890 and circRTTN or EPHA2 was predicted by bioinformatics analysis and verified by dual-luciferase reporter and RNA Immunoprecipitation (RIP) assays. Xenograft assay was used to assess the effect of circRTTN *in vivo*.

Results. CircRTTN and EPHA2 levels were up-regulated, while miR-890 was down-regulated in melanoma tissues and cells. CircRTTN knockdown restrained cell proliferation, migration, invasion and angiogenesis, but promoted cell apoptosis *in vitro*. CircRTTN was an effective molecular sponge for miR-890, and negatively regulated miR-890 expression. The suppressive role of circRTTN knockdown on cell growth, metastasis and angiogenesis *in vitro* was abated by blocking miR-890. MiR-890 directly targeted EPHA2. MiR-890 overexpression elicited a similar anti-tumor role in melanoma cells, which was abrogated by overexpression of EPHA2. In addition circRTTN knowdown markedly attenuated xenograft tumor growth *in vivo*.

Conclusion. Our findings demonstrated that circRTTN mediated melanoma progression via regulating the miR-890/ EPHA2 axis.

Key words: Melanoma, circRTTN, miR-890, EPHA2

Introduction

Malignant melanoma is the most dangerous skin tumor and the leading cause of death from skin cancer (Siegel et al., 2017). In recent years, the global incidence of melanoma has been increasing every year. In 2020, melanoma accounted for 1.7% of all cancer cases and 0.6% of all cancer deaths worldwide (Sung et al., 2021). Melanoma is a multifactorial tumor arising from the interaction of environmental exposure and genetic susceptibility (Shannan et al., 2016), and UV exposure is considered to be possibly the most important risk factor for the disease (Ribero et al., 2016). Although treatment methods for patients with melanoma have been increasingly improved, the treatment effect is still not effective, especially for those with distant metastases (Tsao et al., 2012), and the 5-year overall survival (OS) rate of the disease is still less than 40% (Gershenwald et al., 2017). Therefore, it is very important to explore the molecular mechanism of melanoma to find potential therapeutic targets.

Circular RNA (circRNA) has a covalent closed-loop structure and is a new type of endogenous RNA (Hsiao et al., 2017). Unlike linear RNAs, circRNAs are difficult to be degraded by endonucleases because they are closed-loop structures without 5' and 3' ends (Eger et al., 2018). Therefore, circRNAs can exist in mammalian cells for a long time (Qu et al., 2015). With the development of advanced RNA sequencing (RNA-seq) technology, circRNAs have gradually attracted attention, and they are closely related to many physiological and

Corresponding Author: Xiaojie Ding, Department of Dermatology, Affiliated Hospital of North Sichuan Medical College, No.1, Maoyuan South Road, Shunqing District, Nanchong 637000, Sichuan Province, PR China. e-mail: cbyxydxj@163.com

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pathological processes, including tumor development (Dube et al., 20019). Accumulating evidence suggests that circRNAs are involved in many cellular processes by binding to miRNAs to regulate the level of downstream target genes. For example, up-regulation of hsa_circ_0062270 in melanoma aggravated the malignant progression of melanoma by inducing CDC45 (Hao et al., 2022). Circ_0001951, highly expressed in melanoma, promoted melanoma progression by sponging miR-431-5p to upregulate ROCK1 (Yin et al., 2021). In addition, circ_0082835 promoted distant metastasis of melanoma by binding miR-429 to upregulate EZH2 and activated the Wnt/β-catenin pathway (Sun et al., 2021). Although some circRNAs have been reported to be involved in the development of melanoma, the more subtle regulatory mechanisms of dysregulated circRNAs in melanoma remain a mystery and require further extensive research. CircRTTN (hsa_circ_0108830) was reported to be upregulated in melanoma (Liu et al., 2021). However, the roles and regulatory mechanisms of circRTTN in melanoma are still unknown.

Increasing evidence suggests that miRNAs competes with circRNAs to regulate the expression of downstream genes and are involved in the development of melanoma. For example, circ_0079593 bound miR-573 competitively to induce ABHD2 expression, thereby exacerbating melanoma progression (Zhao et al., 2021). Numerous studies have shown that miR-890 was involved in the development of various cancers, including glioma (Wang et al., 2020) and Triple-negative breast cancer (Wang et al., 2019). It has been reported that miR-890 was significantly reduced in melanoma, and its overexpression inhibited melanoma progression (Xia et al., 2020). Whether miR-890 is involved in the circRTTN-regulated melanoma process needs to be experimentally verified.

EPH receptor A2 (EPHA2) has been reported to be an oncogene in melanoma and was involved in the development of melanoma (Margaryan et al., 2009; Udayakumar et al., 2011; Miao et al., 2015). For example, the upregulation of EPHA2 after ultraviolet radiation can induce apoptosis (Udayakumar et al., 2011). EPHA2 was overexpressed in melanoma cells with the metastatic phenotype and promoted invasion, proliferation, and angiogenesis (Margaryan et al., 2009). EPHA2 inhibitors effectively blocked the tumorigenic growth of melanoma cells, inhibited their activity, and induced apoptosis (Miao et al., 2015). In addition, EPHA2 was regulated by circ_0062270/miR-331-3p to aggravate the malignant progression of melanoma (Chen et al., 2021). In this study, online website predictions indicated that circRTTN had complementary binding sites to miR-890, and similarly miR-890 had targeted binding sites to EPHA2. Based on this, we proposed a circRTTN/miR-890/EPHA2 regulatory network. The purpose of this study was to investigate the regulatory mechanism of circRTTN in melanoma.

Materials and methods

Tissue Collection

53 melanoma and matched normal tissues and 10 benign nevi tissues were collected from Affiliated Hospital of North Sichuan Medical College. All patients did not receive preoperative chemoradiation or radiotherapy, and gave written informed consent prior to surgery. In order to preserve the samples for a longer time, all case samples were frozen at -80°C. Human tissue specimens were utilized with the authorization of the Ethics Committee of Affiliated Hospital of North Sichuan Medical College. The clinicopathological parameters of melanoma patients are shown in Table 1.

Cell culture and transfection

Human Melanoma cell lines including A-375, SK-MEL-2, normal human epidermal melanocytes neonatal (HEMn) and human umbilical vein endothelial cells (HUVECs) were purchased from American Tissue Type Culture Collection (Manassas, VA, USA). A-375, SK-

Table 1. Correlation between CircRTTN expression and clinicopathological parameters of patients with melanoma (n=53).

Variable	n	CircRTTN	
		High (n=26)	Low (n=27)
Age (years)			
≥60	30	14	16
<60	23	12	11
Gender			
Male	28	14	14
Female	25	12	13
Anatomic site			
Acra	40	21	19
Trunk	7	4	3
Other	6	1	5
Ulceration			
Present	10	6	4
Absent	43	20	23
Breslow depth (mm)			
≥2	34	21	13
<2	19	5	14
Clark level			
I-III	25	16	9
IV-V	28	10	18
Distant metastasis			
No	45	20	25
Yes	8	6	2
Lymph node metastasis			
No	37	17	20
Yes	16	9	7
Clinical stage			
I-II	40	17	23
III-IV	13	9	4

*p<0.05.

MEL-2 and HEMn were incubated in DMEM (Dulbecco's modified Eagle's medium) (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). HUVECs were grown in endothelial cell growth medium MV2 with supplement mix (Promo Cell, Heidelberg, Germany). All cells were cultured in a sterile incubator (5% CO_2, 37°C).

The overexpression vector of circRTTN was denoted as circRTTN, and pCD5-ciR was used as its negative control. Similarly, EPHA2 represents the overexpression of EPHA2, and its control was an empty pcDNA3.1 vector. All of the above overexpression vectors are provided by Genepharma (Shanghai, China). In addition, siRNAs against circRTTN (si-circRTTN#1, 2 and 3), siRNA negative control (si-NC), miR-890 mimic, miRNA negative control (NC), miR-890 inhibitor (anti-miR-890) and inhibitor negative control (anti-NC) were obtained from Genepharma. Specific short hairpin RNA (shRNA, Genepharma)-mediated circRTTN silencing was used for *in vivo* assays. In short, Lipofectamine 2000 (Invitrogen) was used to transfect 30 nM oligonucleotide or 600 ng vector into A-375 and SK-MEL-2 cells.

Treatment with RNase R or Actinomycin D

RNase R (Epicentre Technologies, Madison, WI, USA) was added to the RNA sample to reflect their stability. 3 U/μg RNase R (Geneseed, Guangzhou, China) was added to the RNA sample and placed at 37°C for 15 minutes. Next, qRT-PCR was used to detect the levels of circRTTN and EPHA2 mRNA in RNase R-treated samples. Actinomycin D (2mg/ml, Sigma) was added to the cell culture medium to detect the expression of circRTTN and EPHA2 mRNA at different time points.

Quantitative real-time PCR (qRT-PCR)

TRIzol® reagent was added to cells or tissues to obtain total RNA. 1 μg of total RNA was used to synthesize cRNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Vilvoord, Brussels, Belgium). Then, SYBR Premix Ex Taq II (TaKaRa, Dalian, China) was used for qPCR. The above operations were performed according to the instructions. The specific primer sequences are shown in Table 2. The relative abundance was analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). β-Actin (for circRTTN and EPHA2) or U6 (for miR-890) was used as an endogenous control.

Table 2. Primers sequences used for PCR.

Name	Primers for PCR (5'-3')	
hsa_circ_0108830	Forward	CGCTAGCTAACTCTGAGAGCA
	Reverse	ACGTAAGGCAGTGGTAAGGC
RTTN	Forward	GGCTCATCAGGAAACTCGGT
	Reverse	ACGGACGGGAAATTGAACCA
miR-890	Forward	GTATGATACTTGGAAAGGCAT
	Reverse	CTCAACTGGTGTCGTGGAG
EPHA2	Forward	TCACACACCCGTATGGCAAA
	Reverse	ACGTTGCACACGGAGTACAT
GAPDH	Forward	GACAGTCAGCCGCATCTTCT
	Reverse	GCGCCCAATACGACCAAATC
U6	Forward	CTCGCTTCGGCAGCACA
	Reverse	AACGCTTCACGAATTTGCGT
18S rRNA	Forward	AGAAACGGCTACCACATCCA
	Reverse	CCCTCCAATGGATCCTCGTT

Cell Counting Kit-8 (CCK8) assay

The transfected A-375 and SK-MEL-2 cells were transplanted into 96-well plates at a density of 2×10^5 cells per well and cultured for 24, 48, and 72 hours. Subsequently, 10 mL of CCK8 solution (Beyotime, Jiangsu, China) was added to each well of the cell culture medium and incubated for 2h. The absorbance at 450 nm was measured.

Colony formation assay

The transfected A-375 and SK-MEL-2 cells were seeded in 6-well plates a density of 1×10^3 cells per well. This procedure was discontinued after 2 weeks of continuous culture. Finally, the number of clones was detected, and the clone formation rate was calculated.

5-Ethynyl-2'-deoxyuridine (EdU) assay

Commercial EdU cell proliferation kit (Ribobio, Guangzhou, China) was used to detect cell proliferation. The transfected A-375 and SK-MEL-2 cells were seeded on a 96-well plate with 5×10^4 cells in each well and grown for 24h, and then 10 μM EdU was added to culture for 2h. Hoechst 33342 was used for nuclear staining. A fluorescence microscope (Olympus, Tokyo, Japan) was used to observe the cells.

Flow cytometry

Annexin V-FITC/PI apoptosis detection kit (Solarbio, Beijing, China) was used to assess cell apoptosis. Cells were seeded in a six-well plate at a density of 1×10^6 cells per well. After incubation at 48h, cells were centrifuged and harvested, followed by resuspending in binding buffer (0.4 mL). After staining with Annexin V-FITC (5 μL) and PI (10 μL), flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA) was applied for detecting cell apoptosis.

Transwell assays

Transwell chamber (Corning, Tewksbury, MA, USA) was prepared with or without Matrigel matrix (Bedford, Massachusetts, USA) to measure cell invasion and migration, respectively. A-375 and SK-MEL-2 cells were added to the upper chamber with serum-free

medium, and the lower chamber medium was supplemented with 10% fetal bovine serum. It is worth noting that 5×10^4 cells were used for migration assays, while 1×10^5 cells were used for invasion assays. After the cells were cultured for 24 hours, the transmembrane cells were fixed with ethanol (95%, Beyotime), stained with crystal violet (0.1%, Beyotime), and counted under a microscope (Olympus, Tokyo, Japan).

Tube formation analysis

First, 1×10^4 HUVECs serum-starved for 16 h were added to each well of 96-well plates coated with Matrigel (BD Biosciences). Then, the transfected cell culture supernatant was added to the 96-well plates to culture HUVECs. The cells were cultured for 24h, the number of tubes in the field of view was counted, and the tubular structure formation ability of the cells was detected.

Western blot

First, RIPA buffer (Sigma) was added to the cells or tissues to obtain total protein. Then, the proteins were separated by SDS-PAGE gel and electrotransferred to PVDF membrane (Millipore, Billerica, MA, USA). Next, the PVDF membrane was blocked with skimmed milk (5%), and then incubated with the primary antibody. Then the membrane was incubated with the secondary antibody, and the bands were observed with enhanced chemiluminescence reagent (Solarbio). The primary antibody information was as follows: anti-Matrix MetalloProteinase 2 (MMP2, 1 μg/ml; Abcam, Cambridge, UK), anti-Matrix MetalloProteinase 9 (MMP9, 1:1000; Abcam), anti-EPHA2 (1:1000; CST, Boston, MA, USA) and β-Actin (1:1000; Abcam).

Dual-luciferase reporter assay

According to the prediction of the online software Circular RNA Interactome, miR-890 had potential binding sites with EPHA2 3'UTR and circRTTN. There was a mutation at the site where EPHA2 3'UTR or circRTTN bound to miR-890. The mutant type was labeled mut, and the wild type was labeled wt. CircRTTN -wt/mut or EPHA2-wt/mut was cloned into pmirGLO dual luciferase plasmid (Promega, Madison, WI) to construct pmirGLO-circRTTN-wt/mut or pmirGLO-EPHA2-wt/mut recombinant plasmid. Then each recombinant plasmid was co-transfected with miR-890 or NC into A-375 and SK-MEL-2 cells. In order to check the luciferase activity, a dual luciferase assay kit (Promega) was used, and Renilla luciferase was used as a control.

RNA immunoprecipitation (RIP)

Magna RNA immunoprecipitation kit (Millipore)

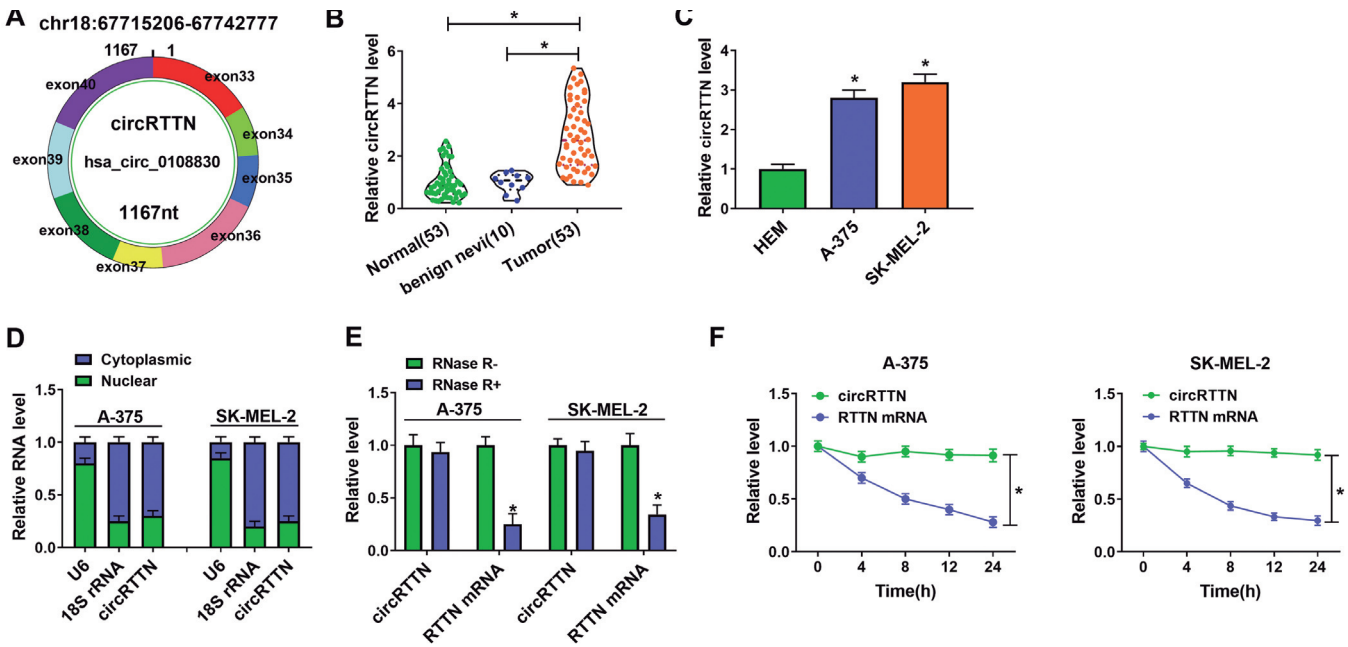

Fig. 1. The expression of circRTTN is upregulated in melanoma. **A.** The information of circRTTN was presented. **B.** The expression of circRTTN in melanoma and normal adjacent tissues determined by qRT-PCR. **C.** QRT-PCR was adopted to measure the expression of circRTTN in two melanoma cell lines including A-375 and SK-MEL-2 along with HEMn cell line. **D.** The nuclear and cytoplasmic separation experiment was used to explore the location of circRTTN in melanoma cells. **E.** After treatment of RNase R, circRTTN and its linear transcript RTTN mRNA were detected by qRT-PCR in melanoma cells. **F.** The levels of circRTTN and RTTN mRNA in melanoma cells treated with Actinomycin D were examined using qRT-PCR at indicated time points (0, 4, 8, 12 and 24h). **P*<0.05.

was used to perform RIP experiments. RIP lysis buffer was added to lyse A-375 and SK-MEL-2 cells (2×10^5 cells/mL). Next, the obtained extract was incubated with RIP buffer coupled with anti-Ago2 (Millipore) or control anti-immunoglobulin G (IgG; Millipore) magnetic beads (Invitrogen). Finally, proteinase K was used to elute the

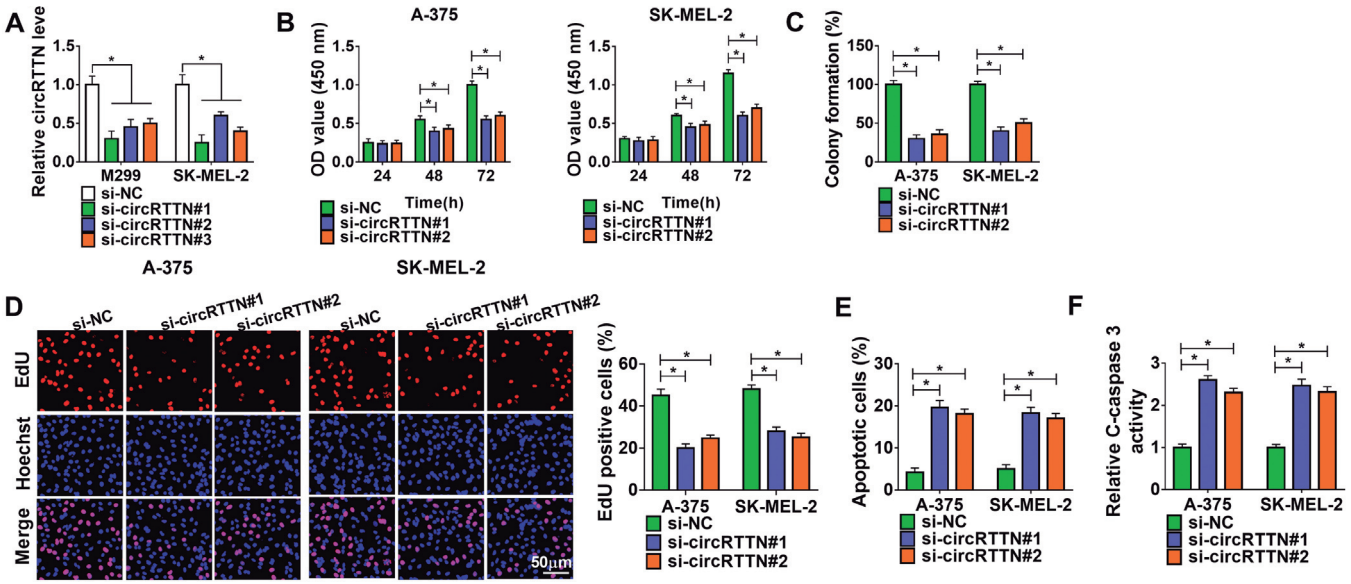

Fig. 2. CircRTTN silencing suppresses melanoma cell growth, and induces cell apoptosis. **A.** CircRTTN expression was measured by qRT-PCR in melanoma cells transfected with si-circRTTN#1, si-circRTTN#2, si-circRTTN#3 or si-NC, respectively. **B-D.** Cell proliferation was measured by CCK-8, colony formation and EdU staining, respectively. **E-F.** Cell apoptosis was measured by flow cytometry and Caspase Activity Assay Kit. **P*<0.05.

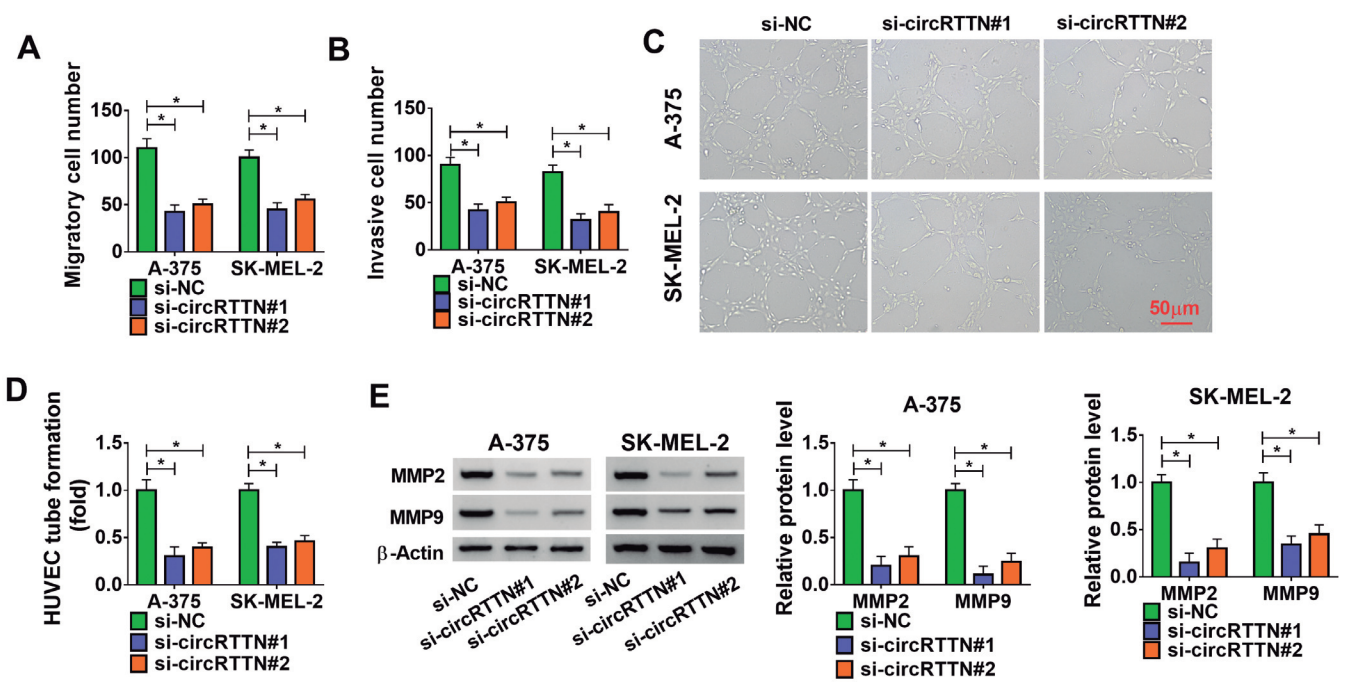

Fig. 3. CircRTTN silencing suppress melanoma cell migration, invasion and angiogenesis. **A-B.** Cell migration and invasion were measured by transwell. **C-D.** Tube formation assay was used for cell tube formation ability. **E.** Western blot was performed to measure the protein expression of marker (PCNA, MMP2 and MMP9). **P*<0.05.

total RNA on the magnetic beads, and then qRT-PCR was used to detect the enrichment of related RNA.

Xenograft tumors in nude mice

Animal experiments were performed according to the guidelines of Affiliated Hospital of North Sichuan Medical College (Approval number: 202061450). 10 BALB/c nude mice were purchased from Vital River Laboratory Animal Technology Company (Beijing, China). Nude mice were randomly divided into two groups. Lenti-sh-hsa_circ_0108830 or Lenti-sh-NC was stably transfected into SK-MEL-2 cells, respectively. 1×10^6 transfected SK-MEL-2 cells were injected into nude mice. The length and width of the tumor were measured with vernier calipers every 5 days and were indicated by a and b, respectively. The equation a×b×b×1/2 was used to calculate the volume of the subcutaneous tumor. After 30 days, all mice were sacrificed, and the subcutaneous tumor tissues were obtained. The tissues were weighed and stored.

IHC (Immunohistochemistry) assay

IHC staining was performed to assess the expression of ki-67 in mouse subcutaneous xenografts. Briefly, tissue sections were subjected to many processes such as antigen retrieval. Subsequently, anti-ki-67 (1:200; Abcam) was incubated with the sections overnight at 4°C. Next, tissue sections were incubated with a

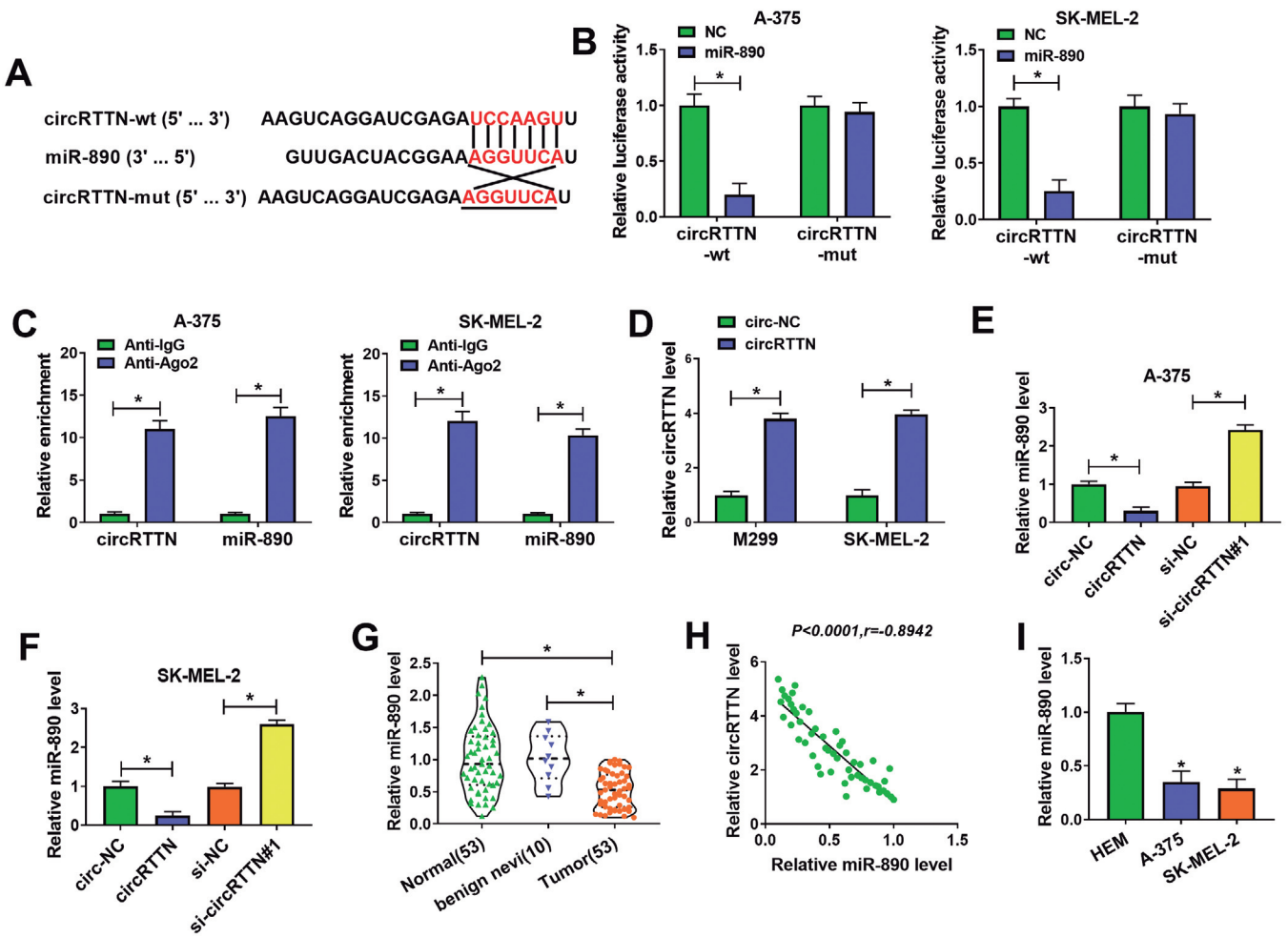

Fig. 4. CircRTTN functions as a sponge of miR-890. **A.** The targets of circRTTN were predicted using bioinformatic database Circular RNA Interactome, and miR-890 was a candidate target of circRTTN. **B-C.** Luciferase activity and RIP assay in melanoma cells were conducted to verify the binding between circRTTN and miR-890. **D.** qRT-PCR was used to analyze the level of circRTTN in A-375 and SK-MEL-2 cells transfected with circ-NC or circRTTN, respectively. **E-F.** The effect of overexpression or knockdown of circRTTN on the expression of miR-890 was examined by qRT-PCR. **G.** The level of miR-890 was determined in melanoma samples and matched normal samples by qRT-PCR. **H.** The correlation between circRTTN and miR-890 expression in melanoma samples was analyzed by Pearson's correlation coefficient. **I.** qRT-PCR was adopted to measure the expression of miR-890 in HEMn cells and melanoma cells. **P*<0.05.

biotinylated secondary antibody (1:100; Proteintech; SA00004-6), and finally tissue sections were stained with 3,3'-diaminobenzidine (DAB) substrate and counterstained with hematoxylin.

Statistical analysis

All results were analyzed on GraphPad Prism 7.0 software (GraphPad, La Jolla, CA, USA) and expressed as mean ± standard deviation (SD). The Pearson correlation coefficient was used to analyze the linear correlation between hsa_circ_0108830, miR-890 and EPHA2 in tissue specimens. Student's t-test or analysis of variance was used to analyze the data. The statistical significance of the difference was represented by $P<0.05$.

Results

CircRTTN was highly expressed in melanoma tissues and cell lines

CircRTTN is generated from 33-40 exons of RTTN gene and is located on chr18:67715206-67742777 (Fig. 1A). It was identifed that circRTTN was significantly more upregulated in melanoma tissues than adjacent normal tissues and benign nevi (Fig. 1B). Additionally, circRTTN was up-regulated in melanoma cell lines (A-375 and SK-MEL-2) compared with HEMn cell line (Fig. 1C). It was clearly identifed that circRTTN was enriched in the cytoplasm through sub-cellular localization analysis (Fig. 1D). The RNase R treatment assay showed that circRTTN was resistant to RNase R

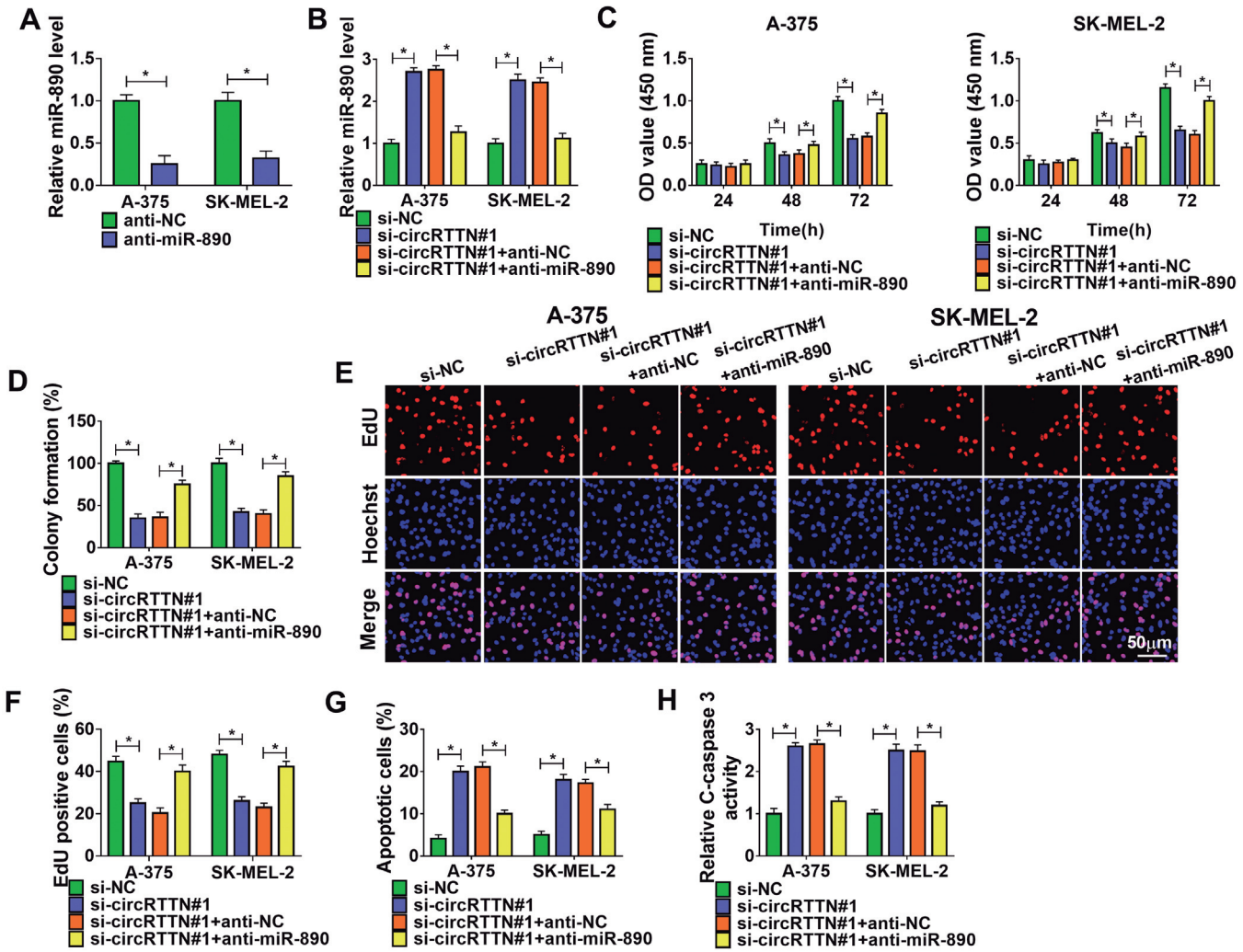

Fig. 5. CircRTTN regulates melanoma cell growth and apoptosis by sponging miR-890. **A.** The knockdown efficiency of miR-890 in melanoma cells was assessed by qRT-PCR. **B-H.** Melanoma cells were transfected with si-circRTTN#1 or si-circRTTN#1+anti-miR-890 along with their matched controls. **B.** The level of miR-890 was determined by qRT-PCR. **C-F.** Cell proliferation was measured by CCK-8, colony formation, and EdU staining. **G-H.** Cell apoptosis was measured by flow cytometry and Caspase Activity Assay Kit. **P*<0.05.

(Fig. 1E). Next, the actinomycin D assay showed that the half-life of circRTTN transcript exceeded 24h, indicating that circRTTN was more stable than the linear RTTN mRNA transcript in melanoma cells (Fig. 1F). These data suggest that circRTTN was a stably upregulated circRNA in melanoma tissues and cells.

Knockdown of circRTTN suppressed melanoma cell growth, and induced cell apoptosis.

Loss-of-function experiment was conducted to investigate the role of circRTTN in melanoma cells *in vitro*. We first suppressed the expression of circRTTN in A-375 and SK-MEL-2 cells by transfecting with siRNA (si-circRTTN#1, si-circRTTN#2 and si-circRTTN#3). CircRTTN expression was markedly suppressed in three knockdown groups (Fig. 2A). CircRTTN knockdown obviously inhibited the proliferation of A-375 and SK-MEL-2 cells, as indicated by the reduced OD value, colony-formation ability, and Edu-positive cells in melanoma cells (Fig. 2B-D). Silence of circRTTN induced apoptosis of two melanoma cell lines (Fig. 2E-F).

Knockdown of circRTTN suppressed melanoma cell migration, invasion and angiogenesis.

Furthermore, reduced circRTTN expression by siRNA inhibited cell migration and invasion of melanoma cells compared with the si-NC group (Fig. 3A-B). The tube formation test showed that CircRTTN knockdown reduced the tube formation compared with the corresponding control (Fig. 3C-D). Besides, circRTTN interference reduced the expression of MMP2 and MMP9 (Fig. 3E). Overall, circRTTN knockdown suppressed melanoma progression *in vitro*.

CircRTTN acted as a sponge for miR-890

Through the analysis of the online website Circular RNA Interactome, we found that miR-890 had a potential binding site with circRTTN, and mutated this site to construct circRTTN-mut (Fig. 4A). To analyze whether circRTTN interacted with miR-890 through the predicted sites, dual-luciferase reporter assay and RIP assay were applied. MiR-890 evidently reduced the luciferase activity of wild-type reporter plasmid (circRTTN-wt), but did not affect the luciferase activity of mutant plasmid (circRTTN-mut) (Fig. 4B). The RIP experiment further verified the binding between circRTTN and miR-890, because circRTTN and miR-890 were enriched in Ago2 immunoprecipitates instead of IgG (Fig. 4C). The expression of circRTTN in A-375 and SK-MEL-2 cells was significantly increased after circRTTN transfection (Fig. 4D). Furthermore, miR-890 level was obviously decreased by circRTTN overexpression, but was notably elevated by circRTTN knockdown (Fig. 4E,F). MiR-890 was notably down-regulated in melanoma tissues compared with that in adjacent normal tissues and benign nevi (Fig. 4G). CircRTTN was negatively correlated with the expression of miR-890 (Fig. 4H). MiR-890 expression was reduced in both melanoma cell lines when compared with HEMn cell line (Fig. 4I). Overall, circRTTN directly interacted

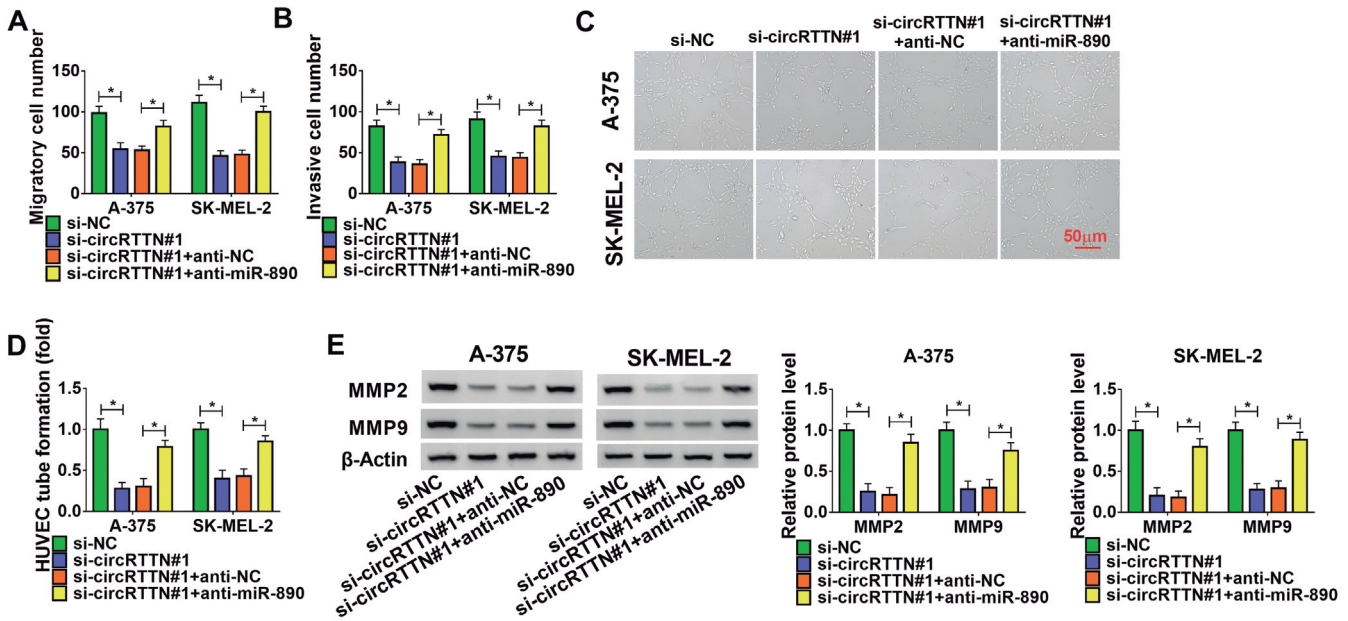

Fig. 6. CircRTTN influences melanoma cell migration, invasion and angiogenesis by sponging miR-890. Melanoma cells were transfected with si-circRTTN#1 or si-circRTTN#1+anti-miR-890 along with their matched controls. **A-B.** Cell migration and invasion were analyzed by transwell. **C-D.** Tube formation assay was used for cell tube formation ability. **E.** Western blot was performed to measure the protein expression of markers (MMP2 and MMP9). **P*<0.05.

with miR-890 in melanoma cells.

Knockdown of miR-890 attenuated the effects of circRTTN knockdown on the growth, and apoptosis of melanoma cells.

The expression of miR-890 was decreased after melanoma cells were transfected with anti-miR-890 (Fig. 5A). To investigate whether circRTTN functioned by targeting miR-890, restoration experiments were performed. Anti-miR-890 reversed the up-regulation of miR-890 by circRTTN silencing in A-375 and SK-MEL-2 cells (Fig. 5B). The CCK8 assay, colony formation assay and EdU assay confirmed that the inhibition of melanoma cell proliferation induced by circRTTN silencing was attenuated to some extent by miR-890 knockdown (Fig. 5C-F). CircRTTN silencing induced cell apoptosis, while anti-miR-890 restored this effect (Fig. 5G-H).

Knockdown of miR-890 attenuated the effects of circRTTN knockdown on the migration, invasion, and angiogenesis.

Also, miR-890 downregulation reversed the inhibitory influence of circRTTN knockdown on metastasis and invasion of A-375 and SK-MEL-2 cells (Fig. 6A,B). The tube formation test showed that compared with the corresponding control, HUVEC cultured with si-circRTTN#1 supernatant reduced tube formation, while the addition of anti-miR-890 attenuated this effect (Fig. 6C,D). Furthermore, the addition of anti-miR-890 largely rescued the expression of MMP2 and MMP9 (Fig. 6E). In sum, these data suggested that circRTTN adsorbed miR-890 to regulate proliferation, metastasis, angiogenesis and apoptosis of melanoma

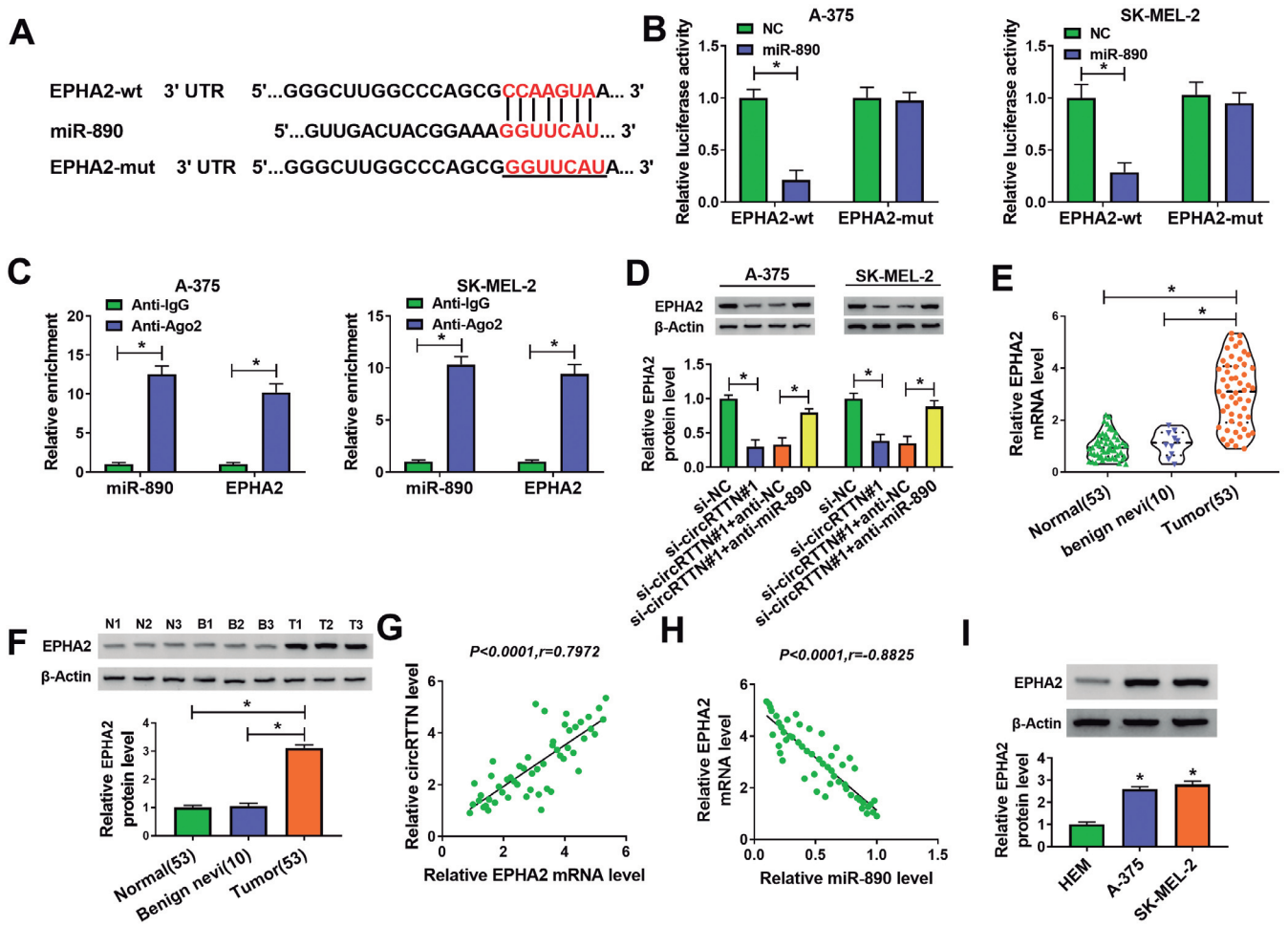

Fig. 7. MiR-890 binds to the 3' UTR of EPHA2. **A.** The putative binding sites of miR-890 in EPHA2 3'UTR. **B-C.** Dual-luciferase reporter assay and RIP assay were performed to confirm the binding between miR-890 and EPHA2. **D.** EPHA2 protein level was examined in melanoma cells transfected with si-NC, si-circRTTN#1, si-circRTTN#1+anti-NC, or si-circRTTN#1+anti-miR-890 respectively by Western blot. **E-F.** The mRNA and protein levels of EPHA2 were determined in melanoma tissues and adjacent normal tissues by qRT-PCR and Western blot. **G-H.** The correlation between EPHA2 mRNA and circRTTN or miR-890 in melanoma samples was analyzed by Spearman's correlation coefcient analysis. **I.** Western blot was used to examine the protein level of EPHA2. **P*<0.05.

cells.

MiR-890 binds to the 3' UTR of EPHA2

The data from TargetScanHuman indicated that the 3'-UTR of EPHA2 contained binding sites of miR-890 (Fig. 7A). Next, the luciferase reporter assay indicated that miR-890 could reduce the luciferase activity of EPHA2-wt cells rather than EPHA2-mut cells (Fig. 7B). RIP assay further verified the binding between miR-890 and EPHA2 (Fig. 7C). EPHA2 expression was down-regulated by circRTTN silencing, and this trend was weakened by the addition of anti-miR-890 (Fig. 7D), indicating that circRTTN positively regulated EPHA2 expression by sponging miR-890 in melanoma cells. EPHA2 mRNA and protein expression was both up-regulated in melanoma tissues compared with adjacent normal tissues and benign nevi (Fig. 7E,F). The linear analysis suggested that EPHA2 mRNA expression was positively related to circRTTN expression (Fig. 7G) but it was negatively associated with miR-890 expression (Fig. 7H) in melanoma samples. Also, EPHA2 protein level was elevated in melanoma cells than HEMn cells (Fig. 7I). Overall, EPHA2 was a target of miR-890 in melanoma cells.

MiR-890 inhibited melanoma cell growth by regulating EPHA2

Transfection efficiencies of miR-890 mimics and EPHA2 plasmid were high in melanoma cells (Fig. 8A,B). To explore if miR-890 exerted a tumor-suppressor role by targeting its target EPHA2, A-375 and SK-MEL-2 cells were transfected with NC, miR-890, miR-890+vector or miR-890+EPHA2, respectively. MiR-890 downregulated EPHA2 protein expression, but addition of EPHA2 upregulated EPHA2 expression in melanoma cells (Fig. 8C). Moreover, EPHA2 restored the inhibitory effect of miR-890 overexpression on the proliferation of melanoma cells (Fig. 8D-F).

MiR-890 suppressed melanoma cell migration, invasion and angiogenesis, but induces cell apoptosis by regulating EPHA2

A-375 and SK-MEL-2 cells were transfected with NC, miR-890, miR-890+vector or miR-890+EPHA2, respectively. MiR-890 markedly induced the apoptosis of melanoma cells, and this tendency was abrogated by EPHA2 (Fig. 9A,B). The migration and invasion of A-375 and SK-MEL-2 cells were inhibited by miR-890 transfection, but EPHA2 overexpression inhibited these effects (Fig. 9C-D). Also, the tube formation test showed that compared with the corresponding control, HUVEC cultured with si-miR-890 supernatant reduced tube formation, while the addition of EPHA2attenuated this effect (Fig. 9E). In addition, miR-890 mimics decreased the expression of MMP2 and MMP9, while the addition of EPHA2 weakened this effect (Fig. 9F). Overall, miR-890 repressed melanoma progression by regulating

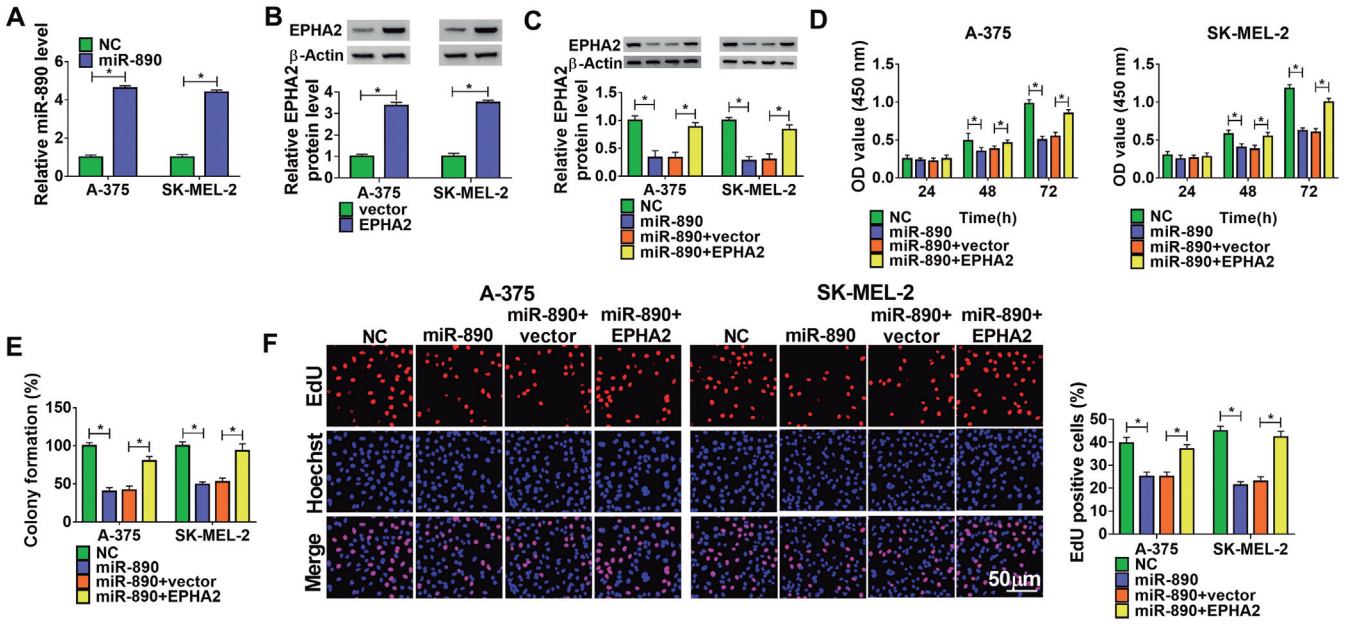

Fig. 8. MiR-890 inhibits melanoma cell growth partly by targeting EPHA2. **A.** Transfection efficiency of miR-890 was analyzed by qRT-PCR. **B.** The protein level of EPHA2 was detected using Western blot in cells transfected with vector or EPHA2. **C-F.** Melanoma cells were transfected with NC, miR-890, miR-890+vector or miR-890+EPHA2, respectively. **C.** EPHA2 protein expression was determined by Western blot. **D-F.** Cell proliferation was assessed by CCK8 assay, colony formation assay and EdU assay. **P*<0.05.

EPHA2 *in vitro*.

CircRTTN knockdown significantly constrained xenograft tumor growth

To probe the functional effect of circRTTN on melanoma development *in vivo*, the xenograft tumor model was established by injecting SK-MEL-2 cells stably transfected with Lenti-sh-circRTTN or Lenti-sh-NC. Compared with the Lenti-sh-NC group, the tumor volume and weight were significantly reduced in Lenti-sh-circRTTN group (Fig. 10A,B). CircRTTN was markedly decreased, while miR-890 was evidently upregulated in Lenti-sh-circRTTN group (Fig. 10C). Additionally, the protein level of EPHA2, MMP2 and MMP9 were significantly decreased in Lenti-sh-circRTTN group (Fig. 10D). Compared with the Lenti-sh-NC group, immunohistochemical staining showed

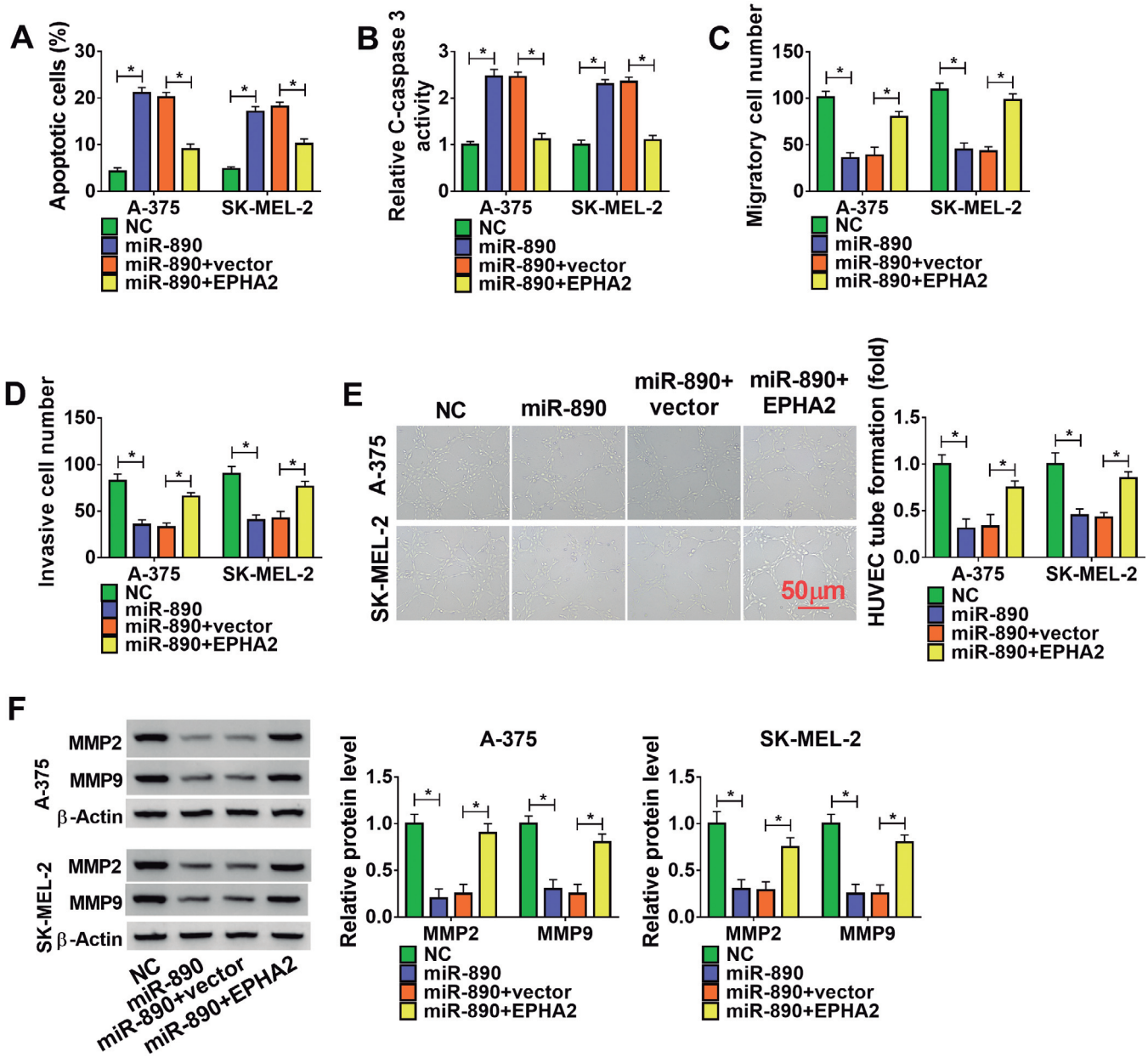

Fig. 9. MiR-890 inhibits the malignant behaviors of melanoma cells partly by targeting EPHA2. Melanoma cells were transfected with NC, miR-890, miR-890+vector or miR-890+EPHA2, respectively. **A-B.** Cell apoptosis was measured by flow cytometry and Caspase Activity Assay Kit. **C-D.** Cell migration and invasion were analyzed by transwell. **E.** Tube formation assay was used for cell tube formation ability. **F.** Western blot was performed to measure the protein expression of marker (MMP2 and MMP9). **P*<0.05.

that the number of ki67 positive cells in xenograft tumors transfected with Lenti-sh-circRTTN was reduced (Fig. 10E). Taken together, circRTTN silencing curbed the growth of melanoma tumors *in vivo*.

Discussion

Malignant melanoma is the deadliest type of skin tumor (Sun et al., 2021). In recent years, numerous studies have shown that circular RNAs (circRNAs) are involved in the dysregulation of certain biological processes, further revealing their close relationship in cancer development (Kristensen et al., 2018). Growing evidence suggests that circRNAs are involved in the malignant development of melanoma (Chen et al., 2021). However, research on circRTTN is still lacking. Our experimental results demonstrated that circRTTN was abnormally upregulated in melanoma tissue samples and cells. Knockdown of circRTTN *in vitro* inhibited cell proliferation, migration and angiogenesis and promoted apoptosis. Likewise, knockdown of circRTTN *in vivo* also inhibited the growth of subcutaneously transplanted tumors. These all indicated that circRTTN was involved in the development of melanoma. Therefore, it was urgent to explore the detailed mechanism by which circRTTN was involved in melanoma development. We predicted that miR-890 and circRTTN had a continuous complementary base through the online bioinformatics website Circular RNA Interactome. Luciferase activity and RIP assays re-examined the binding relationship between miR-890 and circRTTN. miR-890 exerted anticancer effects in triple-negative breast cancer (Wang et al., 2019). It had been reported that overexpression of miR-890 could inhibit the proliferation, migration and invasion of melanoma cells (Xia et al., 2020). Similarly, our experimental results confirmed that miR-890 was lowly expressed in melanoma cancer tissues and cells. Complementary experiments showed that anti-miR-890 attenuated the effect of knockdown of circRTTN on melanoma cell function. This meant that circRTTN directly binded to miR-890 and participated in the progression of melanoma.

The most accepted regulatory mechanism was that miRNAs participate in disease development by interfering with the transcription and translation of target genes (Ayesha et al., 2021). Therefore, we continued to further explore the regulatory mechanism of circRTTN/miR-890.

EPAH2 is a member of the largest RTK subfamily, the EPH receptor family, and is the most frequently altered in cancer (Pasquale, 2010). EPHA2 was upregulated in glioblastoma, and EPHA2 migh be involved in PDGFA signaling in combination with PDGFRA and mediated resistance of GBM cells to PDGFRA inhibitors (Gai et al., 2022). In addition, as an oncogene, EPHA2 was involved in the malignant

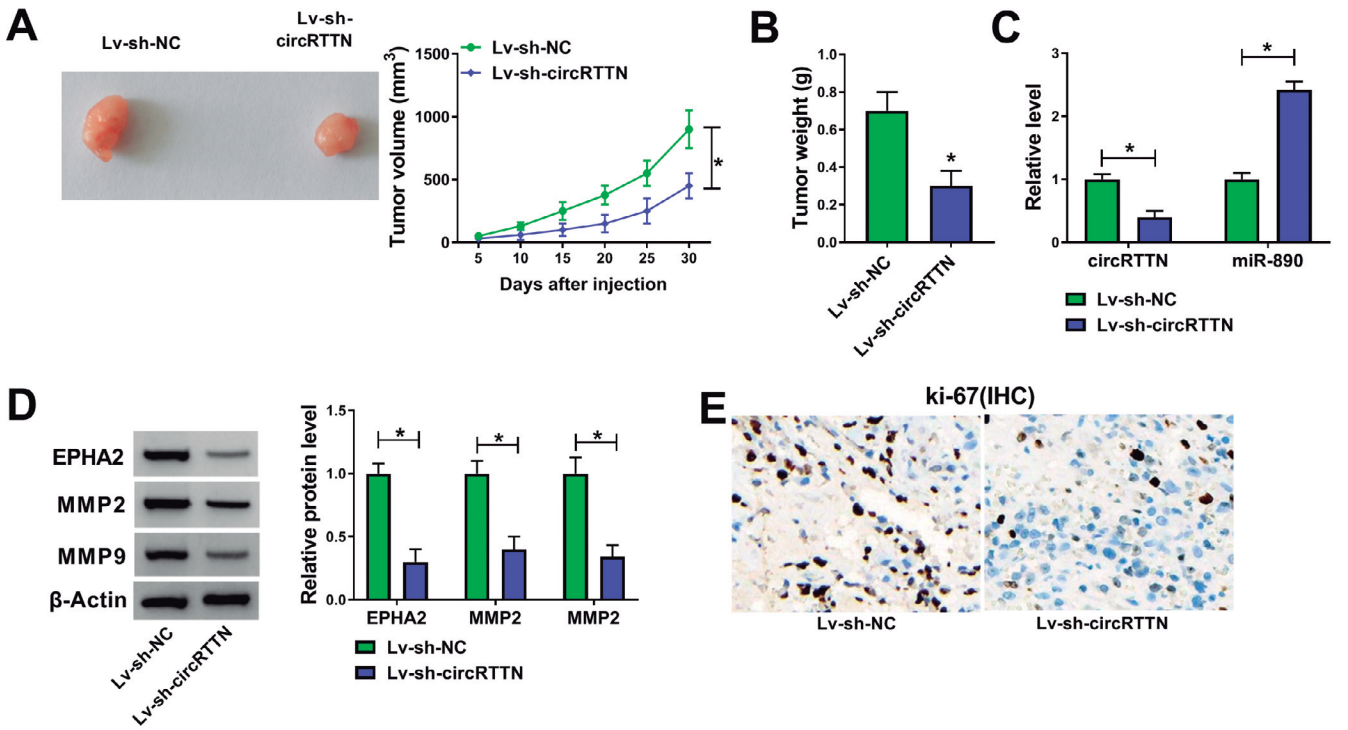

Fig. 10. CircRTTN silencing significantly restrains xenograft tumor growth *in vivo*. SK-MEL-2 cells stable transfected with Lenti-sh-circRTTN or Lenti-sh-NC were injected into the nude mice to establish a xenograft model. Tumor growth was monitored every 5 d for 30 d. **A-B.** Tumor volume and weight, **C.** as well as circRTTN, miR-890 were measured. **D.** Western blot was used to analyze the levels of related proteins in xenograft tumor tissues including EPHA2, MMP2 and MMP9. **E.** IHC assay was utilized to analyze the protein level of ki-67 in resected tumor tissues. **P*<0.05.

development of bladder cancer (Peng et al., 2021). Of course, it also plays a cancer-promoting role in melanoma. Our test results showed that EPHA2 was up-regulated in melanoma. Loss-of-function experiments showed that the addition of EPHA2 could effectively reverse the inhibitory effects of miR-890 on melanoma cell growth and migration. At the same time, we found that circRTTN/miR-890 was able to regulate the expression of EPHA2. It had been reported that the loss of EPHA2 could regulate mitochondrial apoptosis by inactivating the apoptosis-promoting protein BAD induced by p90 RSK/S6K1, thereby inhibiting cell survival (Amato et al., 2014). We also detected that EPHA2 affected caspase-3 activity, indicating that EPHA2 might affect cell apoptosis through the caspase pathway. At the same time, we found that knockdown of crcRTTN down-regulated the expression of EPHA2 to weaken cell proliferation, which might be achieved by blocking the cell cycle. Taken together, circRTTN promoted the development of melanoma by binding to miR-890 and upregulating the expression of EPHA2.

There are limitations to any study. Our experimental results indicated that circRTTN/miR-890/EPHA2 was involved in the development of melanoma. However, the number of tumor samples we obtained was limited and this study was not validated in all melanoma cells. Therefore, the results are for reference only. Despite the limitations, the significance of the findings themselves cannot be ignored. At least, for the first time, we investigated the role of circRTTN in melanoma and dug deep into its mechanism, which might provide therapeutic targets for future melanoma treatment.

Taken together, we found that circRTTN aggravated the malignant development of melanoma cells by inducing the expression of EPHA2 via sponging miR-890.

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Consent for publication. Not applicable.

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Competing interests. The authors declare that they have no financial conflicts of interest

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