Circ_0002770, acting as a competitive endogenous RNA, promotes proliferation and invasion by targeting miR-331-3p in melanoma

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Abstract
Melanoma is a kind of tumor that originates from melanocytes and is characterized by chemoresistance and distant metastasis. Although the complete pathogenesis of melanoma remains unclear, increasing evidence suggests that circular RNAs (circRNAs) may be involved. In the present study, we identified a circular RNA, circ_0002770, which is produced from the well-known oncogene MDM2, and was sharply increased in melanoma and correlated with a poor prognosis. Knockdown of circ_0002770 suppressed melanoma cell invasion, migration and proliferation. Mechanistically, circ_0002770 acted as a sponge of miR-331-3p and could indirectly regulate DUSP5 and TGFBR1. Inhibition of miR-331-3p reversed the inhibitory effect of si-circ_0002770 on melanoma cell proliferation and invasion. In vivo evidence further confirmed that silencing circ_0002770 inhibited melanoma tumor formation. In conclusion, circ_0002770 facilitated melanoma cell proliferation, invasion and migration by sponging miR-331-3p and modulating DUSP5 and TGFBR1.

Introduction
Melanoma, typically occurring in the skin, is a type of malignant tumor that usually originates from pigment-containing cells known as melanocytes.¹ It accounts for only 3% of the overall skin tumor cases diagnosed every year but is responsible for more than 65% of deaths.² Melanoma is a multifactorial tumor arising from an interplay between environmental exposure and genetic susceptibility, and UV exposure is considered to be the most important risk factor. Early detection and timely treatment of melanoma are considered to be the two most critical factors in reducing mortality.³ Compared to other tumors, melanoma has the advantage of cutaneous location, which allows its early detection. Nevertheless, the gold standard for melanoma diagnosis remains pathological examination.⁴

Circular RNAs (circRNAs), characterized by a covalently closed loop structure, are a novel type of endogenous regulatory RNAs that widely exist in mammalian cells.⁵ Unlike linear RNAs, circRNAs have no 5′ and 3′ ends, which enables circRNA resistance to digestion by endonucleases.⁶ Thus, circRNAs could be expressed in mammalian cells for a relatively long time.⁷ In recent decades, circRNAs have been reported to be involved in many human diseases, such as cancer, neurodegenerative disorders, and metabolic disorders.⁸,⁹ In cancer, research studies have shown that circRNAs could be used not only as potential diagnostic agents but also as promising therapeutic targets for diverse human tumors.¹⁰,¹¹ However, little is known about the mechanism of action of circRNAs in melanoma. Circ_0002770 is a newly identified circRNA that originates from the MDM2 gene. It was reported to be upregulated in five uveal melanoma tissue samples compared to matched normal samples by microarray analysis.¹² However, its functional effects on
melanoma remain undetermined. Additionally, circRNAs were recently demonstrated to play a role in many diseases by acting as a miRNA sponge\(^\text{15}\). The circRNA-miRNA-mRNA axis has also been found to contribute to the tumorigenesis of melanoma\(^\text{16}\). However, due to the variety of circRNAs and the complexity of the interaction between circRNAs and miRNAs, the complete regulatory mechanism of the circRNAs/miRNA axis in melanoma remains unclear.

In this study, we identified circ\(_{0002770}\) as a miR-331-3p sponge in melanoma. Then, we aimed to investigate whether circ\(_{0002770}\) plays a role in melanoma tumor growth in vivo. Untreated A375 cells and cells stably transfected with si-NC or si-circ\(_{0002770}\) siRNA#1-3 (Supplementary Fig. S1A) followed by qRT-PCR detection of circ\(_{0002770}\). Circ\(_{0002770}\) was dramatically decreased in the si-circ\(_{0002770}\) group compared to the untreated and si-NC groups (Supplementary Fig. S1B), suggesting that si-circ\(_{0002770}\) could efficiently knockdown circ\(_{0002770}\) in A375 cells. Therefore, si-circ\(_{0002770}\) was used as the si-circ\(_{0002770}\) in the following experiments. The qRT-PCR results indicated that si-circ\(_{0002770}\) could directly block the expression of circ\(_{0002770}\) but not MDM2 mRNA (\(P<0.05\), Fig. 2a).

Wound-healing and transwell assays were performed to study the effects of circ\(_{0002770}\) silencing on A375 cell migration. The results indicated that A375 cell migratory ability was remarkably suppressed in the si-circ\(_{0002770}\) group compared to the untreated and si-NC groups (\(P<0.05\), Fig. 2b, c). Additionally, by using transwell chambers coated with Matrigel, we found that the A375 cell invasive ability was dramatically suppressed by knockdown of circ\(_{0002770}\) (\(P<0.05\), Fig. 2d). We also obtained similar results in SKMel1 cells (\(P<0.05\), Fig. 2e–h), indicating that knockdown of circ\(_{0002770}\) could inhibit cell migration and invasion in both A375 and SKMel1 cells.

**Results**

**Circ\(_{0002770}\) was increased in melanoma**

By analyzing the UCSC dataset, we found that the exons of the well-known oncogene MDM2 encode three circRNAs (circ\(_{0004448}\), circ\(_{0000416}\) and circ\(_{0002770}\)). Then, we designed convergent and divergent primers to verify these circRNAs based on the cDNA and gDNA of melanoma cells. The results indicated that only circ\(_{0002770}\) could be amplified by divergent primers from melanoma cell cDNA (Fig. 1a). The back-spliced junction of circ\(_{0002770}\) was determined by Sanger sequencing (Fig. 1b). After exposure to RNAseq, we found that linear MDM2 mRNA was significantly degraded, while no alteration was observed in circ\(_{0002770}\) (\(P<0.001\), Fig. 1c). In addition, we observed that melanoma patients with high expression of circ\(_{0002770}\) exhibited a shorter survival time than those with low expression of circ\(_{0002770}\) (\(P<0.05\), Fig. 1d). We examined the level of circ\(_{0002770}\) in three melanoma cell lines (SKMel1, A375 and A875) by qRT-PCR. Circ\(_{0002770}\) was strongly increased in SKMel1, A375 and A875 cells compared to normal human epidermal melanocytes (NHEM) (\(P<0.001\), Fig. 1e). Additionally, the qRT-PCR assay results also revealed that circ\(_{0002770}\) was highly expressed in metastatic melanoma tumor tissues compared to primary tissues (Fig. 1f).

**Knockdown of circ\(_{0002770}\) inhibited melanoma cell migration and invasion**

Next, we investigated the role of circ\(_{0002770}\) in melanoma cell migration and invasion in both A375 and SKMel1 cells. First, A375 cells were transfected with negative control siRNA (si-NC) or circ\(_{0002770}\) siRNA#1-3 (Supplementary Fig. S1A) followed by qRT-PCR detection of circ\(_{0002770}\). Circ\(_{0002770}\) was dramatically decreased in the si-circ\(_{0002770}\) group compared to the untreated and si-NC groups (Supplementary Fig. S1B), suggesting that si-circ\(_{0002770}\) could efficiently knockdown circ\(_{0002770}\) in A375 cells. Therefore, si-circ\(_{0002770}\) was used as the si-circ\(_{0002770}\) in the following experiments. The qRT-PCR results indicated that si-circ\(_{0002770}\) could directly block the expression of circ\(_{0002770}\) but not MDM2 mRNA (\(P<0.05\), Fig. 2a).

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**Knockdown of circ\(_{0002770}\) suppressed melanoma cell proliferation**

The role of circ\(_{0002770}\) in melanoma cell proliferation was also studied in A375 and SKMel1 cells by 3D cell culture and colony formation assays. From 3D cell culture assays, we found that the colony number of si-circ\(_{0002770}\)-transfected A375 and SKMel1 cells was significantly decreased compared to that of the si-NC-transfected group and untreated group (\(P<0.01\), Fig. 3a, b). Moreover, in the 3D cell culture, we observed a significant decrease in colony diameter in the si-NC-transfected group and untreated group (\(P<0.01\), Fig. 3c, d). Additionally, by using a colony formation assay, we demonstrated that circ\(_{0002770}\) knockdown resulted in a remarkable decrease in the colony formation rate in both A375 and SKMel1 cells (\(P<0.01\), Fig. 3e, f). These findings suggested that silencing circ\(_{0002770}\) led to the melanoma cell proliferation suppression in A375 and SKMel1 cells.

**Knockdown of circ\(_{0002770}\) inhibited melanoma tumor growth in vivo**

Based on the above in vitro conclusions, we further investigated the role of circ\(_{0002770}\) in melanoma tumor growth in vivo. Untreated A375 cells and cells stably transfected with si-NC or si-circ\(_{0002770}\) were subcutaneously injected into nude mice for 5, 10, 15, 20, 25, 30, and 35 days. Silencing of circ\(_{0002770}\) sharply decreased the tumor volume and weight compared to those in the untreated group and si-NC group (\(P<0.05\), Fig. 4a, b). Tumors were then cut into 35 μm sections and stained with Ki-67 to estimate the effects of circ\(_{0002770}\) silencing on A375 cell proliferation. The results showed that the Ki-67 staining intensity of tissues from the si-circ\(_{0002770}\) group was dramatically weaker than...
that of tissues from the untreated and si-NC groups ($P < 0.05$, Fig. 4c). We also estimated the effects of circ_0002770 on tumor growth in vivo by using SKMel1 cells. Consistent with the previous findings, tumors formed by the si-circ_0002770-transfected SKMel1 cells were significantly smaller than those formed by untreated or si-NC-transfected SKMel1 cells ($P < 0.05$, Fig. 4d, e). Additionally, the Ki-67 staining score in the si-circ_0002770 group was also significantly reduced compared to that in the untreated and si-NC groups ($P < 0.05$, Fig. 4f).

**Circ_0002770 negatively modulated miR-331-3p**

Studies have determined that circRNAs act as miRNA sponges in various cells; therefore, we screened the potential target miRNAs of circ_0002770 through Qian et al. *Cell Death and Disease* (2020) 11:264
bioinformatics analysis (Circular RNA interactome). 12 miRNAs were predicted to have binding sites with circ_0002770, including miR-1248, 197, 331-3p, 338-3p, 513a-3p, 556-3p, 578, 607, 634, 640, 875-5p. To further validate the target miRNAs of circ_0002770, a qRT-PCR was performed by transfection of si-circ_0002770 or si-NC in melanoma cells and miR-331-3p exhibited the highest alteration (Fig. 5a). Transfection of si-circ_0002770 resulted in a dramatic downregulation of circ_0002770 in A375 cells (Fig. 5b). In addition, the miR-331-3p sequence was conserved among species (Fig. 5c). Moreover, a dramatic upregulation of miR-331-3p was observed in si-circ_0002770-transfected A375 cells (Fig. 5d). Bioinformatics analysis revealed a miR-331-3p binding site in circ_0002770 (Fig. 5e). The expression of miR-331-3p in A375 cells was gradually downregulated with the increased concentration of the circ_0002770 plasmid in transfected cells (Fig. 5f). To verify whether circ_0002770 could physically interact with miR-331-3p, RNA pulldown and AGO2 RIP assay were performed in A375 cells. More than six-fold enrichment of circ_0002770 was observed in the bio-miR-331-3p captured fraction compared with the bio-miR-NC group (Fig. 5g); however, no enrichment of circ_0002770 was observed in the bio-miR-331-3p mut group (Fig. 5i, j). Accordingly, in the AGO2 RIP assay, we observed a significant enrichment in the AGO2/IP fraction in the miR-331-3p group compared to that in the miR-NC group (Fig. 5h); however, the miR-331-3p-induced enrichment in the AGO2/IP fraction was abolished when the complementary sequences of miR-331-3p were mutated (Fig. 5k).

Fig. 2 Knockdown of circ_0002770 inhibits melanoma cell migration and invasion. a Circ_0002770 was sharply decreased in si-circ_0002770-transfected A375 cells, while no changes were observed in MDM2 mRNA expression. b, c Transfection of si-circ_0002770 caused a significant suppression of A375 cell migration. d The invasive ability of A375 cells was dramatically inhibited in the si-circ_0002770 group. e Circ_0002770 was significantly downregulated in si-circ_0002770-transfected SKMEl1 cells, while no changes were observed in MDM2 mRNA expression. f, g Transfection of si-circ_0002770 caused a significant suppression of SKMEl1 cell migration. h The invasive ability of SKMEl1 cells was dramatically inhibited in the si-circ_0002770 group. Scale bars, 50 μm, *P < 0.05.
The circ_0002770/miR-331-3p axis regulated melanoma cell proliferation and invasion

We then examined the effects of miR-331-3p overexpression on melanoma cell proliferation and invasion through colony formation and transwell experiments. The colony formation results indicated that miR-331-3p overexpression sharply decreased the colony number of both A375 and SKMel1 cells compared to that of the miR-NC group (P < 0.001, Fig. 6a, c). In the transwell assay, a dramatic reduction in invasive cell number was
observed in miR-331-3p-overexpressing A375 and SKMel1 cells compared to miR-NC-treated cells ($P < 0.001$, Fig. 6b, d). Since miR-331-3p overexpression exhibited similar effects to those of circ_0002770 knockdown on melanoma cells, we wondered whether miR-331-3p inhibition could reverse the effects of si-circ_0002770 on melanoma cells. By cotransfecting A375 cells with si-circ_0002770 and miR-331-3p inhibitor, we demonstrated that si-circ_0002770-induced downregulation of colony formation was abolished ($P < 0.001$, Fig. 6c).
Fig. 6e). Additionally, in the transwell assay, we revealed that circ_0002770 knockdown caused a reduction in invasive cell number that was reversed in A375 cells cotransfected with si-circ_0002770 and miR-331-3p inhibitor (P < 0.001, Fig. 6f). These findings suggested that the miR-331-3p inhibitor could reverse the suppressive effects of circ_0002770 knockdown on A375 cell proliferation and invasion. Similar conclusions were obtained in SKMel1 cells (P < 0.001, Fig. 6g, h).

**Circ_0002770 indirectly regulated DUSP5 and TGFBR1 through miR-331-3p**

Based on the prediction results by TargetScan, we obtained a putative list of target genes of miR-331-3p.
KEGG analysis revealed that the three signaling pathways with the highest scores included the MAPK signaling pathway (Supplementary Fig. S2A). Then, we constructed ceRNA networks (Supplementary Fig. S2B). Among MAPK signaling pathway factors, we focused on the complementary site between DUSP5 or TGFBR1 and miR-331-3p (Supplementary Fig. S2C, Fig. 7a). First, we examined the regulatory relationship between DUSP5 or TGFBR1 and miR-331-3p through qRT-PCR. Compared to the miR-NC group, miR-331-3p overexpression led to a remarkable downregulation of the mRNAs of both DUSP5 and TGFBR1 in A375 and SKMel1 cells (\(P < 0.05\), Fig. 7b, c). By performing a luciferase reporter assay, we validated the interplay between miR-331-3p and DUSP5 or TGFBR1 in A375 and SKMel1 cells. The luciferase activity was dramatically attenuated only in the miR-331-3p+DUSP-wt and miR-331-3p+TGFBR1-wt treated groups (Fig. 7d). Accordingly, Western blot analysis indicated that miR-331-3p overexpression resulted in a significant downregulation of DUSP5 and TGFBR1 proteins in A375 and SKMel1 cells (\(P < 0.05\), Fig. 7e–g). Additionally, we found that the mRNA levels of DUSP5 and TGFBR1 were remarkably decreased in si-circ_0002770-transfected A375 and SKMel1 cells, and their expression could be restored by the transfection of miR-331-3p inhibitor (\(P < 0.05\), Fig. 7h, i). Similarly, Western blot analysis showed that the protein expression levels of DUSP5 and TGFBR1 were also significantly decreased in si-circ_0002770-transfected A375 and SKMel1 cells, and miR-331-3p inhibitor transfection abolished the downregulation of DUSP5 and TGFBR1 induced by si-circ_0002770 (\(P < 0.05\), Fig. 7j–m). These findings demonstrated the presence of the circ_0002770-miR-331-3p-DUSP5/TGFBR1 axis in melanoma cells.


Discussion

In China, melanoma accounts for less than 4% of all skin tumors but is responsible for more than 80% of skin cancer-related deaths. The greatest challenge in melanoma therapy is the relatively low response to currently available treatment modalities. Chemotherapy fails to dramatically improve the prognosis of melanoma patients due to the resistance of melanoma cells to chemotherapy drugs and toxic side effects. To develop more efficient treatment modalities for melanoma patients, it is essential to understand the regulation of melanoma progression. CircRNAs have been shown to regulate melanoma cell proliferation, metastasis, and invasion through diverse molecular mechanisms, indicating that circRNAs could be critical regulators of melanoma.

Circ_0002770 is a recently identified circRNA that originated from a well-known oncogene, the MDM2 gene. Evidence from both in vitro and in vivo studies has shown that MDM2 is often overexpressed in human tumors and is correlated with tumor grade and prognosis. The MDM2/p53 pathway was revealed to be involved in the regulation of melanoma tumor progression. Recently, circ_0002770 was reported to be dramatically increased in melanoma without functional examination. In our study, we found that circ_0002770 was sharply increased in...
melanoma tissues and cells. In vitro, silencing of circ_0002770 inhibited melanoma cell proliferation, invasion and migration. Similarly, knockdown of circ_0002770 in vivo suppressed melanoma tumor growth. Overall, circ_0002770 was shown to be an oncogene of melanoma.

The circRNA-miRNA-mRNA axis is one of the most common molecular mechanisms by which circRNAs exert their function in cancer regulation, including in melanoma\(^1\). For instance, Bian D et al. reported that a novel circRNA, circ_0025039, facilitated melanoma cell growth, invasion and glucose metabolism by directly regulating CDK4 through interaction with miR-198\(^2\). Additionally, circ_0016418 was revealed to be an oncogene in melanoma through the miR-625/YY1 cascade\(^3\). MiR-331-3p was previously reported to participate in the modulation of tumor growth in various human tumors; however, its effects can be opposite in different tumors. For example, miR-331-3p was revealed to be dramatically downregulated in colorectal cancer and to act as a tumor repressor of colorectal cancer by targeting HER2 through the PI3K/Akt cascade\(^4\). In contrast, miR-331-3p was found to be increased in pancreatic cancer and to function as an oncogene in pancreatic cancer\(^5\). However, the role of miR-331-3p in melanoma remains to be identified. In this study, circ_0002770 was demonstrated to act as a sponge of miR-331-3p, and circ_0002770 could negatively regulate the expression of miR-331-3p in melanoma cells. Additionally, a miR-331-3p inhibitor was found to reverse the suppressive effects of circ_0002770 knockdown on A375 cell proliferation and invasion. Furthermore, we demonstrated that DUSP5 and TGFBR1 are two direct target genes of miR-331-3p, and circ_0002770 could indirectly regulate DUSP5 and TGFBR1 through miR-331-3p in melanoma cells.

In conclusion, our findings suggest that circ_0002770 functions as an oncogene in melanoma by indirectly regulating the expression of DUSP5 and TGFBR1 through miR-331-3p, indicating that circ_0002770 might be a promising therapeutic target of melanoma. While the goal of this study was to better understand circ_0002770 function in melanoma, future research is required to explore the role of circ_0002770 in other cancer types and address the therapeutic potential of modulating circ_0002770.

**Materials and methods**

**Melanoma tissues and cell lines**

A total of twenty pairs of melanoma tumor and normal tissue specimens were provided by the First Affiliated Hospital of Zhengzhou University. All tissues were subjected to qRT-PCR analysis. Research protocols were approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University, and all patients provided written informed consent before enrolling. Tissues were stored at \(-80\) °C until use. Normal human epidermal melanocytes (NHEM) and melanoma cell lines (SKMEL1, A375 and A875) were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were grown in DMEM (Gibco, Thermo-Fisher, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, USA) in a 5% CO\(_2\) humidified environment.

**Quantitative real-time PCR (RT-PCR) assay**

TRIzol reagent (Takara, Japan) was employed to prepare total RNA from melanoma tissues and cells, followed by concentration examination using a NanoDrop 2000c (Thermo Scientific, USA). Then, cDNA was synthesized from total RNA (3 μg) using a cDNA synthesis kit (Takara). The reverse transcripts were amplified by qRT-PCR on the ABI 7500 system (ABI Biosystems, USA) using Bestar qPCR MasterMix (DBI Bioscience). The relative level of expression of miRNAs was normalized to the internal control (U6) using the \(2^{-\Delta\Delta C_{t}}\) cycle threshold method. The primers used in the present study are shown in Table 1.

**Cell transfection**

All oligonucleotides, including negative control siRNA (si-NC), circ_0002770 siRNA (si-circ_0002770),

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**Table 1** The sequences of all primers in the RT-PCR experiment of this study.

| Gene   | Forward (5’–3’) | Reverse (5’–3’) |
|--------|-----------------|-----------------|
| GAPDH  | TGTTCTCTACGCTCGGTTAGA | ATGGTGTTGCTCCTATT |
| Circ_0002770 | GTATCCCGAGGATGTTGGGA | ATCAAGGCTCCTTATTC |
| MiR-331-3p | GCTTGCAGGCTGAGATGG | GTGCAGGGTCCGAGGT |
| MDM2   | GGAAGATGAGAAGAACG | ACCGAGCACTGAGAGAG |
| TGFBR1 | GAAGTACCTGCAGTGAATCC | CACCCTTTCAGCTGAGATGG |
| DUSP5  | TCTCCTTGCGTTCGCTGGT | CTTCCTTCAGCTGAGATGG |
| U6     | CTCGCTTCGAGCCACA | AATCTGCAGAATTTCGCTG |

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circ_0002770 overexpression plasmid, miRNA negative control (miR-NC), miR-331-3p mimics and miR-331-3p inhibitor, were designed and provided by Ribobio (Shanghai, China). For cell transfection, A375 and SKMel1 cells were plated into 6-well plates and cultured until approximately 80% confluence, and then the indicated 50 nM oligonucleotides, siRNAs or constructs were transfected into cells by using Lipofectamine 3000 (Invitrogen), according to the manufacturer’s protocol.

Wound-healing assay
Treated A375 and SKMel1 cells (3000 cells/per dish) in exponential growth phase were harvested and seeded into 35-mm glass dishes. Cells were cultured in an incubator at 37 °C until 100% confluence. A sterile pipette tip was used to make a straight wound on the surface of A375 and SKMel1 cells. Images were acquired after 0 and 24 h of wounding, and the width of the wound was examined under a microscope.

Transwell assay
Transwell chambers coated with (40 μl of 2 mg/ml Matrigel) or without Matrigel (Becton, Dickinson and Company, USA) were used to estimate the migratory or invasive abilities, respectively, of A375 and SKMel1 cells. Culture medium without or with FBS was added to the upper or lower chambers, respectively. Transfected A375 and SKMel1 cells were harvested and seeded into the upper chamber followed by incubation at 37 °C in a cell incubator for 24 h. Then, the bottom of the chamber was washed with precooled PBS, and the cells were fixed with 4% paraformaldehyde. After 10 min incubation with 0.5% crystal violet, the number of invasive or migratory cells was counted under a microscope at 10× magnification (Nikon, Japan).

Three-dimensional (3D) cell culture
Culture medium containing one thousand treated melanoma cells (A375 and SKMel1) and 4% Matrigel (BD Bioscience) was added into 96-well plates, which were placed at 37 °C in a cell incubator with 5% CO₂. Matrigel-containing medium was renewed every 2 days until the assay was terminated after two weeks.

Colony formation assay
Transfected A375 and SKMel1 cells were uniformly dispersed and seeded on six-well plates at a concentration of 3 × 10⁴ cells per well. After culturing at 37 °C in an incubator with 5% CO₂ for 14 days, visible colonies were fixed in 10% formaldehyde (Sigma, USA) followed by staining with 1% crystal violet (Sigma) for 10 min. Next, colonies were washed with precooled PBS three times and then counted manually.

In vivo tumor growth assay
All animal experiments were subject to approval by the Institutional Review Board of the First Affiliated Hospital of Zhengzhou University (Zhengzhou, China). Nude male mice, eight weeks old, were kept in the animal room with a twelve-hour day and night cycle. 1 × 10⁴ untreated A375 and SKMel1 cells or A375 and SKMel1 cells stably expressing si-NC or si-circ_0002770 were subcutaneously injected into nude mice randomly for 5, 10, 15, 20, 25, 30, and 35 days. The mice were sacrificed at the end of treatment (day 35) to harvest tumors and tumor volume and weight were measured. Anatomical tumors were subjected to subsequent detection of cell proliferation using Ki-67 staining. The animal study in this study was ethical.

Ki-67 staining
The anatomical tumors were fixed in paraffin and then cut into 4 μm sections by using a freezing microtome. Tissue sections were subjected to nonspecific blockade with 10% donkey serum for 2 h. Next, sections were incubated with anti-Ki-67 antibody (1:500, ab15580, Abcam) overnight, followed by incubation with HRP-conjugated secondary antibody for 2 h.

RNA pulldown assay
A375 cells (1 × 10⁶) seeded in 6-well plates were transfected with biotinylated miR-331-3p (biotin-miR-331-3p) or its mutant (biotin-miR-NC) using Lipofectamine 3000 (Invitrogen). After 24 h of transfection, the cells were collected and centrifuged. Next, the pellets were resuspended in lysis buffer containing protease inhibitors. The cytoplasmic lysates were prepared by centrifugation. Cell lysates were then incubated with bovine serum albumin (Sigma) on the treated beads for 4 h. Finally, RNAs in the beads fraction were extracted with TRIzol and examined with qRT-PCR assay.

RNA immunoprecipitation assay (RIP)
A375 cells were transfected with miR-331-3p mimics or miR-NC for 48 h, and cytoplasmic lysates containing protease inhibitors (Roche) were subjected to immunoprecipitation (IP). AGO2-miRNA complexes were used for IP with a control IgG for 4 h. RNAs bound to the beads were separated using phenol-chloroform extraction (Ambion) and then subjected to ethanol precipitation. Extracted RNAs were examined by qRT-PCR analysis.

Dual luciferase reporter assay
The wild-type and mutant fragments of DUSP5 and TGFBR1 containing the specific miR-331-3p binding sites were amplified and subcloned into the luciferase vector psi-CHECK (Promega, USA) and then named DUSP5 wt/TGFBR1 wt and DUSP5 mut/TGFBR1 mut. A375 cells (1 × 10⁶) were plated into 24-well plates and cultured at...
37 °C overnight. Then, cells were cotransfected with DUSP5 wt/TGFB1 wt or DUSP5 mut/TGFB1 mut and miR-NC or miR-331-3p mimics using Lipofectamine 3000 (Invitrogen). Luciferase activities were detected by the Dual Luciferase Reporter Assay System (Promega, WI, USA) after 48 h of cotransfection.

Western blot analysis
Total proteins were obtained from treated melanoma cells using RIPA buffer and then subjected to protein quantification using a BCA kit (Pierce). Equal protein samples (50 μg) were loaded into and isolated by 10% SDS-PAGE and then transferred to polyvinylidene fluoride membranes. The nonspecific binding sites in the membranes were blocked by incubating with TBST solution containing 5% milk for 2 h. After washing with PBS again three times and then incubated with HRP-conjugated secondary antibodies (1:2000, Santa Cruz) for 2 h. Protein bands were detected by an Odyssey infrared imaging system (LI-COR).

MiRNA targets prediction of circ_0002770
We predicted the specifically binding sites of circ_0002770 interacting with miRNA by using bioinformatic database Circular RNA interactome (https://circinteractome.nia.nih.gov/index.html).

Statistical analysis
Data from the present study are presented as the means ± standard deviation. SPSS (version 20.0. SPSS, Chicago, USA) was employed to analyze the differences between groups using Student’s t-test or one-way ANOVA. A P value less than 0.05 was regarded as statistically significant.

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Competing interests
The authors declare no competing interests.

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