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miR-149 reverses cisplatin resistance of gastric cancer SGC7901/DDP cells by targeting FoxM1

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Drug resistance remains a major unresolved obstacle for gastric cancer (GC) treatment. Recently, increasing studies have showen that microRNAs (miRNAs) are involved in cancer chemotherapeutic resistance and can potentially be applied to reverse drug resistance in cancers. The relationship between miRNA-149 expression and cisplatin (DDP) resistance in GC cells is still unknown. Here, we detected miR-149 expression by using RT-PCR and found that expression of miR-149 was downregulated in SGC7901/DDP cells compared with SGC7901cells, indicating a role of miR-149 in determining cisplatin-resistance of GC cells. Then, SGC7901/DDP cells were tansfected with miR-149 mimics, MTT assay was performed to determine SGC7901/DDP cell viability, and showed that overexpression of miR-149 inhibited the cell viability after cisplatin treatment, suggesting that up-regulation of miR-149 enhanced SGC7901/DDP cell sensitivity to cisplatin. Furthermore, we confirmed that Forkhead box M1 (FoxM1) is a direct target of miR-149 in SGC7901/DDP cells by using luciferase reporter assay. Besides, we also demonstrated that miR-149 enhances SGC7901/DDP cell sensitivity to cisplatin by downregulating FoxM1 expression. In summary, our data provide new insights that miR-149 plays an important role in determining sensitivity of cisplatin-resistant GC cells by targeting FoxM1 and suggest that miR-149 could be a potential target for reversing drug resistance in GC.

1. Introduction

Gastric cancer (GC) is the fifth most frequent malignancy and the third leading cause of cancer-correlated deaths (Ferlay et al. 2015). In 2012, there were 951,600 new GC cases diagnosed and 723,100 deaths worldwide (Torre et al. 2015). The main current strategy for GC therapy is surgery with chemotherapy/radiotherapy, however, relapse and metastasis arise in many patients and result in high mortality of GC due to intrinsic or acquired drug resistance (Bang et al. 2012). Therefore, drug resistance is always a major obstacle for GC treatment and reversing drug resistance has become focus of attentions.

As short endogenous non-coding RNAs, microRNAs (miRNAs) express in all mammalian cells and regulate gene expression at the posttranscriptional level (MacDonagh et al. 2015). Since miRNAs are involved in several cellular processed, including proliferation, differentiation, apoptosis and development by regulating expression level of hundreds of genes (Li and Yang 2013; Ma et al. 2010; To 2013), it is not surprising that miRNAs play an important role in the initiation and progression of human cancer (Cho 2007; Croce 2009). In recent years, studies have shown that miRNAs are strong implicated in cancer chemotherapeutic resistance (Donzelli et al. 2014; Kutanzi et al. 2011; Riquelme et al. 2016; Zheng et al. 2010). Thus, miRNAs can potentially be used as therapeutic target to reverse drug resistance in cancers.

Recent studies have demonstrated that miR-149 is downregulated in many cancers and acts as a tumor suppressor in lung cancer, colorectal cancer, breast cancer and gastric cancer (Chan et al. 2014; Ke et al. 2013; Wang et al. 2012; Xu et al. 2015). However, the relationship between miRNA-149 expression and cisplatin (DDP) resistance in human gastric cancer cells is still unclear.

In this study, we found that miR-149 was significantly down-regulated in SGC7901/DDP cells compared with SGC7901cells. Overexpression of miR-149 significantly inhibited SGC7901/DDP cell viability. Furthermore, we found that miR-149 could enhance SGC7901/DDP cells sensitivity to cisplatin by targeting FoxM1.

2. Investigations and results

2.1. *miR-149 is downregulated in cisplatin-resistant GC cells*

To evaluate the role of miR-149 in cisplatin-resistant GC cells, we measured the expression of miR-149 in SGC7901 and SGC7901/DDP cells and found that miR-149 mRNA was downregulated in

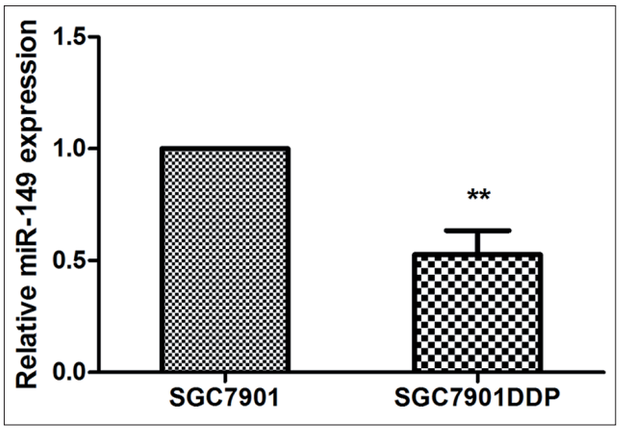

Fig. 1: The expression of miR-149 was significantly down-regulated in cisplatin-resistant GC cells. The expression of miR-149 was measured by using RT-PCR. The relative mRNA level was normalized to GAPDH. ** $P < 0.01$.

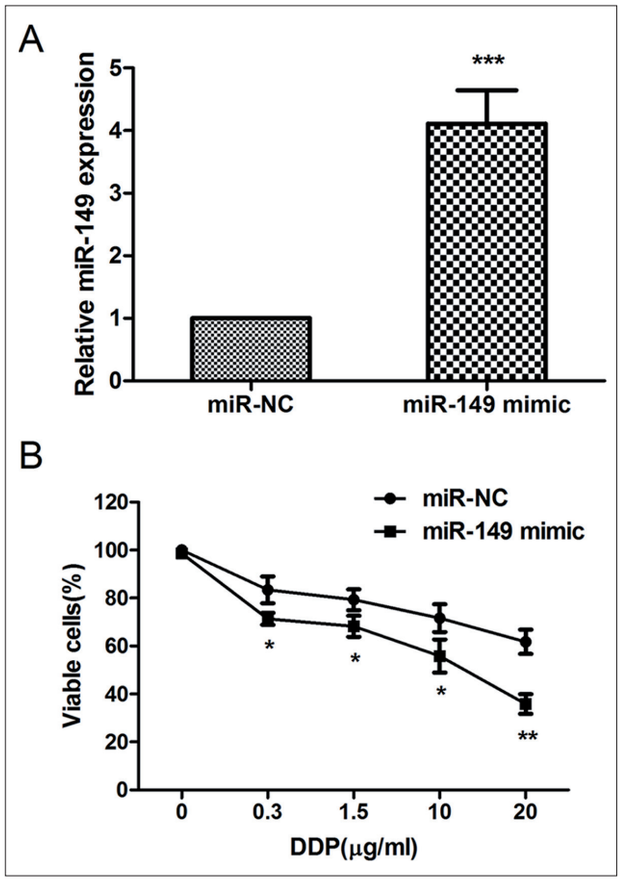

Fig. 2: Up-regulation of miR-149 suppressed SGC7901/DDP cell viability. (A) miR-149 expression in SGC7901/DDP cells was measured by using RT-PCR. (B) Cell viability was determined by MTT assay. SGC7901/DDP cells were transfected with miR-NC or miR-149 mimic. After transfection, cells were exposed to cisplatin at different concentrations. The relative mRNA level was normalized to GAPDH. * $P < 0.05$, ** $P < 0.01$.

SGC7901/DDP cells compared with SGC7901cells (Fig. 1), indicating that a certain expression level of miR-149 may be involved in cisplatin-resistance of GC cells.

2.2. *Overexpression of miR-149 enhances SGC7901/DDP cell sensitivity to cisplatin*

To determine the effect of miR-149 on cisplatin-resistant GC cells, SGC7901/DDP cells were transfected with miR-149 mimics or miR-NC. The transfection efficiency was confirmed by detecting the expression of miR-149 in SGC7901/DDP cells (Fig. 2A). To determine whether miR-149 affects the cisplation resistance of SGC7901/DDP cells, cells were treated with cisplation at the final concentration of 0, 0.3, 1.5, 10, 20 μg/ml. MTT assay was performed to determine the effect of miR-149 on SGC7901/DDP cell viability, and showed that overexpression of miR-149 inhibited the cell viability after cisplatin treatment (Fig. 2B), which suggested that overexpression of miR-149 could enhance SGC7901/DDP cell sensitivity to cisplatin.

2.3. *FoxM1 is a target of miR-149*

By using TargetScan program, FoxM1 was found to have putative miR-149 binding sites within its 3'UTR and selected as a potential direct target of miR-149 (Fig. 3A). A luciferase reporter assay was performed to identify whether FoxM1 is a direct target of miR-149. We cloned the WT 3'-UTR sequence of FoxM1 or the MUT 3'-UTR sequence into the pGL3 luciferase reporter vector and transfected the constructs together with miR-149 mimics or miR-NC into SGC7901/DDP cells. The luciferase activity significantly decreased in SGC7901/DDP cells co-transfected with WT 3'-UTR-FoxM1 and miR-149 mimic compared with those co-transfected with WT 3'-UTR-FoxM1 and miR-NC, whereas the luciferase activity of MUT 3'-UTR-FoxM1 was unaffected with co-transfection with miR-149 mimics or miR-NC (Fig. 3B). Next, we measured the expression level of FoxM1 in SGC7901/DDP cells transfected with miR-149 mimics or miR-NC, and found that up-regulation of miR-149 could significantly inhibit the expression of FoxM1 mRNA (Fig. 3C) and protein (Fig. 3D). These results indicate that FoxM1 is a target of miR-149 in SGC7901/DDP cells.

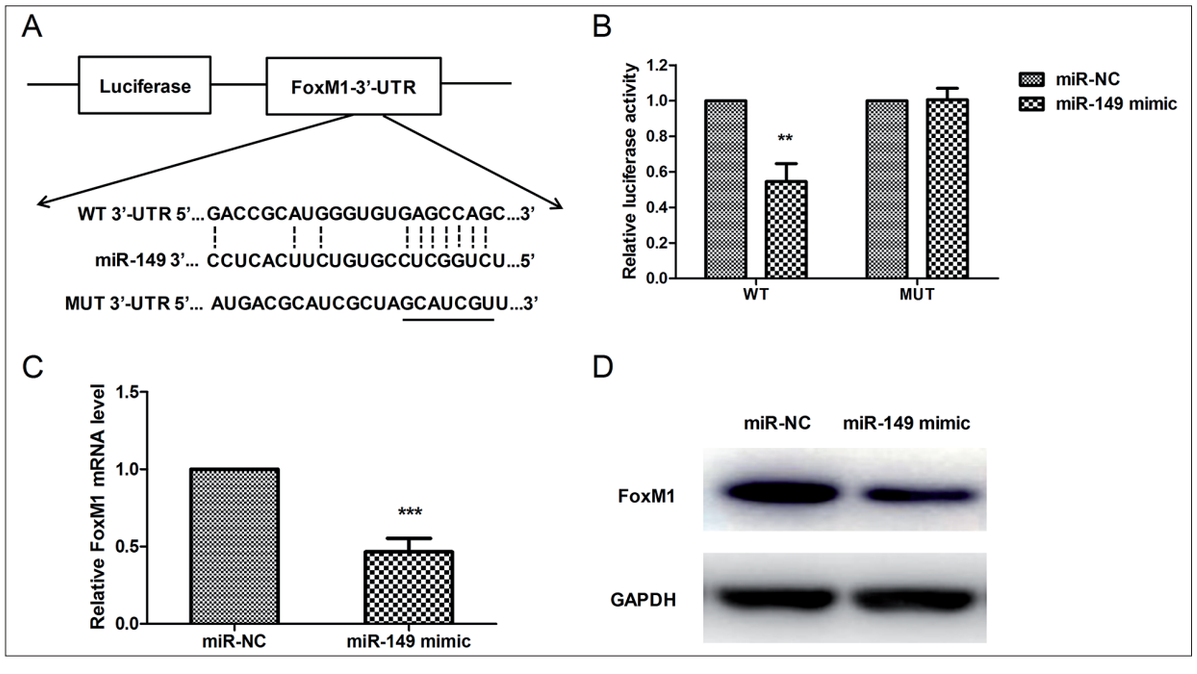

Fig. 3: Validation of FoxM1 as a target of miR-149. (A) Schema of the potential binding site for miR-149 and FoxM1. The mutated sites of mutant 3'-UTR-FoxM1 sequence were highlighted with underline. (B) Validation of miR-149 target using FoxM1 3'UTR luciferase reporter. (C) FoxM1 mRNA (C) and protein (D) expression in SGC7901/DDP cells were assayed by RT-PCR and western blot respectively. SGC7901/DDP cells were transfected with miR-NC or miR-149 mimic. The relative mRNA and protein levels were normalized to GAPDH. ** $P < 0.01$, *** $P < 0.001$.

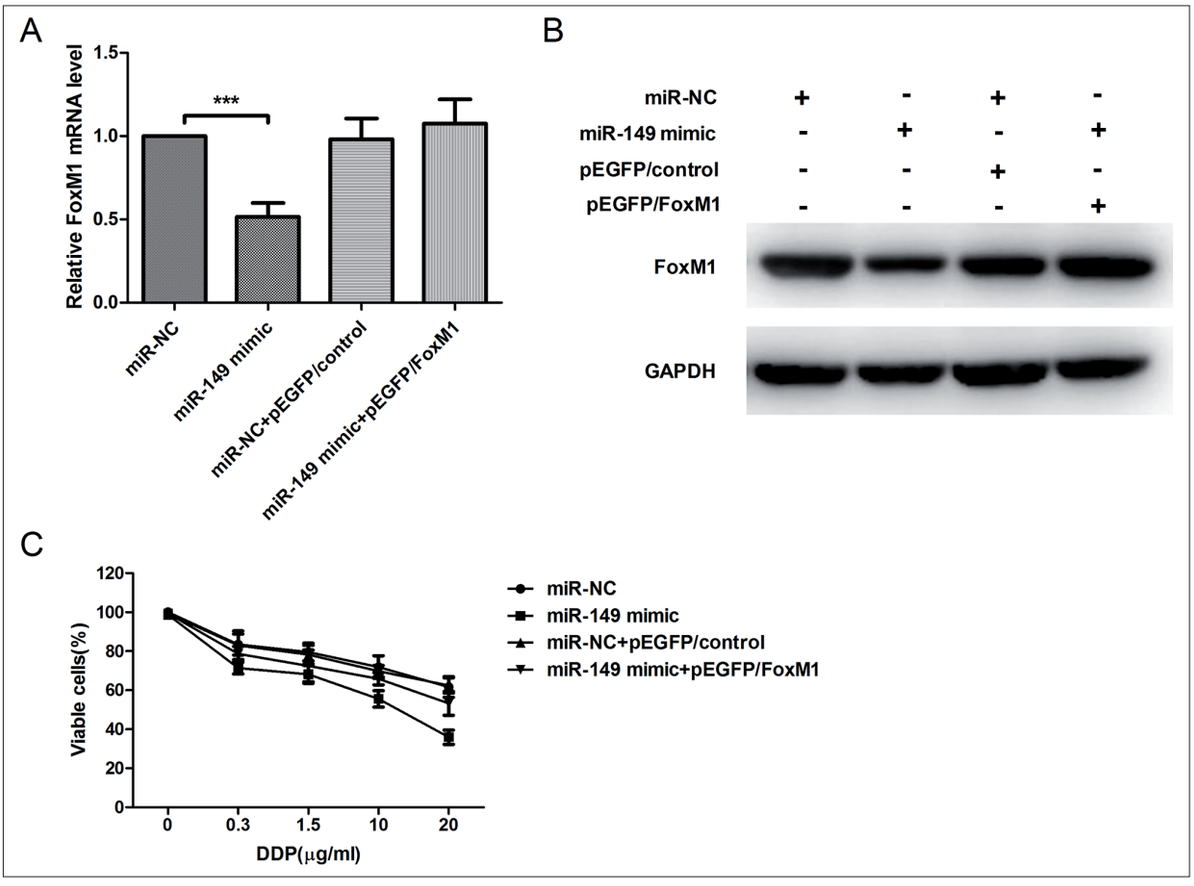

Fig. 4: miR-149 suppressed SGC7901/DDP cell viability by down-regulating FoxM1 expression. FoxM1 mRNA (A) and protein (B) expression in SGC7901/DDP cells were measured by RT-PCR and western blot respectively. (C) Cell viability was determined by MTT assay. SGC7901/DDP cells were transfected with miR-NC, miR-149 mimic, miR-NC + pEGFP/control, or miR-149 mimic + pEGFP/FoxM1 respectively. The relative mRNA and protein levels were normalized to GAPDH. For cell viability assay, cells were exposed to cisplatin at different concentrations after transfection. *** $P < 0.001$.

2.4. miR-149 enhances SGC7901/DDP cell sensitivity to cisplatin by down-regulating FoxM1 expression

To further investigate the regulation of miR-149 on FoxM1, pEGFP/FoxM1 was transfected into SGC7901/DDP cells and used to upregulate the expression of FoxM1 in SGC7901/DDP cells. We found that overexpression of miR-149 suppressed the expression of FoxM1 mRNA and protein while upregulating miR-149 and FoxM1 simultaneously had no effect on FoxM1 expression (Fig. 4A, 4B), which further showed a downregulation of miR-149 to FoxM1. To determine the effect of overexpression of miR-149 and FoxM1 on cisplation resistance of SGC7901/DDP cells, we treated SGC7901/DDP cells with cisplatin at the final concentration of 0, 0.3, 1.5, 10, 20 μg/ml and measured SGC7901/DDP cell viability by using MTT assay. Compared with miR-NC group, SGC7901/DDP cell viability in miR-149 overexpression group (miR-149 mimic) significantly decreased while SGC7901/DDP cell viability was unaffected with co-transfection with miR-149 mimics and pEGFP/FoxM1 (Fig. 4C). These data revealed that miR-149 could attenuate SGC7901/DDP cell viability and enhance SGC7901/DDP cells sensitivity to cisplatin by targeting FoxM1.

3. Discussion

miRNAs play an important role in cancer initiation and development (Li and Yang 2013), and can function as oncogenes or tumor suppressors, which may be used as diagnostic and prognostic markers and therapeutic targets for cancer. Recently, studies have indicated that miRNAs are involved in drug resistance by targeting tumor-related gene transcripts (Aguda 2013; Hong et al. 2014). Overexpression of miR-508-5p significantly reversed resistance of SGC7901/VCR and SGC7901/ADR to VCR, ADR, 5-Fu and CDDP while knockdown of miR-508-5p reduced drug sensitivity (Shang et al. 2014). miR-106a could enhance CDDP resistance in SGC7901 while inhibition of miR-106a in SGC7901/CDDP result in promote CDDP-induced apoptosis (Fang et al. 2013).

Recent studies have demonstrated that miR-149 is involved in progression and development of cancer (Chan et al. 2014; Ke et al. 2013; Wang et al. 2012; Xu et al. 2015). In colorectal cancer, miR-149 inhibited cell migration and invasion and was an independent prognostic factor (Xu et al. 2015). In non-small-cell lung cancer, miR-149 could act as an EMT suppressor (Ke et al. 2013). In breast cancer, miR-149 suppressed breast cancer cell migration, invasion and metastasis (Chan et al. 2014). However, the effect of miR-149 on cisplatin resistance of GC cells remains largely unknown.

In the present study, we have shown that miR-149 is down-regulated in SGC7901/DDP cells compared with SGC7901cells, and upregulation of miR-149 can reduce SGC7901/DDP cell viability after cisplatin treatment, suggesting that miR-149 expression is associated with SGC7901/DDP cells sensitivity to cisplatin. Although it has been reported that miR-149 inhibits EMT of non-small-cell lung cancer and inhibits colorectal cancer cell migration and invasion by targeting FoxM1 (Ke et al. 2013; Xu et al. 2015), whether miR-149 targets FoxM1 to enhance GC cells sensitivity to cisplatin is not unclear.

Forkhead box M1 (FoxM1), a member of the Forkhead family of transcription factors, is correlated with cell cycle progression, apoptosis, angiogenesis and DNA damage repair (Chiu et al. 2015). Overexpression of FoxM1 is found in various cancers, contributing to tumor angiogenesis, invasion and metastasis (Gomes et al. 2013), while downregulation of FoxM1 results in inhibition of cell growth, migration and invasion (Okada et al. 2013). Moreover, patients with high FoxM1 levels were relatively insensitive to chemotherapy (Okada et al. 2013), and FoxM1 knockdown could

sensitize cancer cells to cisplatin (Zhou et al. 2014), which indicated a possible relationship between FoxM1 and drug resistance. In this study, we have confirmed that FoxM1 is a direct target of miR-149, and demonstrated that miR-149 reduced SGC7901/DDP cell viability by down-regulating FoxM1, which suggested that miR-149 enhances SGC7901/DDP cell sensitivity to cisplatin by targeting FoxM1 expression.

In summary, our study showed that miR-149 is associated with cell sensitivity to cisplatin by targeting FoxM1 in SGC7901/DDP cells. These findings provide new insights into the role of miR-149 in the chemosensitivity of cisplatin-resistant GC cells and suggest that miR-149/FoxM1 signaling could be a potential target for reversing drug resistance in GC.

4. Experimental

4.1. Cell culture

Human gastric carcinoma cell line SGC7901 and cisplatin-resistant SGC7901/DDP cells were stored in our lab and cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin, within a humidified atmosphere containing 5% CO_2 at 37 ºC. SGC7901/DDP cells were cultured in the medium contained 1 mg/l DDP.

4.2. Cell transfection

miR-149 mimics and negative control oligonucleotides (miR-NC) were obtained from Ribobio (Guangzhou, China). The open reading frame of FoxM1 was generated by PCR and cloned into pEGFP-C1, named as pEGFP/FoxM1. The transfection were performed by Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol.

4.3. RT-PCR

The total RNA was extracted by using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. First-strand cDNA synthesis was generated by using high-capacity cDNA reverse transcription kit (Roche). Real-time PCR reactions were performed using Power SYBR Green (Takara, Dalian, China). GAPDH was used as an internal control. qRT-PCR and data collection were performed by the LightCycler 3.5 instrument (Roche Diagnostics, Mannheim, Germany).

4.4. Western blotting

Western blots were performed based on the standard procedures. Briefly, the cells were collected and lysed by ice-cold RIPA lysis buffer (Cell Signal Technology, Danvers, MA) supplemented with protease inhibitors. The extracted protein concentrations were determined by bicinchoninic acid (BCA) protein assay. Then, extracted proteins were subjected by SDS-PAGE (10%) for western analysis and transferred to PVDF membranes. Blots were blocked with 5% w/v non-fat dry milk in Tris-buffered saline containing Tween-20 (TBS-T) and then incubated with primary antibodies at 4 °C overnight. The primary antibodies used were anti-FoxM1 (1:1000 dilution; Abcam, Cambridge, MA, USA), HRP-labeled anti-GAPDH (1:10,000 dilution; Abcam, Cambridge, MA, USA). Following washing with TBS-T for 5 min, the blots were incubated with HRP-labeled goat anti-rabbit IgG secondary antibodies (1:10,000 dilution; Abcam, Cambridge, MA, USA) at room temperature for 1 h. Immunobands were visualized using an enhanced chemiluminescence detection system (GE Healthcare Life Sciences).

4.5. Cell viability assay

Cells were seeded onto 96-well plate and incubated overnight in a 5% CO_2-humidified incubator at 37 ºC. Cells were exposed to cisplatin at the final concentration of 0, 0.3, 1.5, 10, 20 µg/ml respectively. 20 µl of MTT solution was added into each well and incubated for 4 h at 37 ºC. Then 150µl DMSO was added into each well, followed by 10 min of incubation at 37 ºC. The OD values were read at 490 nm by enzyme-linked immunosorbent assay (ELISA) reader.

4.6. Target gene prediction

To find the potential target gene of miR-149, TargetScan online software (http://www.targetscan.org/) was applied. http://bibiserv.techfak.uni-bielefeld.de/rnahybrid website was used to analyze the binding free energy and binding sites.

4.7. Luciferase reporter assay

The regulatory effect of miR-149 to FoxM1 was determined by a luciferase reporter assay in SGC7901/DDP cells. The fragment of wild-type (WT) 3'-UTR of FoxM1 containing the predicted miR-149 binding sites or mutant (MUT) 3'-UTR of FoxM1 were synthesized and cloned into the pGL3 luciferase reporter vector (Promega Corporation, Madison WI, USA). Cells were seeded into 24-well plates and co-transfected with 10 ng of miR-149 mimic or vector. The luciferase activity assay was measured according to the manufacturer's instruction (Promega, Madison WI, USA).

4.8. Statistical analysis

Data were expressed as Mean ± SD of three independent experiments. GraphPad Prism 5 software (GraphPad Software Inc.) was used for statistical tests. Comparison between groups was performed using Student *t*-test. $P < 0.05$ is considered as statistically significant.

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Conflicts of interest: None declared.

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