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Original Article

Transfection of adenovirus-mediated mircoRNA-126 gene into infant hemangioma endothelial cells *in vitro*

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Abstract: *Objective:* To investigate the effects of microRNA-126 (miR-126) overexpression on hemangioma endothelial cells (HemECs). *Methods:* An adenoviral vector containing the miR-126 gene was constructed. HemECs were passaged and expanded and adenovirus-mediated green fluorescent protein (GFP) gene was transfected *in vitro*. The infection efficiency of adenovirus vector to HemECs was tested by Ad-GFP infection procedure. GFP expression efficiency was observed using a fluorescence microscope and flow cytometry was used to determine the best virus multiplicity of infection (MOI). The experiment was divided into the blank group, AD-GFP group, and AD-miR-126 group. The miR-126 group was transfected into HemECs *in vitro* with adenovirus-mediated miR-126 gene under optimal MOI conditions. RT-PCR was applied to detect expression of miR-126 gene in cells. The influence of recombinant adenovirus on cell activity was evaluated by CCK-8 assay. Flow cytometry was utilized to detect cell cycle and apoptosis. *Results:* HemECs could be effectively infected by adenovirus containing GFP gene *in vitro*, the transfection efficiency had the dose-effect relationship with multiplicities of infection (MOI). When MOI was 400, the infection efficiency was more than 90%. miR-126 expression in HemECs was significantly enhanced in miR-126 group ($P<0.05$). Compared to the control group, cell proliferation was significantly enhanced ($P<0.05$) and induced S-phase arrest significantly ($P<0.05$) when miR-126 was upregulated. In addition, compared with the control group, the early apoptotic rate was significantly decreased by upregulating miR-126 ($P<0.05$). *Conclusion:* miR-126 overexpression can successfully promote proliferation and inhibit apoptosis of HemECs. This work will provide the theoretical and experimental basis for further transplantation study *in vivo*.

Keywords: mircoRNA-126, HemECs, proliferation and apoptosis

Introduction

Infantile Hemangioma (IH) is the most common benign tumor with an incidence rate as high as 10%. In addition, its natural history is characterized by rapid proliferative growth followed by very slow but nearly inevitable regression over several years [1-3]. Most IH are comparatively small and they cannot pose a serious threat or complication to the baby. However, a small number of IH grow so large that they lead to tissue or organ damage and in some cases become life-threatening [4, 5]. The pathogenesis is unknown and the current view is endothelial cell hyperplasia, the aggregation process, and microvessel lumen formations are the core of vascular tumor growth [6]. microRNAs (miRNAs) have emerged as key regulators of vascular development, being deregulated in many disease processes. Growing evidence suggests the potential involvement of the altered regulation of miR-126 expression in the pathogenesis of cancers and these genes are thought to function as a potential tumor impact factors in osteosarcoma, cervical, lung, gastric, and colorectal cancer [7-11]. To the best of our knowledge, there are no studies on the involvement of miR-126 in IH. In this study, Adenovirus-mediated green fluorescent protein (GFP) gene and miR-126 gene were used to transfect HemECs *in vitro* and observe the effects of miR-126 overexpression on HemECs proliferation and apoptosis, to provide further experimental and theoretical evidence for genetically engineered cells treated with miR-126.

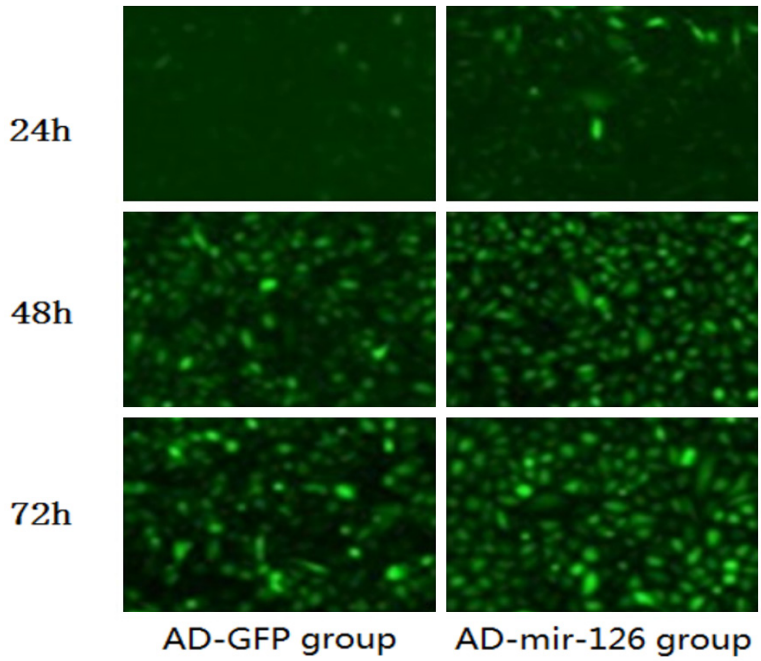

Figure 1. HemECs were transfected by adenovirus. After transfected by 24 h, the green fluorescence could not be seen clearly under the fluorescence microscope. While the strong green fluorescence could be observed after 48 h and the green fluorescence was more obvious after 72 h.

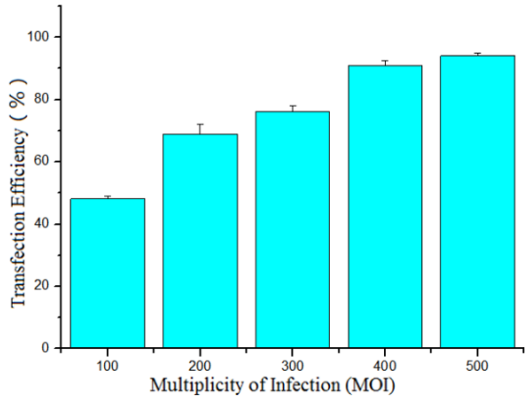

Figure 2. Transfection efficiency of AD-GFP transfected into HemECs. When MOI was more than 400, the transfection rate no longer increased. Therefore, the best MOI is 400.

Materials and methods

Cells and reagents

Hemangioma endothelial cells (HemECs), Green fluorescent protein (GFP) gene, and miR-126 gene recombinant adenovirus were provided by Han Bio Biotechnology Company. Annexin V-PE/7AAD was purchased from BD Pharmingen Company; DMEM/F12 medium was purchased from the American HYClone Company; Fetal bovine serum and Pancreatic enzyme (without EDTA) was from Gibco Company, USA; RT-PCR kit was purchased from TaKaRa company. CCK-8 kit was purchased from Hanbio Biotechnology and DMSO was purchased from WAK-CHEMIE in Germany. The flow cytometry was purchased from the Beckman Company of United States.

Cell culture

The HemECs were cultured in 89% DMEM supplemented with 10% FBS, 1% of penicillin, and streptomycin in a CO_2 incubator with an atmosphere of 5% CO_2 at 37°C. The cells in logarithmic growth phase were used in all of the experiments.

Transfection efficiency

HemECs were transfected with the virus MOI for 100, 200, 300, 400, and 500, respectively, and each MOI value condition was controlled with triplicate sets. Expression of EGFP was observed under fluorescence inverted microscope at 24, 48, and 72 hours after transfection. The proportion of transfected cells was detected by flow cytometry and the best MOI value was detected.

Real-time quantitative RT-PCR for detection of miR-126

Total RNA was isolated using TRIzol Reagent. After quantitation, these RNA samples were reversely transcribed into cDNA (The reaction conditions were: 37°C for 20 minutes and 95°C for 5 minutes). The cDNA was then subjected to qPCR amplification. U6 was used as an internal reference and specific primers for miR-126 were used to amplify miR-126. The PCR conditions were as follows: an initial 95°C for 3 minutes and then 40 cycles of 94°C for 15 s, 60°C for 30 s and 72°C for 30 s and 95°C for 10 s, 65°C for 60 s, 97°C for 1 second. Data were analyzed by using $2^{-\Delta\Delta Ct}$ method.

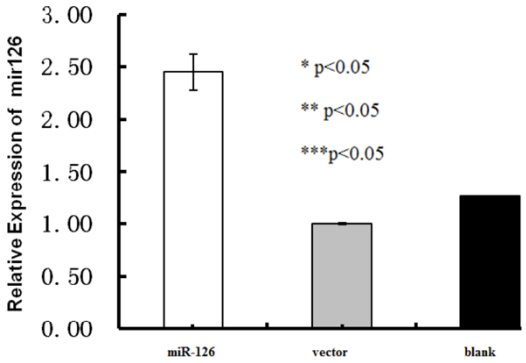

Figure 3. Real-time quantitative RT-PCR for detection of miR-126. *Significant difference between miR-126 expression in vector group and miR-126 group (P<0.05). **Significant difference between miR-126 expression in vector group and blank group (P<0.05). ***Significant difference between miR-126 expression in miR-126 group and blank group (P<0.05).

CCK-8 assays of cell activity

To detect changed cell viability, we performed CCK-8 assay. The cells were digested and collected after transfection of the best MOI and inoculated in 96-well plates for the night. For the time course assay, the incubation time was 24, 48, and 72 hours, respectively. The cells were treated according to the CCK-8 kit and the absorbance value was read in 450 nm wave length.

Flow cytometric analysis of apoptosis

For apoptosis assay, HemECs were seeded in 96-well plates at a density of 1×10^5 cell/well under culture conditions with an atmosphere of 5% CO_2 at 37°C. After incubation with the best MOI for 48 hours, HemECs were harvested and washed. Apoptotic cell death was identified by double supravital staining with Annexin V-PE/7AAD, using the apoptosis detection kit according to the instructions of the manufacturer. Flow cytometric analysis was performed immediately after supravital staining.

Cell cycle analysis

To examine whether miR-126 had an effect on cell cycles, we analyzed cell cycle by flow cytometry. The cells were digested and collected 24, 48, and 72 hours after transfection with the best MOI. Following incubation, the cells were collected and fixed at 4°C overnight. The samples were analyzed with flow cytometry.

Statistical analysis

SPSS software was used for statistical analysis. Paired t-test was performed to compare fold differences in miR-126 expression between miR-126 group, AD-GFP group, and Blank group with $P<0.05$ considered statistically significant.

Results

In determination of transfection efficiency of EGFP gene transfected HemECs and determination of the best MOI after the virus infection, the cell state was observed at 24 hours, 48 hours, and 72 hours, respectively. As the virus infection time prolonged, green fluorescence intensity increased gradually and the fluorescence proportion reached more than 90%. The infection was very obvious (**Figure 1**). Results of flow cytometry showed that the transfection rates were 48.2%, 69.0%, 76.1%, 91.7%, and 94.0% under the conditions of MOI 100, 200, 300, 400, and 500. At MOI 400, the transfection rate tended to be stable and maintained at 90%. When MOI was more than 400, the transfection rate no longer increased, the cells appeared round, fell off, and the number of fluorescence fragments increased. Therefore, the best MOI is 400 (**Figure 2**).

qRT-PCR

Using real-time quantitative RT-PCR, expression levels of miR-126 in HemECs were compared with controlled groups. We found that miR-126 expression in miR-126 group was significantly enhanced with the blank group ($P<0.05$) (**Figure 3**).

miR-126 overexpression promotes the proliferation of HemECs

From the measured absorbance values of 24 hours, 48 hours, and 72 hours, proliferation curves of different treatment groups were drawn. The curves showed that the proliferation ability of miR-126 group cells after infection, compared to the control group, was significantly enhanced ($P<0.05$). A significant difference was observed in the proliferation of HemECs especially in 48 hours ($P<0.01$). These results indicate that miR-126 overexpression

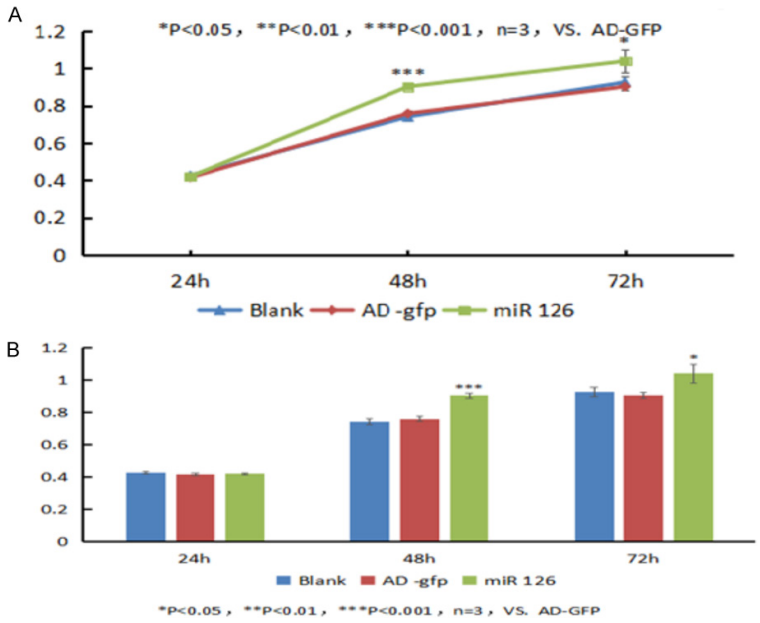

Figure 4. Proliferation is detective after miR-126 is transfected into HemECs. The cell proliferation activity of HemECs transfected with miR-126 increased significantly (P<0.05) and the ability of miR-126 to promote proliferation was the most significant at 48 h (P<0.001).

could promote proliferation of HemECs (**Figure 4**). Absorbance values of cells at 450 nm is shown in **Table 1**.

miR-126 overexpression suppresses apoptosis of HemECs

Three controlled sets of early apoptosis in normal cell group (Blank group) were 2.67%, 2.21%, and 1.98% and the late apoptosis were 2.95%, 3.44%, and 3.57% (**Table 2**) indicating that the activity of HemECs was very good and that there was no problem in the operation process. The three controlled sets of early apoptosis in miR-126 group were 0.86, 0.66, and 0.74 times than those in AD-GFP group. There were significant differences for inhibiting (P<0.05) while the proportion of late apoptosis in miR-126 group were 0.83, 0.98, and 0.93 times higher than those of AD-GFP group, with no obvious difference (**Figure 5**). The proportion of apoptosis indicated that miR-126 overexpression could inhibit the early apoptosis of HemECs.

miR-126 overexpression induces S-phase arrest in HemECs

As we had observed a significant growth-inhibitory effect of miR-126 overexpression on HemECs, we further investigated effects of miR-126 on the cell cycle. To examine whether miR-126 inhibited HemECs growth through blocking cell cycle progression, we treated HemECs with concentrations for 24 hours and subjected them to flow cytometric analysis. The cell number in S phase of miR-126 group was significantly increased with the decreased G2/M phase (**Figure 6**), indicating S phase was blocked (P<0.05). It is speculated that miR-126 overexpression may lead to the effect of the cell cycle. These results indicate that overexpression of miR-126 induces S phase arrest in HemECs, which contributes to the growth inhibitory properties of miR-126.

Discussion

miR-126 has been shown to be either a tumor suppressor or an oncogene depending on the type of cancer, with the exact mechanisms in various cancer types still under intensive investigation. Several studies have shown that miR-126 is frequently down regulated in a variety of malignancies and it could work as a potential tumor suppressor gene [12-14]. Their essential roles in the cell cycle have accordingly become very important areas of investigation. However, miR-126 was not a decrease but an increase in expression and even plays a role in cancer-promoting genes in some tumors. For example, miR-126 expression increased in acute myeloid leukemia cells and can promote cancer cell proliferation and suppress cancer cell apoptosis [15, 16]. Regarding IH, it remains controversial as to whether miR-126 is a tumor-suppressive or oncogenic miRNA. Therefore, it is an urgent task to predict the potential regulators of miR-126 expression to discover the molecular mechanisms involved in the development of IH.

Genetic experiments require appropriate vectors, the most widely used is the adenovirus vector [17, 18]. Green fluorescent protein (GFP) has the ability to produce fluorescence without any substrate or cofactor. It also can be observed in vivo and other characteristics,

Table 1. Absorbance values of cells at 450 nm

	Blank			AD-GFP			miR-126		
	1	2	3	1	2	3	1	2	3
24 h	0.4112	0.4089	0.4235	0.4276	0.4195	0.4135	0.4004	0.3967	0.4142
48 h	0.8053	0.7857	0.8264	0.8101	0.8006	0.7844	0.8909	0.8799	0.9015
72 h	0.9504	0.9378	0.9247	0.9203	0.9010	0.8903	1.1339	1.0216	1.0945

Table 2. The proportion of early apoptosis and late apoptosis in groups

	Blank 1	Blank 2	Blank 3	Ad-GFP 1	Ad-GFP 2	Ad-GFP 3	miR-126 1	miR-126 2	miR-126 3
Early apoptosis level	2.67%	2.21%	1.98%	3.65%	3.37%	3.29%	3.13%	2.23%	2.42%
Later apoptosis level	2.95%	3.44%	3.57%	5.16%	5.52%	5.32%	4.27%	5.39%	4.95%

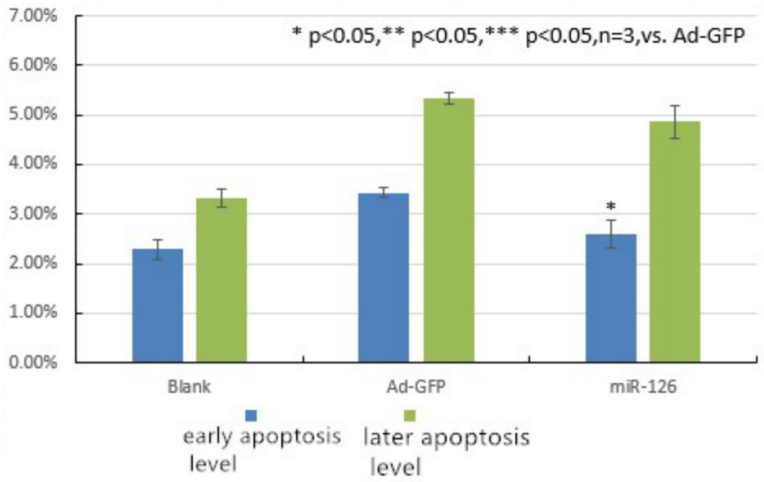

Figure 5. HemECs apoptosis level is changed following transfection of miR-126. Early apoptosis level is inhibited significantly after miR-126 transfection into HemECs (P<0.05). Later stage apoptosis levels were not obviously different after miR-126 transfected into HemECs (P>0.05).

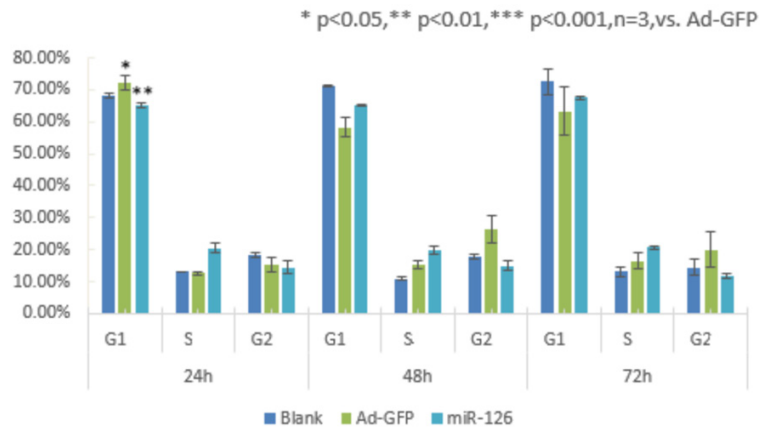

Figure 6. Comparison of the proportion in cell cycle in 24 h, 48 h, and 72 h after infection with miR-126 virus. The cell number in S phase in miR-126 group was significantly increased accompanied by a decrease in G_2 phase (*P*<0.05).

being widely used in gene research [19, 20]. In this study, GFP gene and miR-126 gene were successfully transferred into HemECs by adenovirus vector, which provided an ideal model for gene expression and transfection. Transfection of GFP gene into HemECs was observed under fluorescence microscopy. The morphology of cells was similar to that observed under light microscope. Flow cytometry showed that MOI was stable at a value of 400 and high transfection efficiency which suggests that HemECs are susceptible to adenovirus.

A large number of studies have also confirmed that uncontrolled cell proliferation is the main mechanism for neoplastic progression [21, 22]. The present study indicates that miR-126 gene remarkably induces cell proliferation in HemECs. It can be seen from the changes in cell growth curve that after infection with the virus, the proliferation ability was obviously enhanced in 48 hours. Although the growth rate decreased after 48 hours, there was still a strong ability to proliferate with respect to the control groups. Apoptosis is an important phenomenon

in cytotoxicity induced by antitumor drugs. The execution of apoptosis is associated with characteristic morphological and biochemical changes mediated by a series of gene regulation and cell signaling pathways [23]. With regard to unveiling the underlying mechanism of miR-126 enhancing cell proliferation, we examined HemECs treated with miR-126 for evidence of apoptosis. After treatment with miR-126, the HemECs displayed clear morphological signs of apoptosis in microscopic observation. Flow cytometry showed that there are significant differences for inhibiting the early apoptotic rate in miR-126 group ($P<0.05$), while the proportion of late apoptosis in miR-126 group were 0.83, 0.98, and 0.93 times higher than those of AD-GFP group, with no obvious difference ($P>0.05$). The proportion of apoptosis indicates that miR-126 overexpression could inhibit the early apoptosis of HemECs.

miR-126 genes could decrease the occurrence of HemECs apoptosis, the inhibition of apoptosis. Through the cell proliferation test and apoptosis detection, we found that changes on miR-126 levels have an important effect on the cells. Improving the level of miR-126 in HemECs can significantly increase the proliferation of cells and reduce cell apoptosis, confirming that miR-126 is used as a mediator in the progression of hyperplasia of infantile hemangioma.

Cell proliferation and apoptosis was controlled by the progression of the cell cycle [24]. To investigate how miR-126 induced cell proliferation and inhibited apoptosis, we used flow cytometry to assess the effect of miR-126 on cell cycle distribution. The cell number in S phase cells in miR-126 group was significantly increased along with a decrease in G2 phase ($P<0.05$). It is speculated that overexpression of miR-126 may lead to the effect of the cell cycle. The results show that miR-126 overexpression can induce cell cycle arresting at S phase, which plays an important role in cell cycle regulation.

Therefore, the identification of genetic and epigenetic alterations in proliferating and involution infantile hemangioma lesions will likely contribute to better understanding the underlying molecular mechanisms of development and progression of this disease, a leading cause of morbidity in affected children. The emerging data suggests that while miR-126 may play a crucial role in preserving stemness capability of HemECs, future studies should specifically investigate each of the aforementioned mechanisms.

In order to develop better diagnostic, therapeutic, and prognostic tools for IH enhancement for molecular level, understanding of the disease is crucial. The specificity of miR-126 to IH and the correlation between circulating miR-126 levels implies that miR-126 profiling may be a useful clinical tool in diagnosis anomalies. Therefore, HemeCs transplantation and miR-126 gene therapy are in close connection through the effective target gene into the target cells, which will lay the foundation for the next step transplantation experiments *in vivo*.

Conclusion

The significance of this observation indicates that miR-126 may become an important potential therapeutic agent for infantile hemangioma and suggests that miR-126 may be a potential therapeutic target for the treatment of human hemangioma. Large animal experiments and clinical trials will be necessary to determine whether it can stop growth or shrink the lesions.

Acknowledgements

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Disclosure of conflict of interest

None.

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