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Circular RNA circ_0017247 promotes melanoma migration and invasion *via* targeting miR-145

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Abstract. – OBJECTIVE: The importance of the circular ribonucleic acid (RNA) in malignant tumors causes more attention of researchers. Melanoma is one of the most ordinary malignant tumors. This study aims to identify how circ_0017247 functions in the progression of melanoma.

PATIENTS AND METHODS: Circ_0017247 expression of both melanoma patients' tissue samples and cell lines were detected by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR). Moreover, the function of circ_0017247 was identified by performing Wound healing assay, the transwell assay, the Matrigel assay *in vitro*. Besides, the mechanism assays were performed to uncover the interaction between circ_0017247 and miR-145. In addition, the tumor metastasis assays were also conducted *in vivo*.

RESULTS: In this study, circ_0017247 expression was significantly higher in melanoma tissues compared with that in skin tissue with a melanocytic nevus. The isolated melanoma cells was... circ_0017247 was silenced. Moreover, the number of migrated and invaded melanoma cells were reduced after circ_0017247 was silenced. Further experiments revealed that miR-145 was upregulated *via* knockdown of circ_0017247 and was also a direct target of circ_0017247 in melanoma. Furthermore, the tumor metastasis of melanoma was inhibited *via* knockdown of circ_0017247 in nude mice.

CONCLUSIONS: Our study suggests that circ_0017247 enhances melanoma cell migration and invasion by targeting miR-145 *in vitro* and *in vivo*.

Key words:
Circ_0017247, Melanoma, MiR-145.

Introduction

Melanoma is the most aggressive and fatal skin cancer in the world which is responsible for nearly three-quarters of all skin cancer-related deaths[1]. The incidence of melanoma is increasing steadily every year, approximately 2.8%[2]. The morbidity was highest in Australia, European countries and the United States while lower in South-Eastern Asia[3]. By the end of 2016, it was estimated that almost 76,380 new cases were diagnosed with melanoma and 10,130 patients died because of the development of melanoma in America (http://www.seer.cancer.gov/statfacts/). Though tremendous effects have been made in improving the poor prognosis of the patients, the outcome of the melanoma cases remains depressing[4]. Moreover, the average therapeutic cost of melanoma was increasing rapidly every year when compared to other cancers[5]. Therefore, it is essential to identify the mechanisms underlying the metastasis of melanoma and explore novel treatment targets.

Circular RNAs (circRNAs) are formed by a covalently closed loop which has emerged as a new hot topic in the noncoding RNAs network. Recently, circRNAs have been indicated to be important regulators in multiple physiological and pathological processes of the tumorigenesis. For instance, hsa_circRNA_101996 promotes cell proliferation and cell invasion in cervical cancer by regulating the expression of TPX2[6]. Circ_PTK2 promotes cell proliferation and cell migration in bladder cancer which may be a therapeutic target and a novel potential biomarker for bladder cancer[7]. Circ_LARP4 is significantly down-regulated in ovarian cancer which may serve as a potential biomarker for the prognosis of ovarian cancer patients[8]. The up-regulation of circ-ITCH inhibits cell proliferation and cell metastasis in triple-negative breast cancer by regulating the Wnt/β-catenin pathway[9].

Our work demonstrated that circ_0017247 was remarkably upregulated in melanoma tissues and

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cell lines. Moreover, circ_0017247 knockdown inhibited the metastasis of melanoma *in vitro* and *in vivo*. In addition, we further found that the function of circ_0017247 in melanoma was associated with miR-145.

Patients and Methods

Tissue Specimens

43 malignant melanoma tissues and 35 skin tissues with melanocytic nevus were obtained from patients who underwent surgery during Tangshan Workers Hospital. All tissues were stored at -80°C. This investigation was approved by the Ethics Committee of Tangshan Workers Hospital. The signed written informed consents were obtained from all participants before the study.

Cell Culture

Three melanoma cancer cell lines (WM266-4, SK-MEL-2, and A375), and a human epidermal melanocyte (HEMa-LP), were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maint d in 10% fetal bovine serum (FBS; Inv Carlsbad, CA, USA), Roswell Park Me al Institute-1640 (RPMI-1640; Invitrogen, Carls CA, USA), as well as 1% penicillin/streptomy (Sigma-Aldrich, St. Louis, MO). Beside the cells were cultured in an i at ntaining 5% CO_2 at 37°C.

Cell Transfection

After HCC the c were ed for 24 h on 6-well plates. ells were t cted with circ_0017247 l expressing hairpin RNA (shRN neg control shRNA (NC) using Lipo tamine 300 itrogen, Carlsbad, CA, US nen, shRNA a were synthesized b nePha a (Shanghai, China).

RN n and eal Time-q tative ymerase in Re on -qPCR)

total obtained from the cultured me ma cells or tumor tissues with TRIzol r gen, Carlsbad, CA, USA), was erse transcribed to complementary deoxyribonucleic acids (cDNAs) using Reverse Transion Kit (TaKaRa, Otsu, Shiga, Japan). The pri sequences used for RT-qPCR were as follows: circ_0017247 forward: 5′-ACTGCCAGAAAGTGTGTCCC-3′ and reverse: 5′-TCCTATGAATGAGCCATCTGTCT-3′; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward: 5′-GCACCGTCAAGGCTGAGAAC-3′ and reverse: 5′-TGGTGAAGACGCCAGTGG was performed three times in the f ing s quence: pre-denaturation at 95°C min, followed by 40 cycles at 95°C for 15 C for 30 s, and 72°C for 30 s. The $2^{-\Delta\Delta Ct}$ metho tilized to calculate the relative exp ion.

Wound Healing A

After transfection mel na cell were seeded in 6-well late cubate RPMI-1640 medi vernigh n, th ls were scratched wi plastic tip a ed in serum-free R). Each assa was repeated in triplica indep tly. The relate migrated distance was viewed r a light microscope (Ol Tokyo, Japan) 8 h.

nswell Ass

×10⁴ cells in 0 µL serum-free RPMI-1640 w ransforme the top chamber of an 8 µm por µm pore size, Millipore, Billerica, SA). In the bottom chamber was dded RPMI-1640 and the fetal bovine serum 48 h later, the cotton swab was used to pe top surface of the chambers and immersed for 10 min with precooling methanol, and it was stained in crystal violet for 30 min. The count for the invasion was done in three fields per membrane.

Matrigel Assay

5×10^4 cells in 200 µL serum-free RPMI-1640 were transformed to the top chamber of an 8µm pore size insert (8 µm pore size, Millipore, Billerica, MA, USA) which was lidded with Matrigel (50 µg; BD Biosciences, San Jose, CA, USA). In the bottom chamber was added RPMI-1640 and FBS. 48 h later, the cotton swab was used to wipe the top surface of the chambers and immersed for 10 min with precooling methanol, and it was stained in crystal violet for 30 min. The count for the invasion was done in three fields per membrane.

Luciferase Assay

Circular RNA Interactome (https://circinteractome.nia.nih.gov/) was used to predict the potential target microRNAs and the fragment sequences containing circ_0017247 reaction sites. The circ_0017247 3′-untranslated region (3′-UTR) wild-type (WT) sequence was named circ_0017247-WT and the mutant sequence of

circ_0017247 3′-UTR missing the binding site with miR-145 was named circ_0017247-MUT. The Luciferase reporter gene assay kit (Promega, Madison, WI, USA) was used to detect the Luciferase activity. The Luciferase reporter gene vector was constructed, and the cells were transfected.

Xenograft Model

The transfected cells were injected into the tail vein of NOD/SCID mice (6 weeks old). The mice were sacrificed, and the lung was extracted after 4 weeks. Then, the number of metastatic nodules in the lung was counted. The animal experiments were approved by the Animal Ethics Committee of Tangshan Workers Hospital.

Statistical Analysis

All statistical analyses were performed by the Statistical Product and Service Solutions (SPSS) 21.0 (IBM Corp., Armonk, NY, USA). The independent-sample *t*-test was used to compare the differences between the two groups. Moreover, $p<0.05$ was considered to indicate a statistically significant difference.

Results

Expression Level of Circ_0017247 in Tissues and Cells of Melan

Circ_0017247 expressio melan patients' tissues was detecte RT-q showed that circ_0017 gulated in melanon issue s compared with skin tissues (Figure 1A). Meanwhile, its expression in three melanoma cell lines and a human epidermal melanocyte (HEMa-LP) was also detected. As was shown in Figu circ_0017247 level was significant gher melanoma cells than that in HEM .

Circ_0017247 Knockdown Repressed Cell Migrati nd Invasion in Melanom

In order to explore ffects rc_001 in cell migration a asio the lano-ma cells, the SK EL e was d for the transfectio circ 47 s A. The transfection ency dete RT-qPCR was show e 2A. The und healing assay sh d tha migrated length of SK-MEL-2 cells was re after the transection of 7247 shRNA ure 2B). Moreover, t answell assay showed that the number of rated cells w significantly reduced after the ection of ci 017247 shRNA (Figure 2C). F rmore, th atrigel assay showed that the nu vaded cells was significantly reduced the transection of circ_0017247 hRNA (Figure 2D).

eraction Between MiR-145 and Circ_0017247 in Melanoma

Circular RNA Interactome (https://circinteractome.nia.nih.gov/) was used to find the target microRNAs of circ_0017247. As was shown in Figure 3A, miR-145 was selected from these miRNAs which were interacted with circ_0017247. The RT-qPCR assay showed that the expression

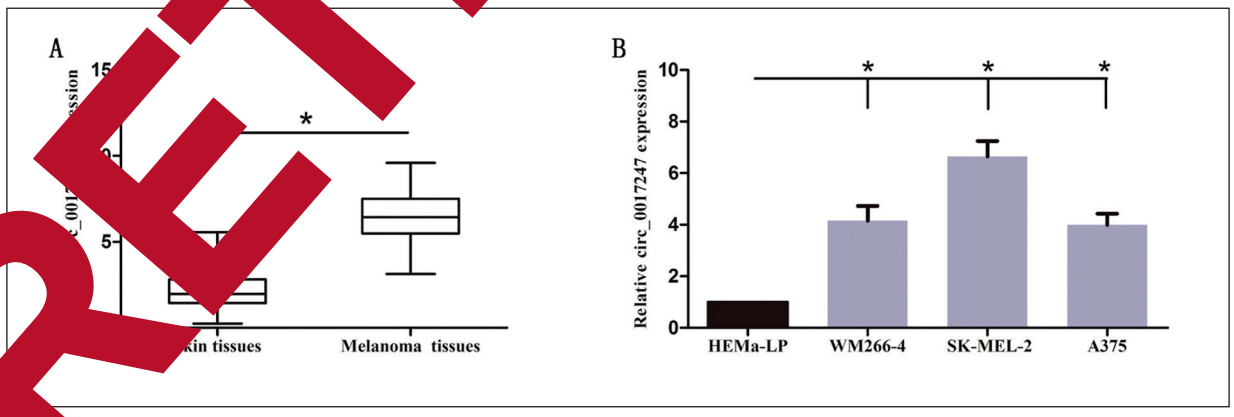

Fig . 1. The expression levels of circ_0017247 were increased in melanoma tissues and cell lines. **A,** Circ_0017247 expression was significantly increased in the melanoma tissues compared with that that in the skin tissues. **B,** The expression levels of circ_0017247 relative to GAPDH were determined in the human melanoma cell lines and a human epidermal melanocyte (HEMa-LP) by RT-qPCR. The data are presented as the mean ± standard error of the mean. *$p<0.05$.

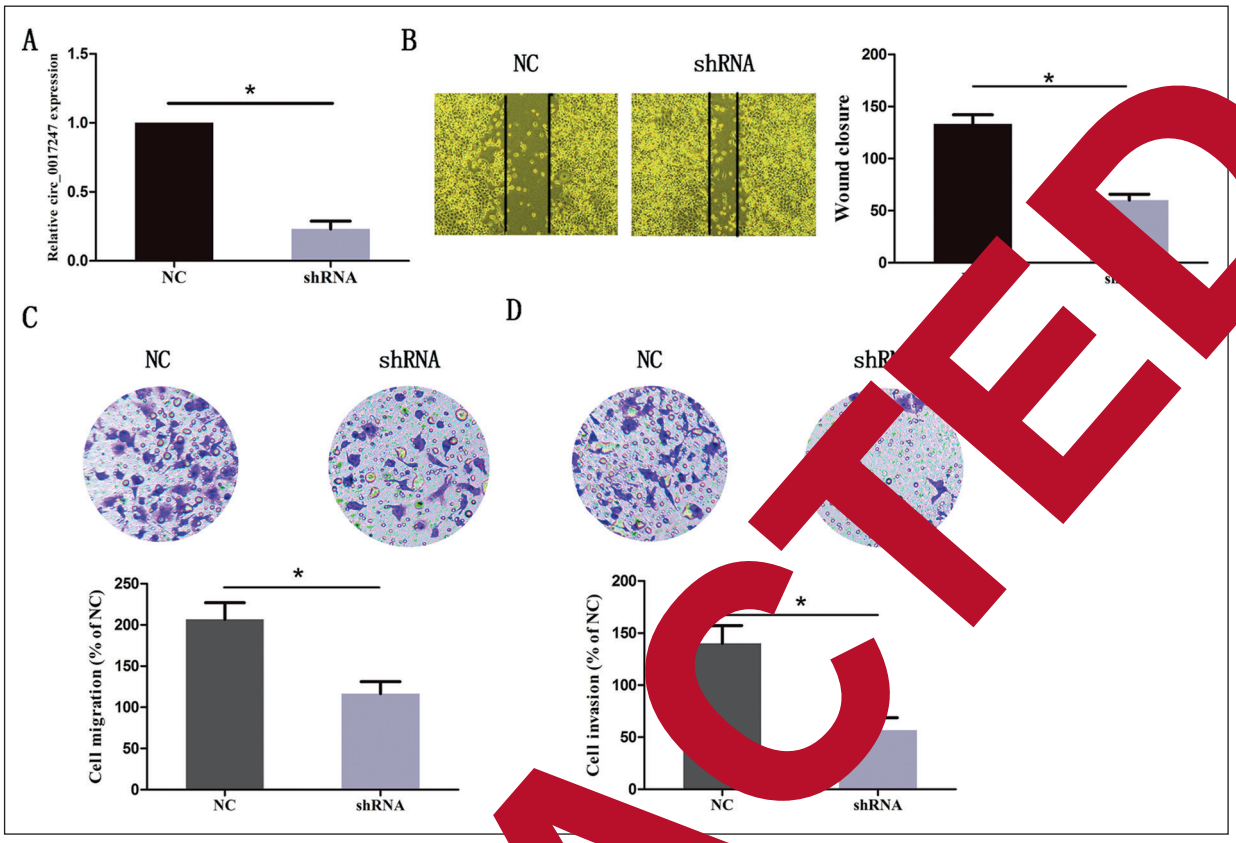

Figure 2. The knockdown of circ_0017247 inhibit[ed] [mel]an[oma] [mi]g[ration] and invasion. **A,** Circ_0017247 expression in melanoma cells transfected with the negative control s[hRNA] (NC) or circ_0017247 shRNA (shRNA) were detected by RT-qPCR. GAPDH was used as an internal control. **B,** The [wound] healing assay showed that the knockdown of circ_0017247 significantly repressed the migrated le[ngth] of melanom[a cells] (magnification: 10×). **C,** The transwell assay showed that the knockdown of circ_0017247 signifi[cantly] repressed cell mi[gration] in melanoma cells (magnification: 40×). **D,** The Matrigel assay showed that the knockdow[n] circ_00[17247] significa[ntly] repressed cell invasion in melanoma cells (magnification: 40×). The results represent the [aver]age of th[ree] independent experiments (mean ± standard error of the mean). *p<0.05, as compared with the control cells.

of miR-145 was [hig]h in the shRNA group than that in the N[C] group (Figure 3B). Meanwhile, the Luciferase assay re[sult]ed that the co-transfection of circ_0017247-W[T] and miR-145 largely increased the Luciferase activity, while the co-transfection of circ_0017247-MUT and miR-145 had no effect on the Luciferase activity either (Figure 3C). Meanwhile, the results of Pearson correlation analysis showed that the expression of miR-145 was negatively correlated to circ_0017247 expression in melanoma tissues (P[<0.05]).

Circ_0017247 Knockdown Repressed Melanoma Metastasis In Vivo

To further identify the inhibited ability of circ_0017247 knockdown in the metastasis of melanoma, we performed tumor metastasis assay in nude mice. As was shown in Figure 4A, the number of metastatic nodules in the lung from the shRNA group was significantly reduced compared to the NC group. RT-qPCR was used to detect the expression of circ_0017247 and miR-145 in those metastatic nodules. The results showed that circ_0017247 was lower-expressed in the shRNA group compared with the NC group (Figure 4B), while miR-145 was higher-expressed in the shRNA group compared with the NC group (Figure 4C).

Discussion

Numerous researches have reported that circRNAs play crucial roles in the progression of melanoma. In fact, circ_0084043, being a sponge of

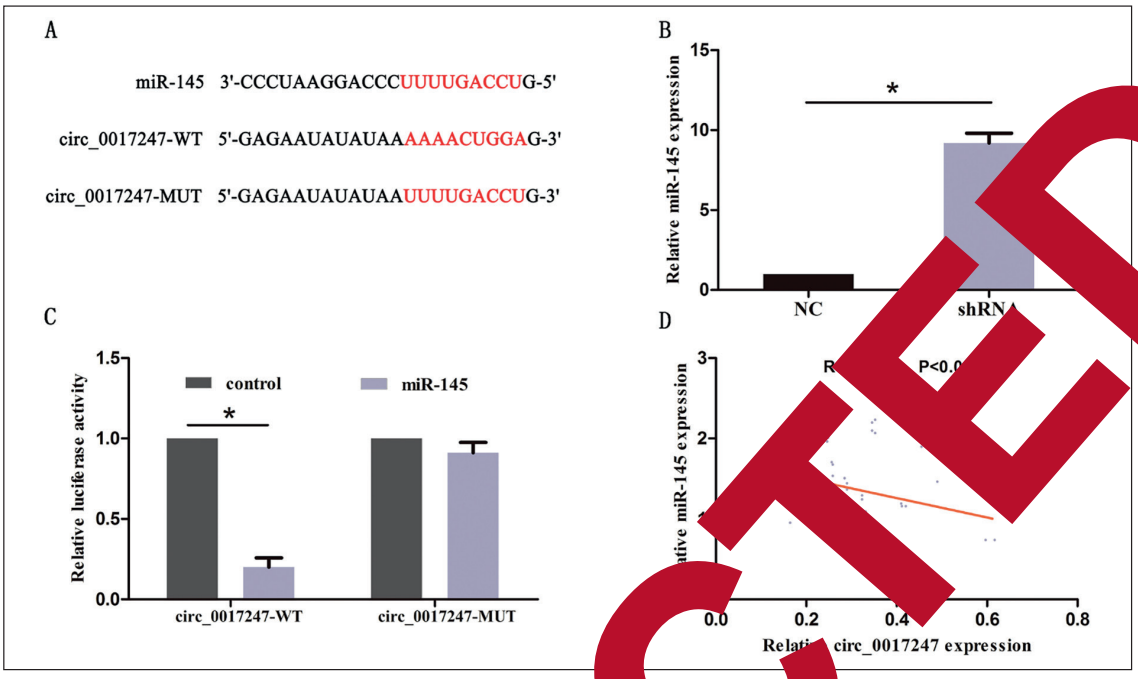

Figure 3. The association between circ_0017247 and miR-145 in melanoma. **A,** The binding area of miR-145 in circ_0017247. **B,** RT-qPCR results showed that the miR-145 expression was increased in the shRNA group compared with the NC group. **C,** The co-transfection of miR-145 and circ_0017247-WT strongly decreased the luciferase activity, while the co-transfection of miR-145 and circ_0017247-MUT did not change the luciferase activity either. **D,** The linear correlation between the expression level of miR-145 and circ_0017247 in melanoma tissues. The results represent the average of three independent experiments. The data are presented as the mean ± standard error of the mean.

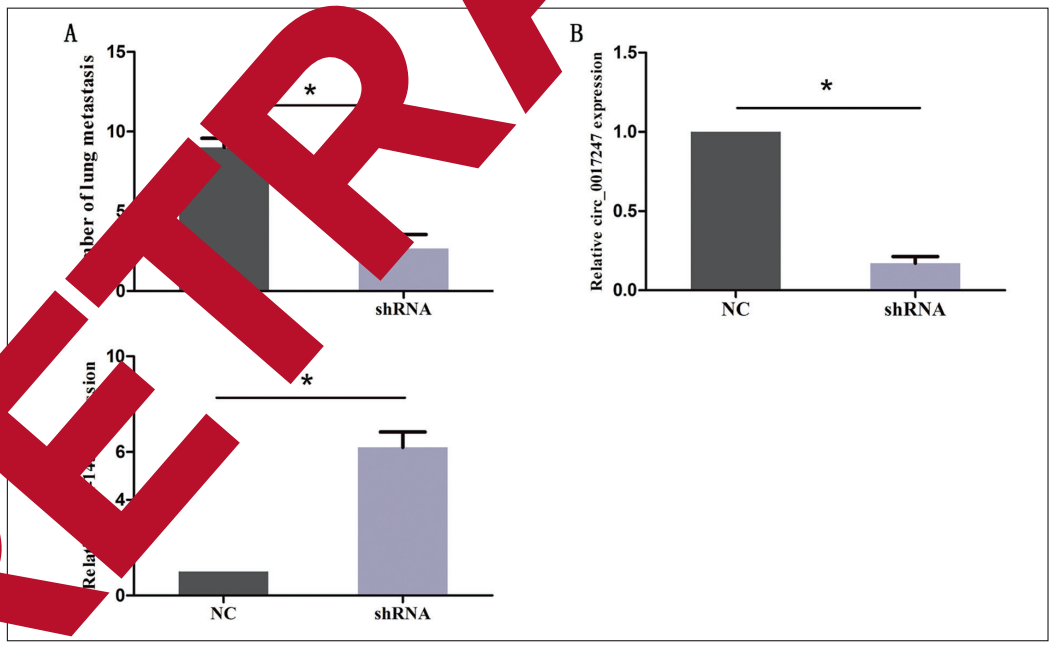

Figure 4. The knockdown of circ_0017247 inhibited tumor metastasis of melanoma *in vivo*. **A,** The number of the metastatic nodules in the lung from the shRNA group was significantly reduced compared to the NC group. **B,** Circ_0017247 of those dissected nodules was lower expressed in the shRNA group compared with the NC group. **C,** MiR-145 of those dissected nodules was higher-expressed in the shRNA group compared with the NC group. The results represent the average of three independent experiments (mean ± standard error of the mean). *$p<0.05$, as compared with the control cells.

miR-153-3p, accelerates cell proliferation and cell migration in malignant melanoma via up-regulating the expression Snail[10]. The knockdown of circ_0025039 inhibits the proliferation, invasion, and colony formation ability of the melanoma cells[11].

Recently, a novel circRNA circ_0017247 was found dysregulated in osteosarcoma[12]. The role of circ_0017247 in malignant tumors remains unknown so far. In the present study, we detected the expression of circ_0017247 in the melanoma tissues and found that it was upregulated compared with the normal skin tissues. To further explore the role of circ_0017247 in melanoma, functional experiments were conducted. The results revealed that circ_0017247 knockdown significantly repressed the ability of cell migration and invasion in melanoma cells, which indicated that circ_0017247 functioned as an oncogene and induced the metastasis of melanoma.

Recent researches discovered that circRNAs function in tumorigenesis of diverse tumors by acting as competing endogenous RNAs (ceRNAs). By regulating the expression of LATS1 and sponging miR-424-5p, circRNA_LARP4 presses the proliferation and invasion of cancer cells[13]. By regulating the express of mir-29a, circ_MYLK functions as an onco and promotes the progression of prostate cance Hsa_circ_0103809 enhances liferatio and inhibits cell apoptosis in ar carcinoma by targeting miR-49 /SOX2 thway[15]. As a miR-1252 sponge, circ_0 bits cell proliferation nd sis in non-small c ung c Therefore, we further explo the potent iRNAs of circ_0017247.

The result th informatics software identified R-145 as a ble target miRNA of circ 247. Previous have depicted that 145 ac as a tumor suppressor in various ncers R-145 decreases the expression em cel elated to transcription factor nation stance, which may be a ther et in colorectal cancer to ove me the ion resistance[17]. By directly targ g ADAM 7, miR-145 inhibits the proli- f hepatocellular carcinoma cells[18]. targeting FSCN1, miR-145 functions as a mor-suppressor in esophageal squamous cell oma[19]. The inhibition ability of miR-145 is also identified in melanoma. The overexpression of miR-145 inhibits cell migration in melanoma by indirectly targeting the following gene[20].

In the present study, the miR-145 expression could be upregulated through the knockdown of circ_0017247. Further experiments revealed that miR-145 was a direct target of circ_001 expression of miR-145 was negative relat to circ_0017247 expression in the anoma tissues. In addition, the knockdown 0017247 also inhibited tumor metastasis *in v* ll these results showed that miR-14 s direct ted by circ_0017247 and fu regulated the stasis of melanoma.

Conclus

Collecti 0017247 co induce melanoma m astasis rgeting miR-145. These findings implied that 0017247/miR-145 axis co ibute to the or melanoma as a p ective target.

Co st

The Au e that they have no conflict of interests.

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