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Original Article

Targeting miR-155 inhibits survival of melanoma cells by upregulating FOXO3a

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Abstract: *Objective:* MicroRNA-155 (miR-155) is frequently up-regulated in various types of human cancer; however, its role in melanoma remains unknown. In the present study, we study the role of miR-155 on growth and apoptosis of melanoma cells through upregulation of FOXO3a in melanoma. *Methods:* Effects of miR-155 overexpression or miR-155 downregulation on growth and apoptosis of melanoma A2058 cells in vitro were detected. Fox03a siRNA or Fox03a cDNA transfection was used to examine the miR-155 signaling pathway. *Results:* Targeting miR-155 inhibited growth and induced apoptosis of A2058 cells in vitro, whereas miR-155 overexpression promotes cell survival. Further, we identified FOXO3a as a direct target of miR-155. Sustained overexpression of miR-155 resulted in repression of FOXO3a protein and mRNA, and targeting miR-155 increases FOXO3a expression. Introduction of FOXO3a cDNA abrogates miR-155-induced A2058 cell survival. Targeting FOXO3a by FOXO3a siRNA rescued miR-155 inhibitor-induced apoptosis. *Conclusions:* Our study reveals a molecular link between miR-155 and FOXO3a and presents evidence that miR-155 is a critical therapeutic target in melanoma.

Keywords: Melanoma, microRNA-155, FOXO3a, apoptosis

Introduction

Melanoma, a skin cancer arising from malignant transformation of melanocytes, is the most aggressive form of skin cancer [1]. There is no curative therapy for advanced stages of melanoma [2]. Therefore, new indicators of prognosis and therapeutic targets are demanded.

MicroRNAs (miRNAs) are small, non-coding, 19-22 nucleotide long RNA molecules, which function as specific epigenetic regulators of gene expression by inhibiting protein translation, leading mRNA to degradation, or both [3, 4]. MiRNAs are involved in the regulation of embryonic development, cell cycle, cell differentiation, apoptosis and angiogenesis [5]. They are also directly implicated in cancer development, progression and metastasis in vitro, in-vivo and reported even in patients [4, 6]. In some cases, cancer is facilitated by the loss of certain miRNAs, such as miR-186 in prostate cancer [7], miR-34a in uveal melanoma [8] and miR-31 in mesothelioma [9]. The loss of these miRNAs enhances invasiveness, migration and proliferation of cancer cells. In other cases, cancer is facilitated by the over-expression of other miRNAs, such as miR-17-92 cluster [10], which promotes migration and invasion in several malignancies.

Currently, our knowledge on the roles of miRNAs in melanoma development and progression is still limited. Overexpression of miR-221/222 in melanoma cells yielded an increased proliferative rate, whereas its inhibition strongly reduced cell growth, invasion and foci formation in vitro and impaired in vivo tumor growth [11]. Moreover, the miR-221/222 cluster can also modulate other regulators of proliferation, including the tumor suppressors PTEN and TIMP3 [12]. Interestingly, miR-221/222 also targets the oncogenic tyrosine kinase receptor c-Kit [13], which is shown to be repressed in certain types of melanoma [14]. Recently, some miRNAs have been shown to contribute to the regulation of MET expression. In particular, miR-34b, miR-34c and miR-199a-3p were found to directly target MET causing a reduc-

tion in its mRNA and protein levels. Accordingly, inhibiting these miRNAs increased MET protein expression and significantly enhanced migration and cell adhesion in cell lines [15].

MicroRNA-155 (miR-155) is processed from the B-cell integration cluster (BIC), a noncoding transcript primarily upregulated in both activated B and T cells [16] and in monocytes/macrophages upon inflammation [17, 18]. Recent gene-targeting studies of miR-155 demonstrate a broad role for miR-155 in the regulation of both immune cell development and function [19, 20], and miR-155 dysregulation is closely related to cancer [21]. Interestingly, another recent study revealed the loss of miR-155 inhibited solid tumor, suggesting that the role of miR-155 may vary depending on the tumor type and the model system. MiR-155 has also been shown to modulate melanoma proliferation in vitro, but no targets or downstream modulators have been reported, the underlying mechanisms remain unclear.

The forkhead box class O3a (Fox03a) is one of the four members (Fox01a, Fox03a, Fox04, and Fox06) belonging to the subfamily of winged-helix forkhead transcription factors (FoxOs), whose functions are negatively regulated by the insulin-phosphatidylinositol 3-kinase (PI3K)-protein kinase B (PKB) signaling [22]. The phosphorylation of FOXO3a by these kinases leads to its translocation from the nucleus to the cytoplasm and loss of the pro-apoptotic function. In the unphosphorylated active form, FOXO3 a resides in the nucleus and induces cell death by up-regulation of apoptotic proteins, such as BIM, p27, BNIP3, and 24p3 [23, 24] and repression of antiapoptotic molecule FLIP and BCL-XL [25]. BIM is a pro-apoptotic BH3-only Bcl2 family member that induces apoptosis via the mitochondria. Among Bcl-2 family members, BIM is remarkable in that it can play a major role in mediating apoptosis on its own [26]. For example, Bim/Bcl2l11 overexpression is sufficient to induce massive T cell death [27]. Bim/Bcl2l11 expression is regulated by FOXO3 [28, 29]. FOXO3 protein levels can be downregulated by miR-155 [30, 31], resulting in a decrease in BIM activity.

In the present study, we assessed the effect of targeting miR-155 on apoptosis and growth of melanoma cells in vitro and vivo, and to explore its molecular mechanisms. We report that miR-155 induces cell survival a in melanoma. Its anti-apoptotic function is mediated by inhibition of FOXO3a. Thus, our findings not only demonstrate regulation of FOXO3a at post-transcriptional levels but also identify miR-155 as a critical therapeutic target in melanoma.

Materials and methods

Cell line and cell culture

The human melanoma cell line A2058 was purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China), and grown in Dulbecco's modified Eagle's medium (DMEM). Cultures were supplemented with 100 μg/mL streptomycin, 100 U/mL penicillin, 1% glutamine, and 10% FBS (pH 7.2 to 7.4) and maintained in a humidified atmosphere containing 5% CO_2 at 37°C.

Oligonucleotides transfection

The miR-155 mimic (miR-155), inhibitor (anti-miR-155), and related negative controls (NCs), siRNA against Fox03a were designed and synthesized and purified by Genepharma (Shanghai, China). pcDNA3-Fox03a and the pcDNA3 vector control were purchased from Addgene. Transfection was performed using Lipofectamine 2000 (Invitrogen, USA), and the concentration of anti-miR-155, miR-155 and siRNAs were 50 nM, 5 nM and 100 nM, respectively. PcDNA3-Fox03a (Addgene) and the pcDNA3 vector control using the GenJet Plus transfection reagent (SignaGen), according to the recommendations of the manufacturer. Cells were used for the necessary experiments 72 h after transfection. Target gene knockdown was confirmed by qRT-PCR and western blot techniques.

Quantitative real-time PCR (qRT-PCR)

For miR155 expression analysis, qRT-PCR was done by using the TaqMan microRNA reverse transcription kit (Applied Biosystems) and TaqMan microRNA assays kit (Applied Biosystems) following the manufacturer's protocols. U6 SnRNA served as the internal control. For mRNA, total RNA was prepared using TRIzol reagent (Invitrogen) and genomic DNA was eliminated with TURBO DNA-free Kit (Ambion). One μg of total RNA was reverse transcribed using iScript reverse transcriptase (Bio-Rad).

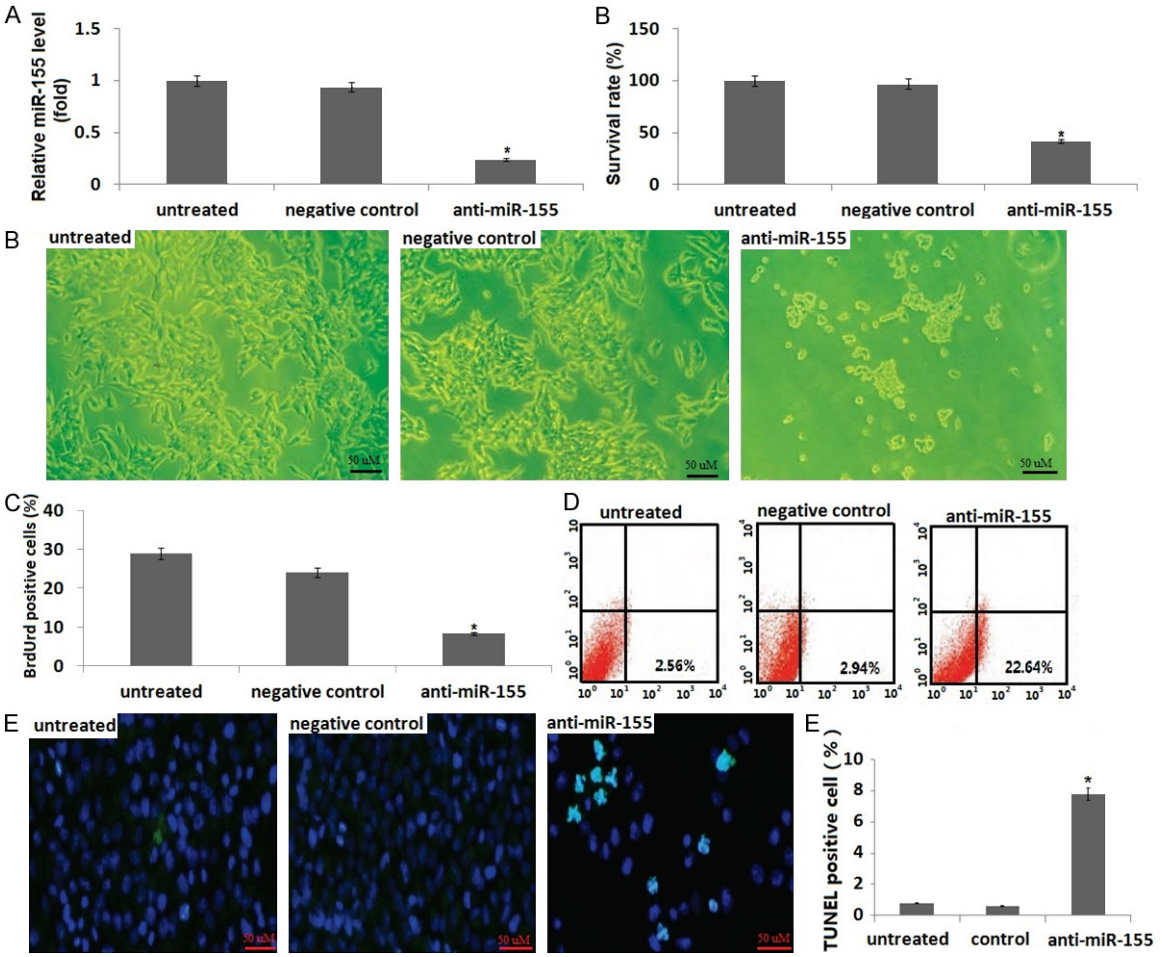

Figure 1. Targeting miR-155 on growth, apoptosis, and proliferation. Anti-miR-155 was transfected into A2058 cells for 72 h. A. Targeting miR-155 in A2058 cells by qRT-PCR assay. B. Growth rate of A2058 cells transfected with anti-miR-155 or control were determined by MTT assays. C. Quantification of the BrdUrd incorporation assay in the indicated cells. D. Effects of anti-miR-155 or negative control on cell apoptosis, as analyzed by flow cytometry. E. Effects of anti-miR-155 or negative control on cell apoptosis, as analyzed by TUNEL assay. Vs control, *P<0.01.

Omission of reverse transcriptase served as a negative control. CDNA was amplified using Platinum PCR SuperMix (Invitrogen). PCR was performed as follows: 5 min at 94°C, 32 cycles of 60 s at 94°C, 60 s at 58°C, and 60 s at 72°C, followed 5 min at 72°C. Real-time quantitative RT-PCR analysis was done using FoxO3a and β-actin primers from Applied Biosystems or Integrated DNA Technologies.

Western blot assay

Whole cell lysates from transfected A2058 cells were prepared using RIPA buffer. Proteins were analyzed by SDS-PAGE, transferred to nitrocellulose membranes and probed with the FoxO3a antibody (Santa Cruz). Western blots were quantified by densitometry.

Cell-viability analysis (MTT assay)

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay was used to determine cell viability. For the MTT assays, A2058 cells in different groups (~6,000 cells/well) were seeded in 96-well plates, using Dulbecco's Modified Eagle's Medium, and cultured for 72 hours. On the day of the viability assay, fresh medium (~100 μL/well) and MTT solution (5 mg/mL in phosphate-buffered-saline [PBS]) was added to each well, and the plates were incubated at 37°C for at least 3 hours. At the end of the incubation period, the medium and MTT solution were removed from each well and dimethyl sulfoxide was added. The 96-well plates were then gently shaken for 30 minutes. In this type of experi-

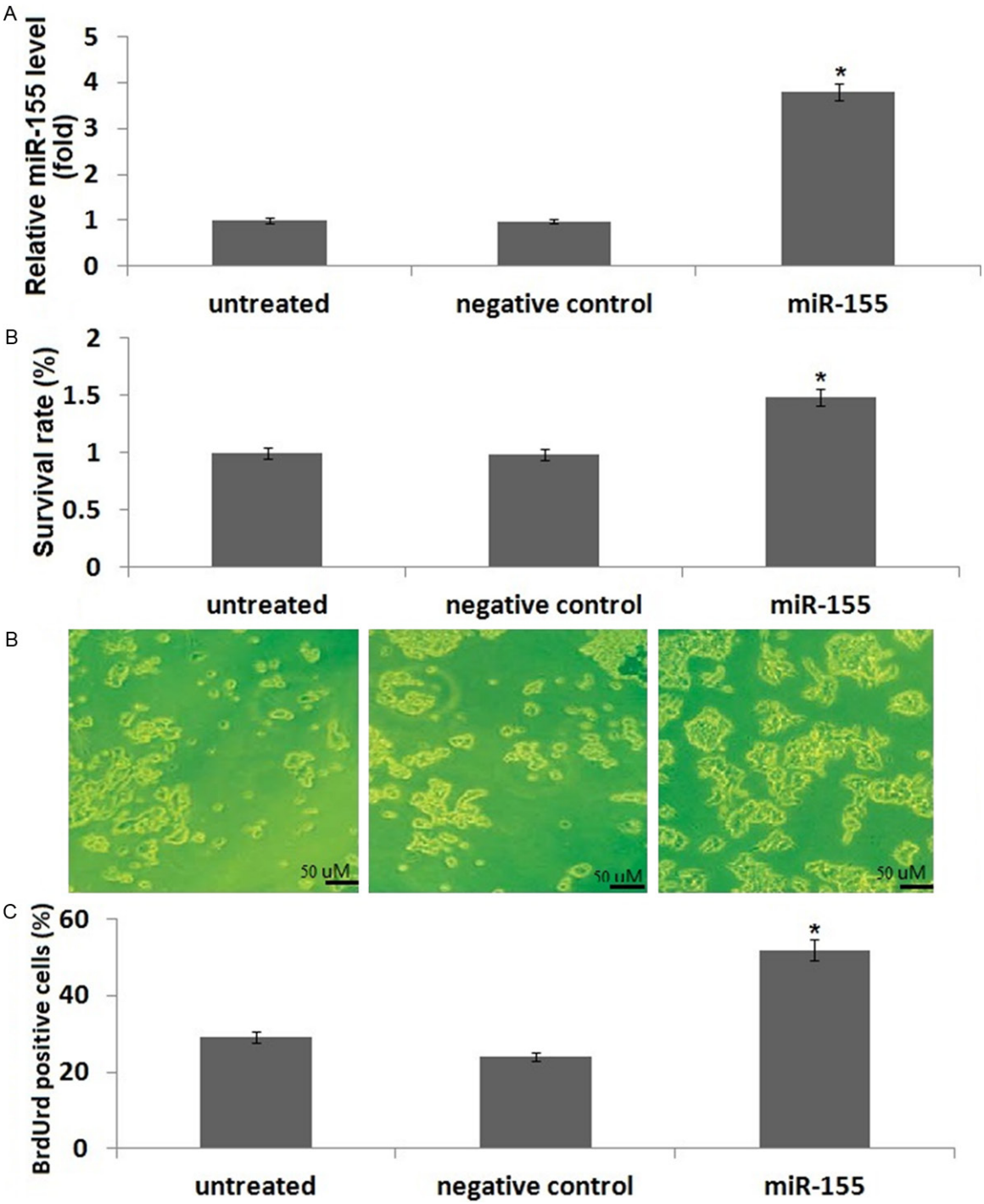

Figure 2. Effect of miR-155 overexpression on growth and proliferation. MiR-155 mimics was transfected into A2058 cells for 72 h. A. MiR-155 levels in A2058 cells by qRT-PCR assay. B. Growth rate of A2058 cells transfected with miR-155 or control were determined by MTT assays. C. Quantification of the BrdU incorporation assay in the indicated cells. Vs control, *P<0.01.

ment, optical density was measured at 590 nm. The percentage of viability was calculated compared with untreated control (100% viability). The experiment was repeated several times. In all instances, similar results were obtained.

Proliferation assay

A2058 cells in different groups were incubated with 10 μM BrdU for 60 min before harvesting the cells at designated time points after plat-

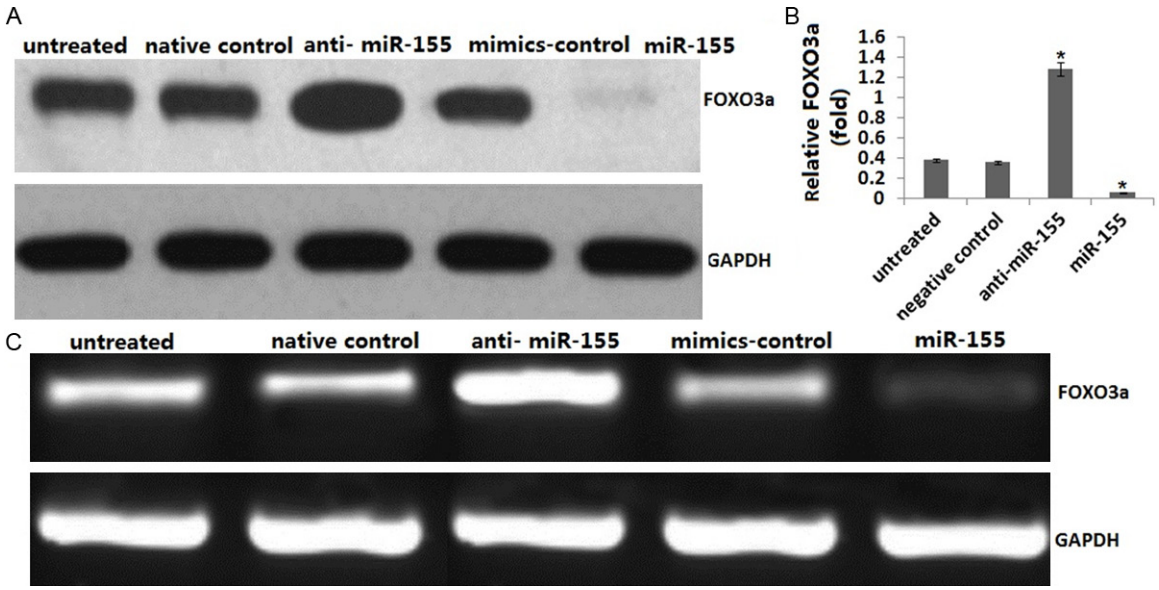

Figure 3. *FOXO3a* was regulated by miR-155 in A2058 cells. A2058 cells were transiently transfected anti-miR-155 or miR-155 or negative control for 72 h. A. The expression levels of *FOXO3a* was analyzed by western blotting. B. *FOXO3a* mRNA expression was analyzed by Real-time PCR; C. *FOXO3a* mRNA expression was analyzed by semi-quantitative RT- PCR. GAPDH was used as a control. Vs control, *P<0.01.

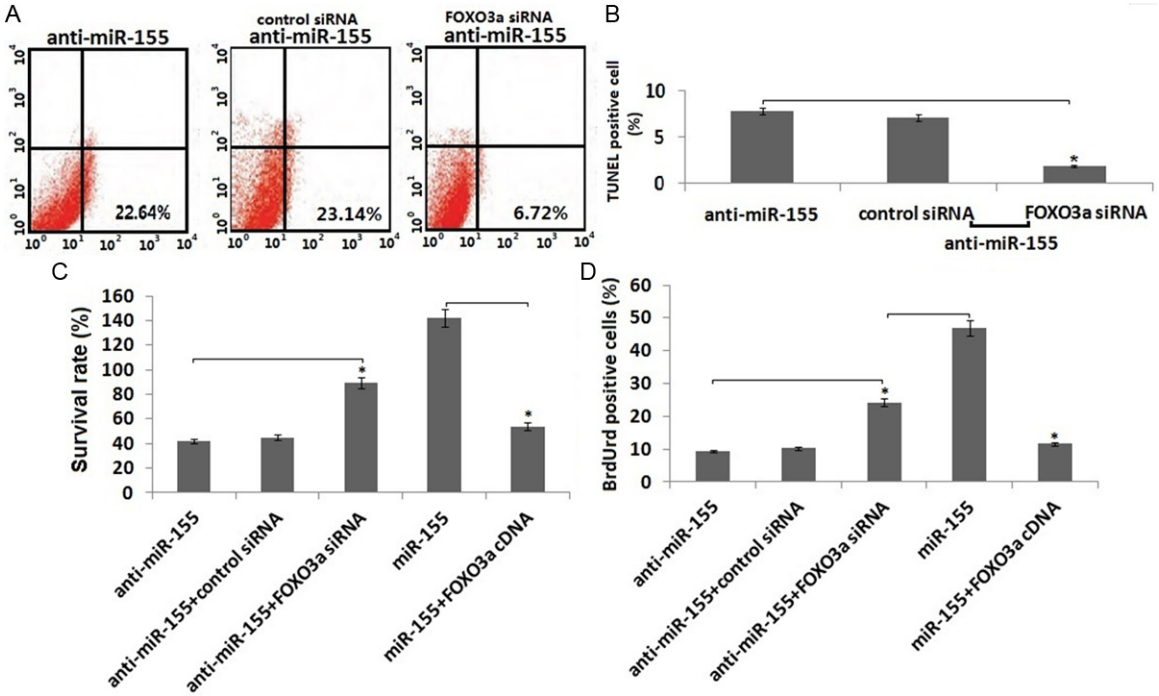

Figure 4. *FOXO3a* mediates anti-miR-155-induced apoptosis and growth inhibition. A2058 cells were transiently co-transfected with anti-miR-155 or/and *FOXO3a siRNA*, or A2058 cells were transiently co-transfected with miR-155 or/and *FOXO3a cDNA for 72 h. A.* Cell apoptosis was detected by flow cytometry assay; B. Cell apoptosis was detected by TUNEL assay; C. Cell viability was detected by MTT assays; D. Cell proliferation was detected by BrdU incorporation assay. *P<0.01.

ing. After the 60-min BrdU incubation, A2058 were washed twice with PBS, removed from the plates with TrypL Express (Invitrogen, Hangzhou, China) and centrifuged at 500×g for 5

min, then fixed in ice-cold 70% ethanol. DNA was then denatured using 2N HCl for 30 min and then BrdU incorporation was detected with a FITC-conjugated monoclonal antibody raised against BrdU (5 μg/mL; Roche Applied Sciences, Hangzhou, China) in PBS with 0.1% bovine serum albumin (BSA). A2058 (20,000 cells) were analyzed using a FACScan flow cytometer and Cellquest Pro software, both from BD Biosciences (San Jose, CA).

DNA fragmentation with TUNEL

Cells were transfected with Lipofectamine 2000 (Invitrogen, Hangzhou, China), and TUNEL assays were performed by using the classical terminal deoxy ribonucleotidyl transferase-mediated uridine 5'-triphosphate-biotin nick-end labelling test, all according to the instructions of the manufacturer for the in situ technique.

Flow cytometry analysis

Following the treatment protocol described above, cells in early and late stages of apoptosis were detected with an annexin V-FITC apoptosis detection kit from Bio Vision (Mountain View, CA) as the manufacture's instruction.

Statistics

All analyses were done with statistical software SPSS 17.0 (SPSS Inc.).

Results

Effect of targeting miR-155 on growth, apoptosis, and proliferation

Anti-miR-155 was transfected into A2058 cells for 72 h. A~ 4-fold down-regulation of miR-155 was confirmed by qRT-PCR analysis using miR-155 and U6 SnRNA as normalization controls (**Figure 1A**). To determine the effect of targeting miR-155 on viability and proliferation of A2058 cells, MTT and BrdU assay was used. As shown in **Figure 1B**, targeting miR-155 caused a significant decrease in cell viability and growth. Furthermore, targeting miR-155 increases BrdU incorporation in A2058 cells (**Figure 1C**), indicating that targeting of miR-155 in A2058 cells inhibits the DNA synthesis.

Next, we examined whether the inhibition of cell growth by targeting miR-155 was also accompanied by the induction of apoptosis. We first analyzed the apoptotic rate of cells following transfection with anti-miR-155 for 72 h by annexin V-FITC/PI staining method. As shown in **Figure 1D**, the apoptotic rate was 2.56±1.38% in controls, and 22.6±3.8% in anti-miR-155 transfected cells ($P<0.01$), suggesting that targeting miR-155 promotes apoptosis of A2058 cells. TUNEL assay has the same results as annexin V-FITC/PI staining (**Figure 1E**). Thus, targeting miR-155 can inhibits proliferation and induces apoptosis.

Effect of miR-155 on growth and proliferation

To evaluate the effects of miR-155 overexpression on A2058 cells, the miR-155 mimics was transfected into A2058 cells. The expression of miR-155 was significantly increased in miR-155 transfected A2058 cells relative to cells transfected with the miR-155 negative control (**Figure 2A**). Interestingly, the overexpression of miR-155 had a significantly stimulatory effect on cell growth in A2058 cells by MTT (**Figure 2B**) and BrdU assay (**Figure 2C**).

FOXO3a is regulated by miR-155 in A2058 cells

We first investigated the effects of miR-155 on FOXO3a expression in A2058 cells. Anti-miR-155 transiently transfected into A2058 cells for 72 h significantly induced FOXO3a protein expression (**Figure 3A**) and mRNA expression (**Figure 3B**, **3C**) in A2058 cells. However, miR-155 mimics transiently transfected into A2058 cells for 72 h significantly inhibited FOXO3a protein expression (**Figure 3A**) and mRNA expression (**Figure 3B**, **3C**) in A2058 cells. Taken together, these data suggest that FOXO3a is regulated by miR-155 in the A2058 cells.

FOXO3a mediates anti-miR-155-induced apoptosis and growth inhibition

Next we determined the role of FOXO3a in anti-miR-155-induced apoptosis and growth inhibition using siRNA targeting FOXO3a. Targeting miR-155 induced significant apoptosis in A2058 cells by annexin V-FITC/PI staining and TUNEL assay (**Figure 4A**, **4B**), and inhibited cell survival and proliferation by MTT and BrdU assay (**Figure 4C**, **4D**), which was associated with the activation of FOXO3a (**Figure 4E**). However, targeting FOXO3a by siRNA transfection reve-

rsed anti-miR-155-induced apoptosis and growth inhibition in the A2058 cells (**Figure 4A-D**). Although miR-155 promoted growth and proliferation of A2058 cells by FOXO3a inhibition, FOXO3a overexpression by FOXO3a cDNA transfection reversed the effect of miR-155 (**Figure 4C**, **4D**). Collectively, these results demonstrate an essential role of FOXO3a in anti-miR-155-induced apoptosis in A2058 cells.

Discussion

These results demonstrate that increased miR-155 expression may enhance tumor growth and proliferation in melanoma A2058 cells. Over-expression of miR-155 resulted in a decrease in FOXO3a protein and mRNA expression. Importantly, FOXO3a overexpression by FOXO3a cDNA transfection recapitulated the results obtained with miR-155 overexpression. In addition, targeting miR-155 inhibited tumor growth and proliferation, and induced apoptosis in melanoma A2058 cells, followed by a increase in FOXO3a protein and mRNA expression. However, siRNA silencing of FOXO3a expression recapitulated the results obtained with targeting miR-155. The effects of targeting miR-155 on melanoma growth therefore appear to be mediated by up-regulation of FOXO3a.

As a member of the forkhead transcriptional factor family, FOXO3a is a key tumor suppressor in melanoma [32]. It has been reported that upregulation of FOXO3a in melanoma cells exerts a general inhibitory effect on cell growth [33]. Consistent with these reports, downregulation of FOXO3a could significantly augment mTORC2-mediated tumor growth in nude mice and lead to apoptosis resistance, suggesting that FOXO3a is a determinant of tumor progression and chemotherapeutic response [34]. Collectively, all these studies indicate that activation of FOXO3a may be a potential therapeutic intervention strategy for melanoma.

The role of miR-155 has been extensively studied in the immune system. The importance of miR-155 in the immune system was evident by the phenotype of the miR-155 knockout mice [35], which were immunodeficient [36]. Mice lacking miR-155 are viable and fertile but are deficient in lymphocyte development and generation of B- and T-cell responses after B-cell receptor or T-cell receptor activation. Also, dendritic cells in miR-155-deficient mice have been shown to have an impaired antigen-presenting function [37], supporting the importance of miR-155 in the immune cells. Many known targets of miR-155 (more than 40 so far), such as PU.1 [37], c-MAF [38], and SOCS-1, have been shown to affect cell differentiation and function. Interestingly, SOCS-1 is also reported to be a target of miR-155 in breast cancer cells [39]. On the other hand, FOXO3a, which affects cell survival and invasion, is also reported to be a target of miR-155 [40]. These results suggest that the repression of multiple targets by miR-155 may contribute to melanoma formation. In the current study, we found that ectopic expression of miR-155 drastically increased the growth rate of A2058 cells, while suppression of miR-155 inhibited cell proliferation and induced apoptosis, indicating that upregulation of miR-155 may function as an onco-miRNA.

Through bioinformatics analysis, the tumor suppressor FOXO3a gene was indicated as a theoretical targeted gene of miR-155. Western blotting analysis showed that overexpression of miR-155 resulted in the decrease of FOXO3a protein and mRNA, and it's the same the other way round. Furthermore, expressing FOXO3a significantly abrogated the miR-155-induced proliferation, and targeting FOXO3a significantly abrogated the anti-miR-155-induced apoptosis. The biological function of miR-155 in protection against apoptosis and cell survival, as related to FOXO3a function, is currently under investigation in our laboratory.

Recently, several miRs were found to be involved in the regulation of the FOXO family of transcription factors. It was reported that miR-27a, miR-96, and miR-182 coordinately regulate the expression of FOXO1 through directly targeting the FOXO1 3'-UTR [41]. MiR-155 was shown to regulate cell survival, growth and hemosensitivity by downregulating FOXO3a in breast cancer [42]. Wang and colleagues reported that upregulation of FOXO3a results in a decrease in miR-21 expression and suppressing its oncogenic activity, which further supports the notion that FOXO3a functions as a tumor suppressor [43].

In summary, the current study provides, for the first time, an important link between anti-miR-155-mediated anti-proliferation and pro-apoptosis of melanoma cells and upregulation of FOXO3a. Our findings suggest an essential role of miR-155 in the regulation of melanoma cells proliferation and apoptosis. Understanding the

precise role played by miR-155 in melanoma progression will not only increase our knowledge of the biology of the tumor but its inhibition may also allow development of a novel therapeutic strategy.

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Disclosure of conflict of interest

None.

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