Research Article

MicroRNA-365 is a negative regulator of endothelial cell proliferation

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Abstract

Recent studies have shown that human microRNA miR-365 has significant inhibitory effects on proliferation of transformed cancer cells and vascular smooth muscle cells. However, the effects of miR-365 on proliferation and migration of vascular endothelial cells remain unknown. Using human umbilical vein endothelial cells and in vitro assays, we demonstrated that miR-365 was a suppressor of endothelial cell proliferation, whereas cell migration was not affected by miR-365. We also identified that the expression level of the serine/threonine protein kinase serum- and glucocorticoid-regulated kinase-1 (SGK-1) was regulated by miR-365. The cytostatic effect of miR-365 was mimicked by the specific SGK-1 inhibitor GSK 650394. We further demonstrated that microvesicles isolated from plasma of patients with intracerebral hemorrhage, in which the level of miR-365 was elevated, decreased the expression level of SGK-1, and this effect was abolished in cells pretreated with miR-365 antagomir. However, we did not observe a significant effect of the microvesicles on cell proliferation. It is suggested that miR-365 may have important roles in vascular physiology and/or pathophysiology by modulating endothelial cell proliferation.

Introduction

Mounting evidence has suggested that microRNAs (miRNAs) are important epigenetic regulators of physiology and pathophysiology of the cardiovascular system (Condorelli *et al.* 2014, Hata 2013). The expression of a number of miRNA species has been found to be enriched in vascular endothelial (Santoro & Nicoli 2013) and smooth muscle cells (Bonauer *et al.* 2010), and cardiac muscles (Thum *et al.* 2008). Not surprisingly, aberrant expression or function of different miRNAs has been linked to multiple cardiovascular pathologies, including cardiac hypertrophy and remodeling, myocardial infarction, heart failure, arrhythmia, hypertension, atherosclerosis, aneurysm, and stroke (Condorelli *et al.* 2014, Romaine *et al.* 2015), at least in animal models. Inside the cell, miRNA molecules regulate gene expression primarily via sequence-specific interaction with target mRNAs, leading to mRNA degradation or translational repression. Individual miRNAs may regulate the expression of multiple genes, while the expression of an individual gene may be regulated by multiple miRNAs (Romaine *et al.* 2015).

In addition to intracellular miRNA molecules, miRNAs can also exist in the extracellular fluids such as plasma or serum. Recent studies have suggested that circulating miRNAs may be used as new biomarkers for cardiovascular disease (Wang *et al.* 2014). Moreover, it has been shown that microvesicle-encapsulated miRNAs secreted by cells can be internalized by other cells and modulate biological functions in the recipient cells (Muller *et al.* 2011, Valadi *et al.* 2007, Zhang *et al.* 2010). Hence it is thought that microvesicle-encapsulated miRNAs in the circulation are biologically functional and may be involved in long-distance cell-cell communications, raising the possibility that circulating miRNAs may represent novel therapeutic targets for human diseases (Wang *et al.* 2014).

Genomic profiling studies have revealed that changes of the circulating miRNA profile are associated with different cardiovascular diseases in humans, such as coronary arterial disease (Fichtlscherer *et al.* 2010), myocardial infarction (Wang *et al.* 2010), heart

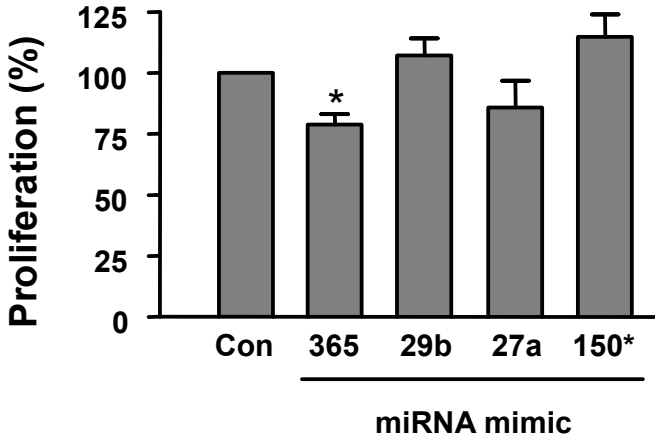

Figure 1. Effects of various miRNA mimics on proliferation of human umbilical vein endothelial cells (HUVECs). Cells transfected with a non-targeting miRNA mimic were used as control (Con). Cell proliferation was assayed 48 hours after transfection. Data represent mean ± SEM. * $P < 0.05$ *vs* control, one-way ANOVA followed by Tukey's test, $n = 3$ experiments.

failure (Goren *et al.* 2012, Tijsen *et al.* 2010), aortic aneurysms (Kin *et al.* 2012), and hypertension (Li *et al.* 2011). In addition, altered circulating miRNA levels have also been reported in patients with cerebral vascular diseases including intracranial aneurysms (Li *et al.* 2014) and stroke (Guo *et al.* 2013, Li *et al.* 2015). In our previous study in intracerebral hemorrhage patients, we identified a group of 30 circulating miRNAs that were selectively upregulated in both male and female patients as compared to healthy controls (Guo *et al.* 2013). Bioinformatic analysis revealed that these miRNAs were overrepresented in biological processes associated with inflammation (Guo *et al.* 2013). One of these specifically changed miRNAs is miR-365, which has been shown to have an important role in regulating cell proliferation in transformed cancer cells (Chen *et al.* 2015, Kang *et al.* 2013, Nie *et al.* 2012). Currently, however, the effects of miR-365 on functions of the vascular endothelial cell remain unknown. Therefore, in the present study, we investigated the impacts of miR-365 on endothelial cell proliferation.

Materials and Methods

Reagents

Synthetic miRNA mimics and antagomirs were purchased from GenePharma (Shanghai, China). GSK 650394 was obtained from Tocris Bioscience (Bristol, UK).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from American Type Culture Collection. HUVECs were cultured in Endothelial Cell Medium (ECM) supplemented with 1% endothelial cell growth supplement (ECGS) (ScienCell Research Laboratories, Carlsbad, CA, USA), penicillin/streptomycin (Invitrogen), and 5% fetal bovine serum (FBS) (Invitrogen). Cells were cultured in 95% air/5% CO_2 at 37°C. For experimentation, cells within passage 10 were used.

Transfection of miRNA mimics and antagomirs

Twenty four hours before transfection, cells were subcultured into 24-well plates at a density of 1.5 x 10^5 cells per well. Cells were incubated with miRNA (final concentration 30 nM) mixed with Lipofectamine RNAiMAX Reagent (Invitrogen) for 6 hours and then changed to fresh medium. In our preliminary experiments, we characterized the gene silencing efficacy of transfected miR-365 mimic at different time points. We found that the optimal effect was obtained at 24 hours after transfection, whereas the effects at 48 or 72 hours were much smaller. We therefore carried out western blot and qPCR experiments 24 hours post transfection. Cell proliferation was assayed 48 hours after transfection.

Cell proliferation

Cell proliferation was assessed with a tetrazolium-based non-radioactive assay kit (CellTiter 96 Aqueous from Promega, Madison, WI, USA) as used previously (Datla *et al.* 2007).

Cell wound healing assay

Cell migratory activity was tested with the wound healing assay. Cells were cultured in 24-well plates to 100% confluency. Wound healing assay was performed by scratching the cell monolayer with a 200 µl pipette tip (Datla *et al.* 2007), and the cells were further cultured in reduced serum (1%) ECM for 6 - 24 hr. The recovery rate of the denuded area was monitored by taking digital pictures at different time points, and the rate was quantified as percentage recovery of the total denuded area.

Quantitative real-time PCR (qPCR)

Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. cDNA was reverse transcribed from 1 µg of total RNA using random hexamer primer and TaqMan Reverse Transcription Reagents (Applied Biosystems, Carlsbad, CA, USA). Thermal cycling parameter settings for reverse transcription were: 25°C for 10 min; 37°C for 60 min;

95°C for 5 min. Real-time PCR was performed using the Taqman Gene Expression Assay primer-probe set for human SGK-1, and the TaqMan Gene Expression Master Mix (all from Applied Biosystems). An ABI Prism 7500 system (Applied Biosystems) was used for real-time PCR amplification. Thermal cycling parameters were as follows: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 s plus 60°C for 1 min. 18S rRNA was used as the house-keeping gene. The $2^{-\Delta\Delta CT}$ method was used to assess the relative mRNA expression level. The results were expressed as fold of control.

Western blot

Total proteins were separated by 10% SDS-PAGE and transferred to Immobilon-P PVDF membranes (from Millipore, Billerica, MA, USA). The membrane was blocked with 5% non-fat milk and then incubated with primary antibodies at 4°C overnight. The blots were developed with an ECL-Plus (enhanced chemiluminescence) reagent (GE Healthcare, Giles, UK). All primary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). HRP-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA, USA).

miRNA target gene prediction

Potential miRNA target genes were predicted using five independent bioinformatics tools: miRanda, MirTarget2, PITA, RNAhybrid and TargetScan.

Isolation of microvesicles from human plasma

Collection of human samples was approved by the hospital Human Ethics Committee, and informed consents were obtained from all subjects. Peripheral blood was collected from patients with intracerebral hemorrhage (diagnosed by multi-detector-row computed tomography scanning). Isolation of microvesicles from plasma was performed as described (Caby *et al.* 2005). Briefly, fresh plasma was centrifuged at 500 g for 30 min, followed by 12,000 g for 45 min. The supernatant was then transferred to a fresh tube and microvesicles pelleted by ultra-centrifugation at 110,000 g for 2 hours. For microvesicle treatment in cultured cells, microvesicles isolated from 4 ml of plasma were washed and resuspended in 1 ml of culture medium, and cells were incubated for 24 hrs. Equivalent amount of bovine serum albumin was used as control.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Differences between groups were analyzed with unpaired *t*-test (two groups) or one-way analysis of variance (ANOVA) followed by *post hoc* Tukey's test (for multiple groups) unless stated otherwise. Statistical analysis was performed with GraphPad Prism. A value of $P < 0.05$ was regarded as statistically significant.

Results

To determine whether miR-365 had any effect on proliferation of vascular endothelial cells, we transfected HUVEC cells with a miR-365 mimic construct and

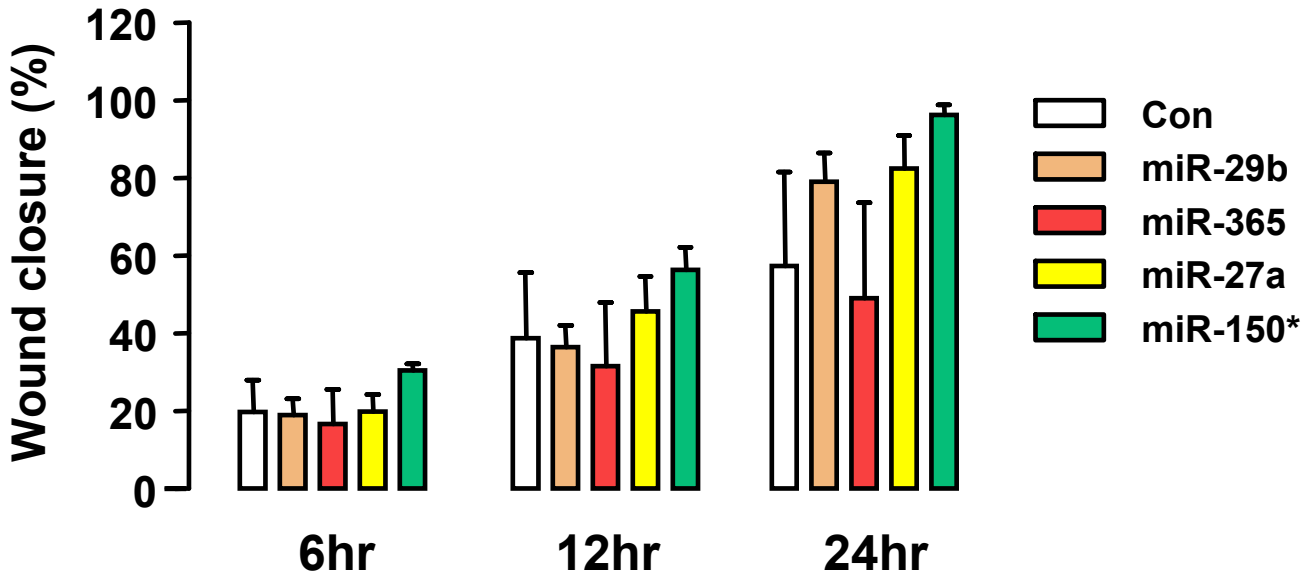

Figure 2. Effects of various miRNA mimics on migration of HUVECs assessed with the wound healing assay. Wound healing response was expressed as % recovery of the total denuded area. Data were mean ± SEM. * $P < 0.05$ *vs* control (Con), one-way ANOVA followed by Tukey's test, $n = 3$-4.

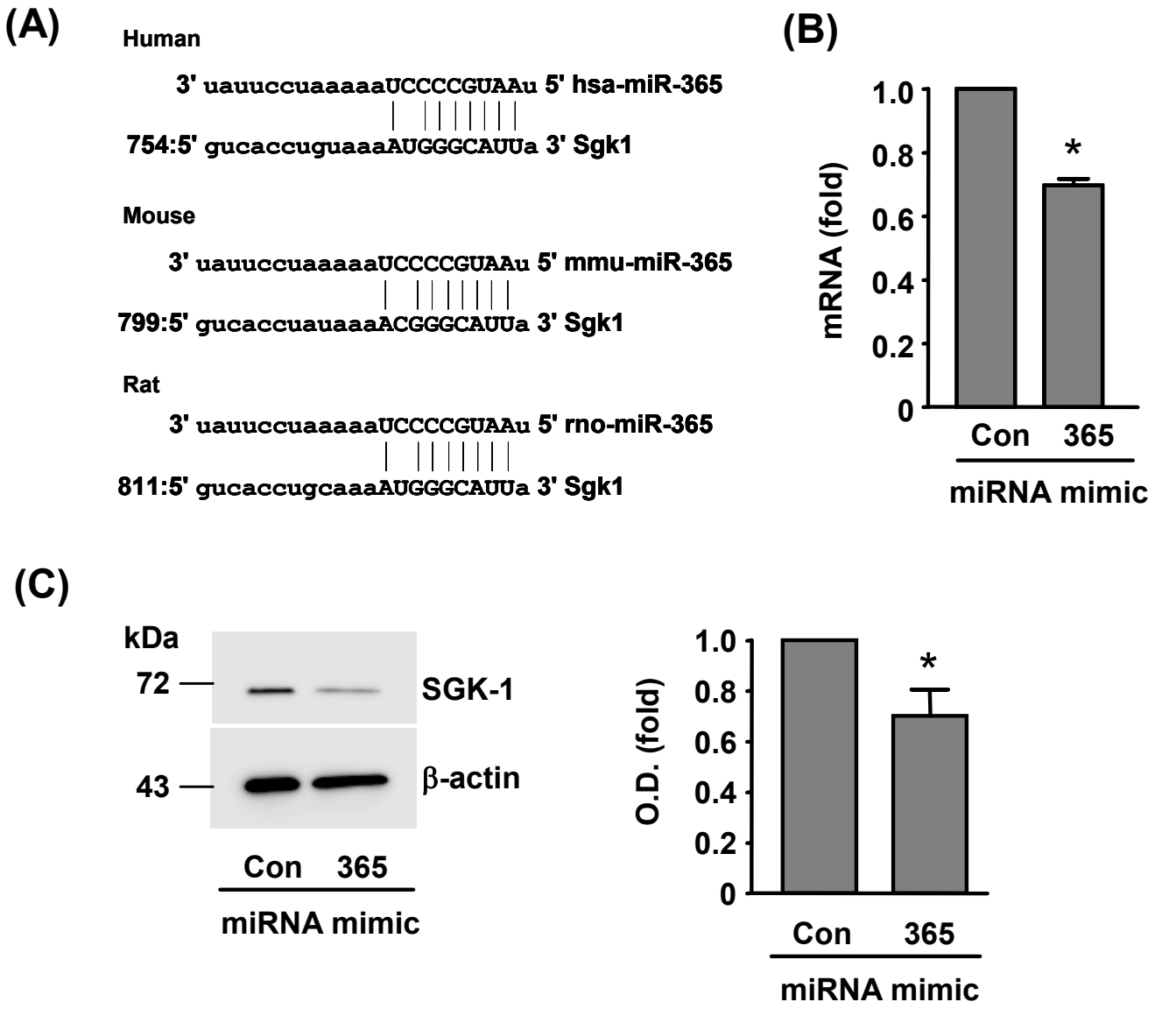

Figure 3. Regulation of the expression of serum- and glucocorticoid-regulated kinase-1 (SGK-1) by miR-365. (A) Conserved miR-365 targeting sequence in SGK-1 genes of human, mouse and rat. (B) Transfection with the miR-365 mimic in HUVECs decreased the mRNA level of SGK-1 as measured by qPCR, which was carried out at 24 hours after transfection. (C) Western blot and densitometry data showing the effect of miR-365 mimic on protein expression of SGK-1 24 hours after transfection. Data were mean ± SEM. * $P < 0.05$ *vs* control (Con), unpaired *t*-test, $n = 3$ - 6.

measured the rate of cell proliferation. As compared to the non-specific control sequence, the miR-365 mimic significantly reduced the rate of proliferation of HUVECs (Figure 1). In addition, we measured the effects of miR-29b, miR-27a and miR-150*, which were also specifically upregulated in the plasma of intracerebral hemorrhage patients (Guo *et al.* 2013). In contrast, we found that miR-29b, miR-27a or miR-150* had no significant effects on HUVEC proliferation. To test whether miR-365, as well as miR-29b, miR-27a and miR-150* had any effects on HUVEC migratory activity, we transfected mimics of these miRNAs and measured the cell monolayer wound healing response. We found that these miRNAs showed no significant effects on HUVEC migration (Figure 2).

To understand the mechanism by which miR-365 modulated endothelial cell proliferation, we first performed bioinformatics analysis to predict target genes for miR-365. As listed in Table 1, several potential targets for miR-365 were identified by 5 independent bioinformatic tools. Among these target genes, we focused on serum- and glucocorticoid-regulated kinase -1 (SGK-1), a serine/threonine protein kinase with high homology in amino acid sequence and biological functions to Akt (Webster *et al.* 1993). Sequence analysis revealed that the target sequence for miR-365

Table 1. Predicted target genes for miR-365

Gene Symbol	Description	MFE (kcal/mol)
CSK	c-src tyrosine kinase	-24.2
ANKRD11	ankyrin repeat domain 11	-23.1
LAMP2	lysosomal-associated membrane protein 2	-20.7
SOCS5	suppressor of cytokine signaling 5	-24.9
EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	-24.5
SGK3	serum/glucocorticoid regulated kinase family, member 3	-21.3
TMOD3	tropomodulin 3 (ubiquitous)	-19.7
DTNA	dystrobrevin, alpha	-23.3
ARRB2	arrestin, beta 2	-21.2
MYLK	myosin light chain kinase	-22.7
SGK1	serum/glucocorticoid regulated kinase 1	-27.0
OAZ2	ornithine decarboxylase antizyme 2	-24.2
GALNT4	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 4 (GalNAc-T4)	-22.5
MAP-K1IP1L	mitogen-activated protein kinase 1 interacting protein 1-like	-23.3
PAX6	paired box 6	-23.4
HMGCR	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	-21.9
NR3C2	nuclear receptor subfamily 3, group C, member 2	-24.9
CREB5	cAMP responsive element binding protein 5	-22.5
USP48	ubiquitin specific peptidase 48	-24.0

in the SGK-1 gene was totally conserved in human, mouse and rat (Figure 3A). We next performed qPCR analysis and showed that treatment with the miR-365 mimic significantly decreased the mRNA level of SGK -1 (Figure 3B). To confirm the effect of miR-365 on the expression level of SGK-1, we did western blot experiments and demonstrated that the miR-365 mimic also significantly decreased the SGK-1 protein in HUVECs (Figure 3C). We noted that the tyrosine kinase c -Src was also identified as a potential target of miR-365 (see Table 1).

Since c-Src is tightly involved in modulating cell proliferation (Roskoski 2004), we tested whether miR-365 could also modulate the expression level of c-Src. As shown in Figure 4, however, we found that transfection with miR-365 mimic did not show any effect on Src expression. To confirm the role of SGK-1 in modulating endothelial cell proliferation, we treated

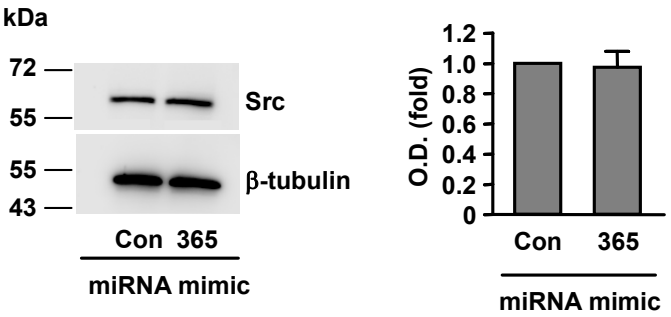

Figure 4. Western blot and densitometry data showing that the miR-365 mimic had no effect on the protein expression level of Src in HUVECs. Data were mean ± SEM, n = 3. Con, control.

HUVECs with a specific SGK-1 inhibitor GSK 650394. We showed that GSK 650394 at 100 mM significantly suppressed HUVEC proliferation (Figure 5). To further establish that modulating SGK-1 expression had a causal role in mediating the miR-365 effect, we tested the effect of miR-365 mimic in the presence of GSK 650394. We found that in cells pretreated with GSK 650394, miR-365 mimic had no significant effect on proliferation (101.6 ± 5.5% versus control miRNA, $P > 0.05$, $n = 3$), supporting a causal role of SGK-1 in mediating the miR-365 effect.

Previous studies have shown that miRNA-rich microvesicles can be captured by endothelial cells, while the encapsulated miRNA molecules may produce biological effects once they are internalized into the cells (Zhang *et al.* 2010). To test this possibility for miR-365, we treated HUVECs with concentrated native microvesicles isolated from the plasma of intracerebral hemorrhage patients in the presence or absence of the miR-365 antagomir. As shown in Figure 6A, microvesicles slightly but significantly ($P < 0.05$ with unpaired t-test) decreased the expression level of SGK-1, and this effect was abolished in cells pretreated with miR-365 antagomir. However, we failed to detect an effect of the microvesicles on endothelial cell proliferation either with or without miR-365 antagomir (Figure 6B).

Discussion

Several recent studies have shown that increased miR-365 expression has inhibitory effects on the proliferation of various cancer cells (Chen *et al.* 2015, Kang *et al.* 2013, Nie *et al.* 2012). In the present study, we have identified miR-365 as a suppressor of cell proliferation in primary human vascular endothelial cells, indicating that miR-365 may be involved in modulating cell proliferation not only in transformed cells, but also in normal cells. Our results are in line with two recent reports showing that miR-365 inhibits prolifera-

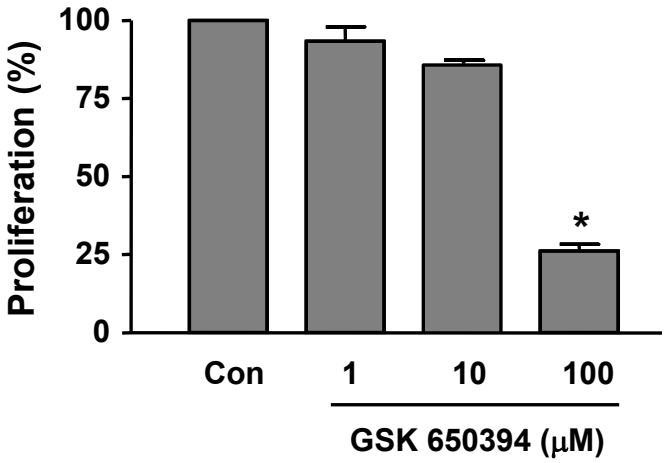

Figure 5. Effects of the SGK-1 inhibitor GSK 650394 on HUVEC proliferation. DMSO was used as vehicle control (Con). Data were mean ± SEM. * $P < 0.05$ *vs* Con, one-way ANOVA followed by Tukey's test, $n = 4$.

tion of vascular smooth muscle cells (Kim *et al.* 2014; Zhang *et al.* 2014). In one study, the authors found that the expression level of miR-365 was significantly reduced in the rat carotid artery after balloon injury, a pathological process characterized by aberrant proliferation of the medial smooth muscle cells (Zhang *et al.* 2014). In the other study, it was demonstrated that mitogens such as platelet-derived growth factor, angiotensin II and serum all downregulated miR-365 expression in vascular smooth muscle cells, whereas miR-365 overexpression induced cell cycle arrest (Kim *et al.* 2014). Moreover, Qin et al. provided evidence showing that treatment of endothelial cells with oxidized low-density lipoproteins increased miR-365 expression, which in turn mediated an apoptotic response by repressing Bcl-2 expression (Qin *et al.* 2011). These data suggest that miR-365 may have important roles in regulating vascular physiology and may be involved in the pathogenesis of vascular disease.

Evidence suggests that miR-365 may target multiple molecular targets to regulate proliferation in a cell-specific manner. For example, in human colon cancer cells, the cytostatic effect of miR-365 appears to be mediated by targeting and repressing the expressions of Cyclin D1 and Bcl-2 (Nie *et al.* 2012). On the other hand, it was shown that the transcription factor NKX2-1 was the target of miR-365 in suppressing proliferation of lung cancer cells (Kang *et al.* 2013). In vascular smooth muscle cells, both groups identified that miR-365 exerted its anti-proliferative actions by inhibiting the expression of cyclin D1 (Kim *et al.* 2014, Zhang *et al.* 2014).

In the present study, we showed that the serine/threonine protein kinase SGK-1 appeared to be a target of miR-365. This notion has also been reported by others (Xu *et al.* 2011). We clarified that miR-365 mimic significantly decreased SGK-1 expression at both mRNA and protein levels in endothelial cells. We further demonstrated that the SGK-1 inhibitor GSK

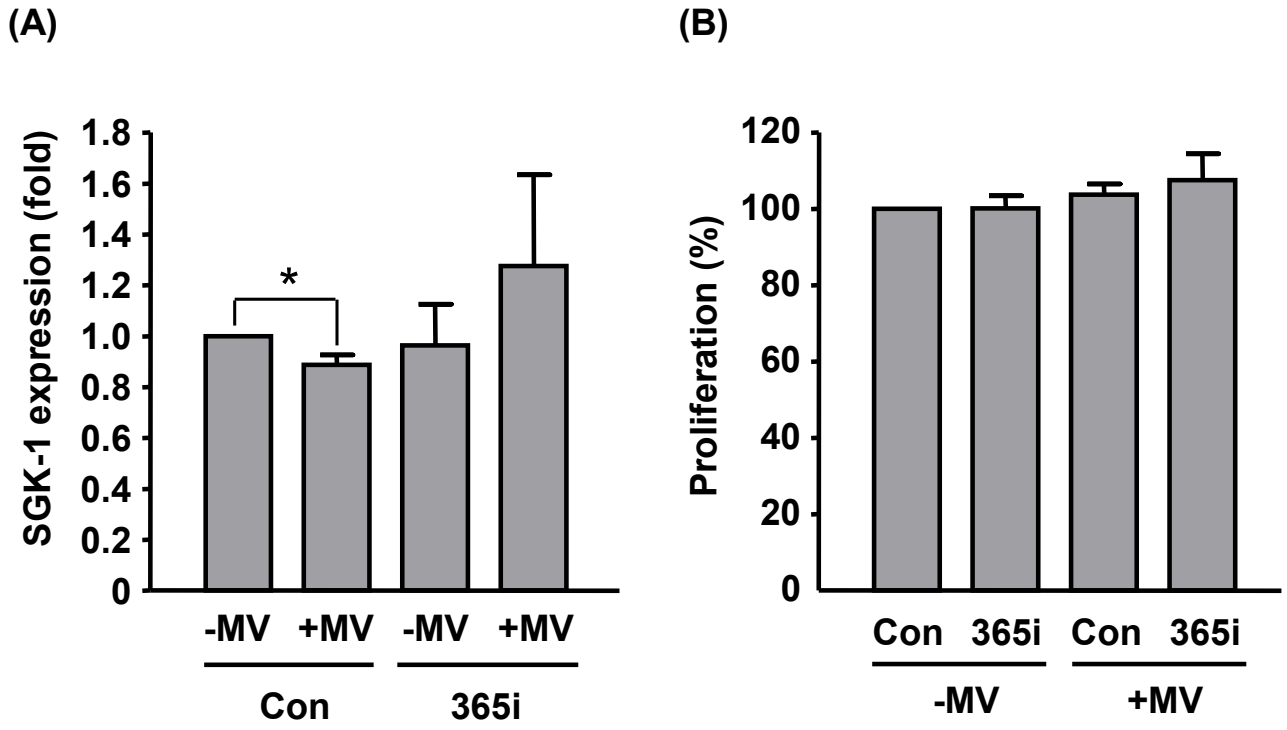

Figure 6. Effects of microvesicles (MV) isolated from the plasma of intracerebral hemorrhage patients, with or without miR-365 antagomir (365i) on (A) SGK-1 expression (measured by qPCR) and (B) proliferation in cultured HUVECs. A nonspecific antagomir molecule was used as control (Con). Data were mean ± SEM. * $P < 0.05$ (only with unpaired *t*-test); $n = 3 – 5$.

650394 mimicked the effect of miR-365 expression on endothelial cell proliferation, whereas in the presence of GSK 650394, miR-365 mimic had no significant effect, supporting a causal role of SGK-1 in mediating the miR-365 effect. We noted, however, that GSK 650394 was reported to have potential off-target effects other than inhibiting SGK-1 (Burgon *et al.* 2014). Upon stimulation with mitogens such as insulin or insulin-like growth factor-1, SGK-1 is activated by phosphorylation by phosphoinositide-3-kinase (PI3K) or mammalian target of rapamycin complex (mTORC), and the activated SGK-1 shares common downstream substrates with the prosurvival kinase Akt (Bruhn *et al.* 2010). Interestingly, a recent study has shown that SGK-1 can promote vascular smooth muscle cell proliferation by inducing phosphorylation of glycogen synthase kinase-3β and β-catenin activation (Zhong *et al.* 2014). Taken together, these findings support the notion that SGK-1 is a critical regulator of cell proliferation, including vascular cells (Bruhn *et al.* 2010). Nevertheless, a limitation of the present study was that we could not exclude an involvement of other miR-365 targets in the anti-proliferative effects.

It is thought that circulating miRNAs in the plasma are encapsulated in membranous microvesicles (Reid *et al.* 2010), which can be internalized by other cells and subsequently modulate the functions of recipient cells (Zhang *et al.* 2010). We treated endothelial cells with native microvesicles isolated from the plasma of intracerebral hemorrhage patients. We found that microvesicle treatment decreased the expression level of SGK-1, although the effect was relatively small. Moreover, we showed that inhibiting the miR-365 function with an antagomir abrogated this effect. These results indicate that miR-365 contained in the microvesicles can exert biological functions in vascular endothelial cells, supporting the notion that microvesicle-mediated microRNA transportation may represent a novel means of remote cell-cell communications (Valadi *et al.* 2007; Zhang *et al.* 2010). However, although we showed that microvesicle treatment reduced SGK-1 expression, it failed to affect the rate of proliferation of endothelial cells. A possible reason to explain this observation is that microvesicles contain numerous biologically active molecules, while the effect of miR-365 may be masked by those of other molecules.

Given that the levels of multiple miRNAs are significantly elevated in the plasma of intracerebral hemorrhage patients (Guo *et al.* 2013), we were inspired to explore whether these altered miRNAs have any effects on the regenerative functions (e.g. proliferation and migration) of vascular endothelial cells. Based on the present experiemnts, however, we could not directly link miR-365 with complications or outcomes following intracerebral hemorrhage. Instead, our results have provided new clues for molecular mechanisms of vascular diseases with impaired endothelial proliferative functions. Although our results and those from other groups indicate that miR-365 may be actively involved in modulating vascular cell functions, the pathophysiological significance of this miRNA in influencing vascular disease progression and/or the vascular healing process in vivo warrants more investigations.

In summary, we have identified that miR-365 is a suppressor of endothelial cell proliferation, and this effect is related to inhibition of SGK-1 expression by miR-365. Together with the data from previous studies (Qin *et al.* 2011), it is suggested that miR-365 may have important roles in vascular physiology and/or pathophysiology by modulating endothelial cell functions.

Conflicts of interest

None.

Authors' contributions

Xiao Wu performed the experiments. Fan Jiang conceived the study and wrote the manuscript.

Acknowledgements

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