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# MicroRNA-92a Regulates Expression of Kruppel-like Factor2 in Rabbit Model of Intracranial Aneurysm

X. Wu[1*], J. Zhang[2*], Q. Huang[1✉], P. Yang[1], J. Chen[1] and J. Liu[1✉]

[1] Department of Neurosurgery, Changhai Hospital, Shanghai, China 200433
[2] Department of Neurosurgery, PLA 455 Hospital, Shanghai, China 200052

**Corresponding author:** Dr. Qinghai Huang and Dr. Jianmin Liu, Department of Neurosurgery, Changhai Hospital, 168 Changhai Road, Yangpu district, Shanghai, China 200433. Email: ljmjianmingliu@126.com
* These authors contributed equally to this work.

**Abstract**

To explore role of miRNA-92a in regulation of KLF2 expression in intracranial aneurysm model, real time PCR, IHC, ISH and luciferase activity were used to test relationship of miR-92a and KLF2. The results demonstrated that KLF2 showed a time-dependent increase following development of IA model, but the miR-92a showed a time-dependent decrease, co-expression of miR-92a was found to effectively down-regulate luciferase expression in constructs with 3′UTRs of KLF2, to determine the functions of miR-92a in rabbit ECs, we transfected the pre-miR-92a into rabbit ECs for over-expression, the results showed that the protein level of KLF2 was significantly down-regulated when over-expression of miR-92a. In conclusion, our research demonstrated that miR-92a and KLF2 were negative correlation in intracranial aneurysm model, and miR-92a could directly target KLF2 in endothelial cells through complementary sequence of 3′UTR region.

***Key words:*** MiRNA-92a, target gene, Kruppel-like Factor 2, intracranial aneurysm, rabbit model.

## Introduction

Intracranial aneurysm (IA) is a common vascular disorder, which frequently leads to fatal vascular rupture leading to subarachnoid hemorrhage (SAH). Recent genome-wide association studies have identified several genes that were associated with the risk of harboring IA. The Krüppel-like family of transcription factors (KLFs) are a set of zinc finger DNA-binding proteins that regulate gene expression. Krüppel-like Factor 2 (KLF2), also known as lung Krüppel-like Factor (LKLF), is a member of the Krüppel-like factor family of zinc finger transcription factors, and it has been implicated in a variety of biochemical processes in the human body, including lung development, embryonic erythropoiesis, epithelial integrity, T-cell viability, and adipogenesis (1). In addition, KLF2 play a role in maintaining the development of endothelial cells and the normal structure and functions of blood vessels (2). Most importantly, KLF2 can be regulated by intravascular wall shear stress (WSS), corresponding to the change of its expression. In recent years, it has become clear that KLF2 is a central regulator of endothelial and monocyte/macrophage proinflammatory action (3-5). Although the effect of KLF2 in macrophage activation predicts that it likely inhibits vascular inflammation, the mechanisms of action of KLF2 in this process remain uncertain.

MiRNAs are small single-stranded non-coding RNAs(18-25 nucleotide) transcribed in the nucleus, processed by the enzymes Drosha (DROSHA) and Dicer (DICER1) and incorporated in RNA-induced silencing complexes that mediate the translational inhibition or degradation of target messenger RNAs (6). Many miRNAs have been identified that play key roles in physiological and pathophysiological processes, including atherosclerosis (7). microRNA-92a (miR-92a) is a crucial miRNA that inhibits endothelial cells angiogenesis and impairs endothelial cells function(8, 9). In this research, IA models were established in rabbit to research the relationship of KLF2 and miR-92a in development of IA.

## Materials and Methods

### *Experimental animals*

A total of 144 four-month-old male New Zealand rabbits (2.0-2.5 kg) were enrolled in the study, and all animal experiments were performed in accordance to the guidelines established by the Animal Care and Experimentation Committee of The Changhai Medical Research Institute. The rabbits were randomly divided into three groups, namely sham group, unilateral ligation of CCA group (UL group), and bilateral ligation of CCA group (BL group) (n=48 for each group). Animals in each group were tested at week 1, 2, 3 or 4 after ligation (n=6 for each time point).

### *Intracranial Aneurysm model of rabbit induced by hemodynamic*

The animals were anaesthetized with 0.2 ml/kg xylazine hydrochloride (Sigma, USA) solution by intramuscular injection, and after quietness, 1% pentobarbital sodium (Sigma, USA) (20 mg/kg) was injected via the ear vein until eyelash reflex disappeared. After anesthesia, the four limbs, in the dorsal position, were fixated onto a small animal fixation station. The hair from suprasternal fossa to the middle of lower-jaw horizontal neck was shaved, followed by iodophor disinfection solution and hole towel spread to the rabbit. At the cervical median incision, the skin in front of the trachea was cut open, and the muscles in anterior cervical region were cut wide along the neck white line until reaching the trachea. The

**Table 1.** The primer sequences of KLF2, miR-92a, GAPDH and U6.

| Name | Sequence | Length (bp) | Tm°C |
|---|---|---|---|
| KLF2 | F:5' GAGCCTATCTTGCCGTCCTT 3'<br>R: 5' AGCACGCTGTTTAGGTCCTC 3' | 119 | 60 |
| GAPDH | F: 5' AGACACGATGGTGAAGGTCG 3'<br>R: 5' TGCCGTGGGTGGAATCATAC 3' | 164 | 60 |
| miR-92a | F: 5' UAUUGCACUUGUCCCGGCCUG3' | 98 | 60 |
| U6 | F:5' CTCGCTTCGGCAGCACAR 3' | 111 | 60 |

neck muscles were opened and separated at the lateral trachea, exposing the bilateral CCAs. Then the cervical arterial sheath was separated, and the cervical arteries were stripped off, without injury of the vagus. Rabbits were treated without ligation (Sham group), with right-side ligation (UL group) and with bilateral ligations (BL group), respectively. The basilar artery was selected after four weeks to test expression of miR-92a and KLF2 using real time PCR and IHC.

***Real time PCR***

RNA was extracted from cells using Trizol reagent (Invitrogen, USA). Total RNA was reverse transcribed, followed by 30 PCR cycles using RNA PCR kit ver 3.0 (Taraka, China). Information of gene-specific primer pairs is listed in Table 1. PCR was performed in 50 µl of mixture containing 10 µis5f PCR Buffer (Taraka), 28.5 µCRdd$H_2O$, 0.25 µ, Ex-Taq (Taraka), 0.5 µx-Taq (Taraka), 0.5 28.5rmed in 50 µx-template cDNA. The cycling conditions consisted of an initial 2-min cycle at 94°C, followed by 30 30-s cycles at 94°C (denaturation), one 30-s cycle at 50–60°C (annealing), and one 2-min cycle at 72°C (extension). PCR products were detected by 2.5% agarose gel electrophoresis. Real-time PCR was performed in a 20-µ(extensre containing 10 µl SYBR premix Ex Taq buffer (Takara), 0.4 µl ROX Reference Dye, 0.8 µM each of forward and reverse primers (Table 1), 1 µl template cDNA, and 7 µl dd$H_2O$. The cycling conditions consisted of an initial 10 s at 95°C, followed by 40 cycles of two-temperature cycling: 5 s at 95°C (for denaturation) and 34 s at 60°C (for annealing and polymerization). Each experiment was performed in duplicate in 96-well plates and repeated three times. Gene expression was detected on an ABI 7500 real-time PCR system (USA). The expression level was calculated using the $2^{-\Delta\Delta Ct}$ method to compare relative expression.

***Endothelial cells culture***

Mouse cardiac vascular endothelial cells were isolated according to the procedure for endothelial cells isolation as described previously, with some improvements.(10) (11) (12) The blood vessel was washed 3 times with phosphate buffer saline (PBS) containing 100 IU/mL penicillin and 100 µg/mL streptomycin to remove the blood. The blood vessel tissue pieces were digested with 0.25% (v/v) trypsin in PBS for 30 min at 37 °C. Enzymatic activity was then neutralized with the 5 mL FBS (Gibco, USA). The cell suspension containing the majority of $CD31^+$ vascular endothelial cells were obtained after being purified through flow cytometry. Mouse cardiac vascular endothelial cells were cultured in EGM-2 MV medium (LONZA, Switzerland).

***Assay of luciferase activity***

The3′UTRfragmentsforKLF2weregeneratedbyPCR using the following primers: 5′-AGAGGGTCTCCCTC-GATGAC-3′ and 5′-CTAGTGTAGACCCGGTGGGA-3′and cloned into the psiCHECK-2 vector (Promega) downstream from the Renilla luciferase cassette. The predicted miR-146 binding site was mutated using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). HEK293T cells were grown in a 96-well plate and co-transfected with the luciferase reporter vector together with a miRNA precursor or a negative control (20 nM; Ambion) using Attractene (Qiagen) according to the manufacturer's instructions. Activities of firefly and Renilla luciferase were analyzed using the Dual-Luciferase Reporter Assay System (Promega) 24 h after transfection.

***Expression of miR-92a by recombinant lentivirus***

The pre-miR-92a sequence was synthesized by Sangon Biotech (Shanghai, China), cloned into a lentiviral vector, and then used to recombine lentivirus in HEK293F cells. The lentivirus, designated as pCMV-SM30, also expressed eGFP as a marker for monitoring infection efficiency. Analogous lentivirus expressing only monomeric GFP was used as a control. Real-time PCR was used to evaluate the expression level of miR-92a after virus infection. miRNAs were isolated from cells using a microRNA isolation kit (Applied Biosystems, USA) according to the manufacturer's instructions. cDNA synthesis was performed using the High-Capacity cDNA Synthesis Kit (Applied Biosystems, USA) with 2 ng RNA as a template. The miRNA sequence-specific reverse-transcription PCR primers for miR-146 and endogenous control U6 were purchased from Ambion (USA). Real-time PCR analysis was performed using the Applied Biosystems 7500 real-time PCR system. The gene expression threshold cycle (CT) values of miRNAs from each sample were calculated by normalizing the samples to the internal control U6, and relative quantitation values were plotted.

***Western blotting***

KLF2, the target gene of miR-92a, was detected by western blot analysis following overexpression of miR-92a. Cells were lysed using M-PER Protein Extraction Reagent (Pierce, USA) supplemented with a protease inhibitor cocktail (DMSF). Protein concentrations of the extracts were measured using the BCA assay (Pierce, USA) and equalized with extraction reagent. Equal amounts of extracts were loaded and subjected to SDS-PAGE, followed by transfer onto nitrocellulose membranes. Specific antibodies (KLF2, abcam, USA, 1:500; GAPDH, abcam, USA, 1:5000) and horseradish peroxidase-coupled secondary antibodies (1:1000) were purchased from Santa Cruz, USA. Membranes were probed using ultra-enhanced chemiluminescence western blotting detection reagents. GAPDH was used as

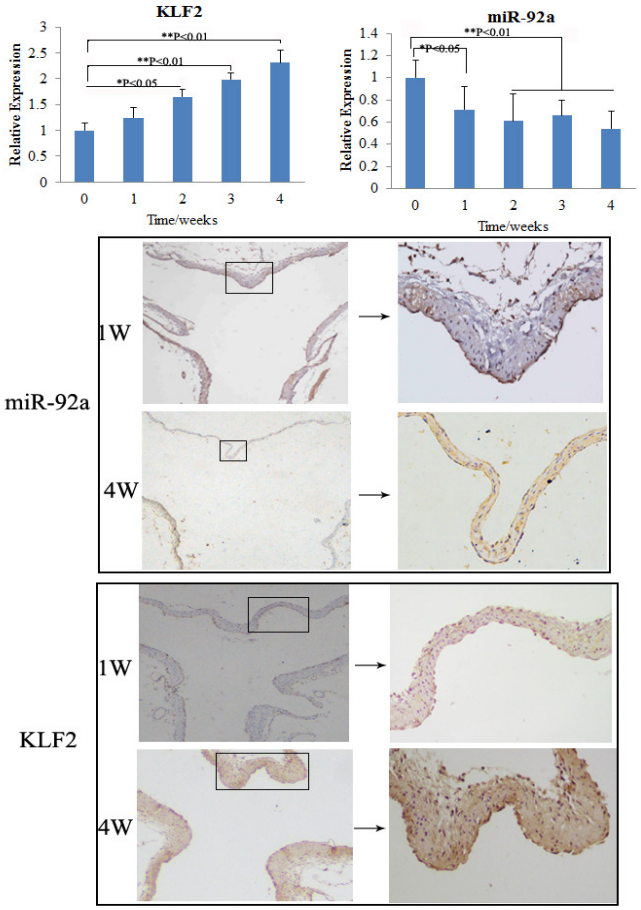


**Figure 1.** Expression level of KLF2 and miR-92a in Intracranial Aneurysm model. The real time PCR results showed that KLF2 showed a time-dependent increase following development of IA model, but the miR-92a shoed a time-dependent decrease. The IHC and ISH also demonstrated that the expression of miR-92a and KLF2 were negative correlation.

an internal control.

### *Immunohistochemistry (IHC)*

The basilar artery sections were dewaxed, rehydrated, blocked endogenous peroxidase activity, and antigen retrieval, then treated with 10% normal goat serum and incubated with primary antibody at 4°C for overnight. The sections were then incubated second antibody at room temperature (RT) for 3h. Subsequently, the slides were colored with DAB for 3-5 minutes at RT, all the images were obtained and photographed using a microscope.

### *In Situ Hybridization (ISH)*

The basilar artery was fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) overnight at 4°C and then treated with proteinase K for 30 min. In situ hybridization was performed as previously described (13). Briefly, Locked nucleic acid (LNA) probes were designed and synthesized by Sangon Biotech. The sequence of the LNA probe complementary to mature miR-92a was ACAGGCCGGGACAAGTGCAATG. LNA probes were labeled with digoxigenin using the DIG Oligonucleotide 3′-end Labeling Kit (Roche, USA) and purified using Sephadex G-25 MicroSpin columns (Amersham, Sweden), all the images were obtained and photographed using a microscope.

### *Statistical Analysis*

Statistical analyses of the data were performed with a one-way ANOVA followed by the Tukey-Kramer honestly significant difference (HSD) test for the three sets of results. A P-value of less than 0.05 was considered significant. Statistical analyses were done with a JMP® Statistical Discovery Software (SAS Institute, Cary, NC).

## Results

### *Expression of miR-92a and KLF2 in IA model*

The basilar arteries in the Sham group had smooth tip lumina, complete endothelial cells and complete internal elastic membranes, but no fracture, thinning or aneurysm formation. But the UL group had slight outward bulging, though it was not very severe in the tip of basilar artery, and the BL group had aneurysmal bulges. The expression of miR-92a and KLF2 were analyzed using real time PCR and IHC in basilar artery. The real time PCR showed that KLF2 showed a time-dependent increase following development of IA model, but the miR-92a shoed a time-dependent decrease (Figure 1). The IHC and ISH also demonstrated that the expression of miR-92a and KLF2 were negative correlation (r=-0.768, $P<0.01$, Figure 1).

### *KLF2 is a direct target of miR-92a*

miRNA target genes are likely to have relatively long and conserved 3′UTR (14). We noticed that KLF2 has a long evolutionarily conserved 3′UTR, so we used the TargetScan algorithm (15) to search for miRNAs that could potentially regulate . The miR-92a has putative target binding sites in KLF2 in the rabbit genome (Figure 2). To directly test whether miR-92a targets KLF2, we cloned the 3′UTRs of KLF2 downstream of a luciferase reporter, and co-transfected these reporter constructs along with miRNA precursors into the human cell line HEK293T. Co-expression of miR-92a was found to effectively down-regulate luciferase expression in constructs with these 3′UTRs (Figure 2). Muta-

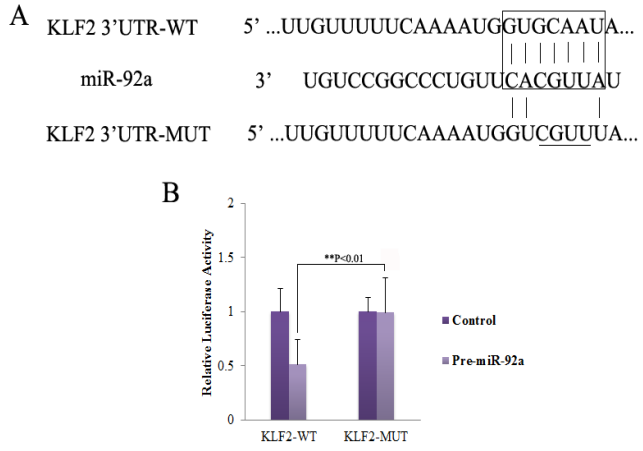


**Figure 2.** KLF 2 is the direct target of miR-92a. miR-92a complementary sites with 3′UTR of KLF2. The mutant sequence (KLF2-MUT) is identical to KLF2-WT construct except for four point mutations disrupting base-pairing at the 5′end of miR-92a (indicated with a bar). Mutating the miR-92a target site in the 3′UTR of KLF2 abolishes inhibition of luciferase activity by endogenous miR-92a in 293 cells.

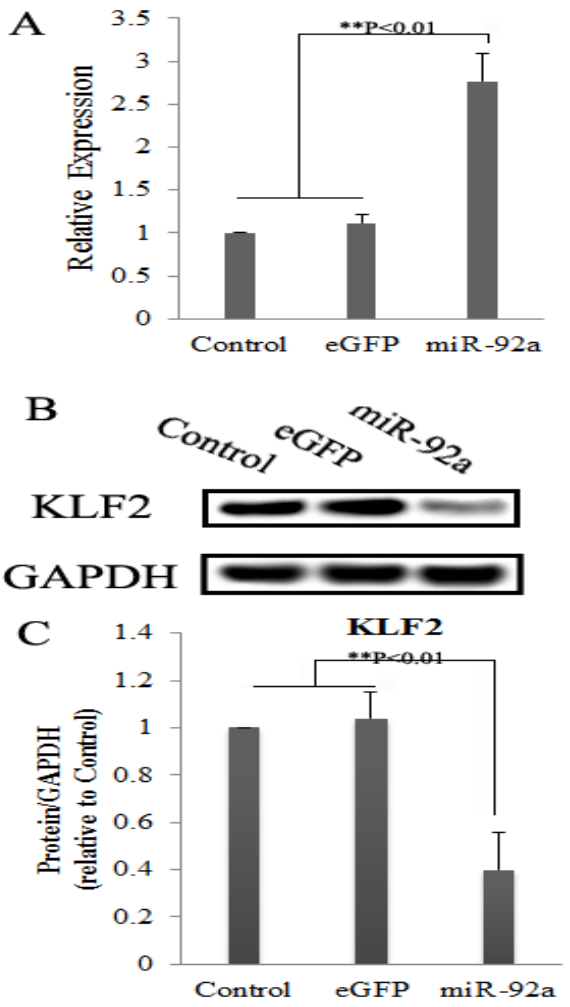


**Figure 3.** Rabbit ECs were transfected with lentivirus as described in the methods section, and the expression of miR-9 was quantified by real time PCR. A The expression of miR-92a was significant increase after transfection. B Effect of miR-92a on protein level of KLF2. C the protein expression level of KLF2 were analyzed by Image J tools.

tions in the seed sequence of the predicted miR-92a binding sites within KLF2 abolished the inhibitory effects of miR-92a on luciferase expression.

***miR-92a expression after transfection in rabbit ECs***

To determine the functions of miR-92a in rabbit ECs, we transfected the pre-miR-92a into rabbit ECs for over-expression. The expression of miR-92a was quantified by real time-PCR at 72th h after transfection. As shown in Figure 3, miR-92a levels were significantly elevated by the pre-microRNA transfection. We then analyzed the miR-92a sequence and its target gene sequence, confirmed that KLF2 was target genes for miR-92a. Protein expression of KLF2, the putative target gene, was performed on miR-92a-transfected cells using the Image J tools comparative method (Figure 3), and the results showed that the protein level of KLF2 was significantly down-regulated when over-expression of miR-92a.

## Discussion

An intracranial aneurysm (IA, also called cerebral or brain aneurysm) is a cerebrovascular disorder in which weakness in the wall of a cerebral artery or vein causes a localized dilation or ballooning of the blood vessel. Aneurysms in the posterior circulation (basilar, vertebral and posterior communicating arteries) have a higher risk of rupture. Basilar artery aneurysms represent only 3%-5% of all intracranial aneurysms but are the most common aneurysms in the posterior circulation. Kruppel-like factor 2 (KLF2) is a pivotal in maintaining the development of endothelial cells and the normal structure and functions of blood vessels. Most importantly, KLF2 can be regulated by intravascular wall shear stress (WSS), corresponding to the change of its expression. The shear-responsive transcription factor KLF2 is a critical regulator of the patterns of endothelial gene expression induced by atheroprotective flow. In our research, the KLF2 were up-regulated after established IA model in basilar arteries. Of considerable interest, recent evidence indicates that significant interactions exist between KLF2 and miRNAs in endothelial cells (16).

miRNAs are small, noncoding RNAs that are cleaved from 70-100 nucleotide (nt) hairpin pre-miRNA precursors in the cytoplasm by RNaseIII Dicer into their mature form of 19-25 nt. Single-stranded miRNAs bind messenger RNAs of potentially hundreds of genes at the 3′UTR region with perfect or near-perfect complementarity, resulting in degradation or inhibition of the target messenger RNA (17). miRNAs are fundamental biological molecules that have been shown to play important roles in biological development(18). There are some reports about KLF2 was regulated by miRNA in other research, miR-126 has been reported to be up-regulated by flow in a KLF2-dependent manner in zebrafish embryos (19). Additionally, miR-143/145 are regulated by KLF2 in endothelial cells and may contribute to the vasculo-protective functions of KLF2 (20). Although KLF2 is known to exert anti-inflammatory effects and inhibit the proinflammatory activation of monocytes, whether KLF2 also affects the expression of miRNAs in macrophages and their role in preventing the pro-inflammatory activation of macrophages has remained elusive to date. In our research, artificial flow causes a down-regulation of miR-92a in basilar arteries, which in turn elevates KLF2 mRNA, these results demonstrated the relationship of miR-92a and KLF2 were negative correlation in IA model. And then the endothelial cells were obtained from basilar arteries to test the regulation of miR-92a and KLF2. The luciferase activity was used to test whether miR-92a targets KLF2, the result showed miR-92a directly target 3'UTR of KLF2. The KLF2 transcription factor has previously been shown to modulate miRNA expression in several cell types. For example, KLF2 binds to the promoter of the miR-143/145 gene cluster to up-regulate the expression of vascular protective genes in endothelial cells (20). Additionally, KLF2 also mediates the expression of miR-126 in endothelial and glioma cells (19, 21). Lingrel JB and colleagues recently observed reduced expression of miR-124a and miR-150 in macrophages from myeKlf2-/- mice, thus indicating that KLF2 directly mediates the expression of these two miRNAs in macrophages (22).

Our current results demonstrate that miR-92a plays an important role in development of IA model and that KLF2 is a key target of miR-92a in endothelial cells. Each miRNA could have multiple target genes (15, 23); indeed, several target genes have been predicted and

some tested for miR-92a, including those that encode the transcription factors TGF, DKK, and PPCS, as well as components of the wnt signaling pathway(24-26). One of the questions addressed here is whether the flow rate or cell dysfunction effect mediated by miR-92a in ECs is directly related to repression of KLF2 expression. Results suggest that miR-92a regulates cell dysfunction through repression of KLF2 expression. Although KLF2 is an important target gene of miR-92a, other targets may also play a role in miR-92a function in ECs.

In conclusion, our research demonstrated that miR-92a and KLF2 were negative correlation in intracranial aneurysm model, and miR-92a could directly target KLF2 in endothelial cells through complementary sequence of 3′UTR region.

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