Vascular Smooth Muscle Cell Proliferation Is Influenced by let-7d MicroRNA and Its Interaction With KRAS

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Background: Several microRNAs (miRNAs) have been reported to regulate cardiovascular biological and pathological processes through inhibiting the translation of certain RNA transcripts. However, little is known about the association between miRNAs and vascular smooth muscle cell (VSMC) proliferation. The aim was to investigate the role of miRNAs in VSMC growth and the potential mechanism.

Methods and Results: Primary VSMCs were isolated from the medial layer of the thoracic aorta obtained from spontaneously hypertensive rats (SHR) and Wistar Kyoto rats (WKY). miRNA microarrays were used to analyze the difference in miRNA expression between VSMCs of SHR and WKY rats and were validated using TaqMan real-time PCR. Of the potentially related genes under the influence of let-7d identified through literature search, KRAS was verified by western blot and functionally analyzed using miRNA mimics transfection and analysis of transfectants by cell enumeration was made using CCK-8 and flow cytometric analysis of cell cycle progression. let-7d-transfected VSMCs from SHR, WKY and human coronary arteries expressed significantly lower amounts of KRAS protein, displayed reduced cell growth and led to a greater number of cells in the G1 phase than the G2/M phases of the cell cycle.

Conclusions: let-7d was significantly downregulated in VSMCs as an important regulator of cell proliferation. RAS might be involved in the proliferation regulation by let-7d. (*Circ J* 2011; **75**: 703–709)

Key Words: Genes; Hypertension; Vessels

Hyperplasia and/or hypertrophy of the vascular smooth muscle cells (VSMCs) are central factors in cardiovascular diseases such as hypertension and atherosclerosis. Abnormal gene regulation is strongly implicated in the pathogenesis of hypertension, heart failure and arteriosclerosis characterized by abnormal proliferation and hypertrophy of the VSMCs, such as the KRAS/extracellular signal-regulated kinase (ERK) pathway, one of the most important pathways involved in VSMCs proliferation.^{1,2}

MicroRNAs (miRNAs) are highly conserved endogenous short noncoding RNAs, regulating gene expression at the post-transcriptional level by inhibiting the translation of protein from mRNA or by promoting the degradation of mRNA. Their regulation is involved in many physiological and pathological conditions, including metabolism, differentiation development, apoptosis, proliferation and oncogenesis.³

Increasing evidence has demonstrated that several specific miRNAs function as a regulator of VSMC proliferation, apoptosis and differentiation by targeting the post-transcrip-

tional expression of multiple genes.^{4–7} For example, it has been reported that Krüppel-like factor 5 (KLF5) is a target gene of miR-145 that is responsible for its regulatory effect on VSMC phenotypic modulation and VSMC proliferation.⁵ Others, such as mir221 and mir222, have been indicated to be novel regulators for VSMC proliferation and neointimal hyperplasia by inhibiting the expression of p27(Kip1) and p57(Kip2).⁷ These findings may represent new promising therapeutic targets for the treatment of a number of proliferative vascular diseases, such as atherosclerosis and hypertension. Genetically, spontaneously hypertensive rats (SHR) and Wistar Kyoto rats (WKY) strains share a common lineage, differing notably in their vascular phenotype.⁸ Since proliferation activity was much higher in VSMCs of SHR rats (SHR-VSMCs) than that of WKY rats (WKY-VSMCs),⁹ we speculated that miRNA expression profiles in SHR-VSMCs might be responsible for the proliferation characteristics of SHR-VSMCs. Therefore, the present study was performed to screen the aberrantly expressed miRNAs in

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SHR-VSMCs and also to investigate the possible related genes of these miRNAs.

Methods

Cell Culture

Rat aortic smooth muscle cells were isolated from the medial layer of the thoracic aorta obtained from female SHR and WKY rats (10 weeks old) and cultured in Dulbecco's modified Eagles medium (DMEM, Gibco BRL, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA), penicillin (10 U/ml, Gibco BRL, USA), streptomycin (10 U/ml, Gibco BRL). The cells of passage 3–6 were used. The animal experiment was performed in accordance with the US National Institutes of Health guidelines and approved by the animal ethical committee of the Second Military Medical University. HCASMCs (Casecade, USA) were cultured in HCASMC PROLIF (M231/SMGS) (Invitrogen, USA) with the same treatment mentioned above.

Analysis of the Expression of miRNAs by miRCURY LNA™ miRNA Arrays

Total RNA of SHR-VSMCs and WKY-VSMCs were harvested using RNeasy mini kit (Qiagen, USA) according to the manufacturer's instructions. After RNA was quantitatively measured on the Nanodrop instrument and confirmed

by gel electrophoresis, the samples were labeled using the miRCURY™ Hy3™/Hy5™ Power labeling kit and hybridized on the miRCURY™ LNA Array (v.11.0) (Exiqon, USA). Scanning was performed using the Axon GenePix 4000B microarray and the GenePix pro V6.0 was used to read and quantify the raw intensity of the image. The intensity of the green signal was calculated after background subtraction and averaged from 4 replicated spots of each probe on the same slide. The Median Normalization Method was used to obtain "Normalized Data" [Normalized Data=(Foreground–Background)/Median]. The median was the 50 percent quantile of the miRNA intensity that was greater than 50 in all samples after background correction. Fold-changes were calculated to identify the differentially expressed miRNAs.

Candidate Validation by miRNA Real-Time Quantitative PCR

Validation of candidate gene(s) from the miRNA microarray expression analysis was performed using real-time PCR with TaqMan® MicroRNA Assays (Applied Biosystems, USA). The PCR reaction consisted of 40 cycles (95°C for 15 s, 61°C for 15 s, 72°C for 10 s) after an initial denaturation step (95°C for 3 min). The PCR products were analyzed by electrophoresis on 2% agarose gels. GAPDH was used as an endogenous control. The results of real-time PCR were analyzed using the $\Delta\Delta C_t$ method.¹⁰

Table. Differentially Expressed miRNA Lists

ID	Name	Fold change	Foreground		Foreground-background		Normalized	
		SHR/WKY	SHR	WKY	SHR	WKY	SHR	WKY
17718	miR-92b	7.67	404	83	371	49	1.11	0.14
11105	miR-378	2.61	250	118	216	85	0.64	0.25
42834	miR-219-2-3p	2.17	107	68	73	35	0.22	0.10
14294	miR-1	0.41	71	128	37	94	0.11	0.27
27263	let-7a	0.42	279	6,799	2,756	6,765	8.20	19.59
9938	let-7i	0.42	136	280	101	246	0.30	0.71
29153	miR-34b	0.43	575	89	22	54	0.07	0.16
42670	miR-500	0.41	56	91	23	58	0.07	0.17
46437	let-7d	0.25	215	784	181	750	0.54	2.17
11182	miR-98	0.35	65	126	32	93	0.10	0.27
46435	let-7f	0.27	1,256	4,670	1,222	4,636	3.64	13.43

Only miRNAs with changing fold higher than 2 or lower than 0.5 was showed.

Transfection of let-7d miRNA Mimics

The let-7d miRNA mimics and negative control (NC) (Genepharma, China) were diluted in the medium at a final concentration of 100nmol/L. Cells were incubated with let-7d mimics and NC for 48h using the fast transfection protocol recommended by Invitrogen. Efficiency of transfection was determined by comparing it against the NC provided by Genepharma.

Cell Counting Kit-8 (CCK-8)

Transfectants (SHR-VSMC/NC, SHR-VSMC/let-7d, WKY-VSMC/NC, WKY-VSMC/let-7d, HCASMC/NC and HCA-SMC/let-7d) were resuspended in 200 μ l of cell culture medium and seeded in 96-well microtiter plates at 2.5×10^4 cells/ml and incubated for 48h or 72h. Next, 10 μ l of CCK-8 reagent (Dojindo, Japan) was added to each well 2h before the end of incubation. The optical density value (OD) of each sample was measured at a wavelength of 450nm on a microplate reader (Multiskan MK3, Thermo Lab Systems). The results of cell viability measurement were expressed as the absorbance at OD450.

Flow Cytometric Analysis

Transfectants were trypsinized, washed with PBS twice and then fixed with 70% ethanol on ice for 1h. The fixed cells were spun down, resuspended in PBS at 1×10^6 cells/ml. After incubation with ribonuclease (RNase A) at a final concentration of 3,000units/ml at 37°C for 30min, trypsinized cells were filtered through a nylon mesh (BD Biosciences, USA). The cell suspension was stained with propidium iodide before analysis on a FACS Aria flow cytometer (BD Biosciences).

Western Blot Analysis

SHR-VSMCs, WKY-VSMCs and HCASMCs were transfected with let-7d miRNA mimics and NC. Cells transfected for 72h were lysed in lysis buffer (Beyotime, China) for western blot on ice, and after being heated for 5min at 95°C, 20 μ g of denatured protein for each reaction was used to load a 10% polyacrylamide SDS (H₂O, 1.5mol/L Tris-HCl (pH 8.8), 10% SDS, 30% Acrylamide/Bisacryl, 10% Am Persulfate, TEMED) for electrophoresis. Polyvinylidene difluoride membranes (Whatman, UK) were used for transfer by a wet transferring system (Bio-Rad, USA). The membrane was probed with primary antibodies against K-Ras-2A (diluted 1:100 in TBST, Santa Cruz, USA) at 4°C overnight, and with

secondary HRP-conjugated anti-rabbit antibody (Santa Cruz) for 1h at room temperature. After washing with $1 \times \text{PBS} + 0.1\%$ Tween, the bands were detected by chemiluminescence (ECL detection kit, USA) and photographed with Kodak film (BioMax® Light-1, 18*24cm, USA). Twenty photos of each membrane were taken with an exposure interval of 15s. GAPDH was used as an endogenous protein for normalization. The ratio of KRAS/GAPDH was used for semi-quantification and comparison between different groups.

Statistical Analysis

All data were presented as mean $\pm$ SD. One-way ANOVA was used to compare between samples. $P < 0.05$ was considered statistically significant. All experiments were performed at least 3 times.

Results

miRNA Expression Profile Comparison Between SHR-VSMCs and WKY-VSMCs

The differential expression of specific miRNA between SHR-VSMCs and WKY-VSMCs suggest a potential difference in cell proliferation. Some miRNAs were decreased, such as let-7d and miR-1, which are shown as shades of green in the heat map. While some (eg, miR-92b, miR-378) were significantly increased as depicted by the shades of red in the heat map ($P < 0.05$, [Figure 1A](#), [Table](#)). The entire miRNA expression profiles are provided in the supplemental file ([Figure S1](#)). To verify the accuracy of the microarray results, we made further identification using TaqMan real-time quantitative PCR. Results were validated using TaqMan real-time quantitative PCR, demonstrating a $\Delta\Delta\text{Ct}$ of 2.1 cycles or 0.23-fold change (equivalent to a 4.29-fold decrease) when comparing SHR-VSMCs ($\Delta\text{Ct} = 4.2 \pm 1.2$) with WKY-VSMC ($\Delta\text{Ct} = 6.3 \pm 1.5$) ($n = 5$, $P < 0.05$, [Figure 1B](#)). Thus, let-7d was further studied to determine its potential biological function.

Inhibition of KRAS Expression in VSMCs via let-7d Transfection

According to the literature, let-7d reportedly downregulated KRAS in human as well as rat VSMCs.^{11,12} In the present study, SHR-VSMCs, WKY-VSMCs and HCASMCs were transfected with let-7d mimics and the rat/human KRAS protein levels were quantified by western blotting. Western blot analysis showed reduced expression of KRAS in the 3

Figure 4. Effect of let-7d mimics on cell cycle of vascular smooth muscle cells (VSMCs) of SHR rats (SHR-VSMCs), VSMCs of WKY rats (WKY-VSMCs) and HCASMCs. (A) Flow cytometric analysis of SHR-VSMCs transfected with let-7d mimics or negative controls. (B) Percentages of cells at different phases in different groups of SHR-VSMCs. (C) Flow cytometric analysis of WKY-VSMCs transfected with let-7d mimics or negative controls. (D) Cell cycle progression of cells at different phases in different groups of WKY-VSMCs. (E) Flow cytometric analysis of HCASMCs transfected with let-7d mimics or negative controls. (F) Percentages of cells at different phases in HCASMCs transfected by let-7d mimics or negative controls. N=4 for each group. SHR, spontaneously hypertensive rats; WKY, Wistar Kyoto rats.

types of vascular cells transfected with let-7d (Figure 2).

Effects of let-7d Overexpression on VSMC Proliferation

SHR-VSMCs, WKY-VSMCs and HCASMCs were transfected with let-7d miRNA mimics prior to the proliferation assay. CCK-8 assay was employed to measure cell proliferation. The results showed that exogenous expression of let-7d inhibited proliferation of VSMCs significantly (Figure 3). After incubation for 48h and 72h, there were significant inhibitory effects in the transfected group compared with the controls. In SHR-VSMCs, WKY-VSMCs and HCASMCs, the inhibitory rates were 28.11% ($P<0.05$), 18.77% ($P<0.05$)

and 17.32% ($P<0.01$) after incubation for 48h, and 25.19% ($P<0.05$), 18.53% ($P<0.01$) and 18.27% ($P<0.01$) after incubation for 72h, respectively, which suggested overexpression of let-7d inhibited proliferation of VSMCs in vitro.

Effects of let-7d Overexpression on the Cell Cycle of the VSMC

Cell cycle analysis was performed to determine whether there was any cell cycle alteration in the 2 kinds of VSMCs mentioned above. The result showed that compared with control group the percentage of cells at the G1 phase was increased from 54.25% to 71.82% (SHR-VSMCs, $P<0.01$), 71.23% to

91.21% (WKY-VSMCs, $P<0.01$) and 44.13% to 56.81% (HCASMCs, $P<0.01$), respectively, whereas the cells at the S phase decreased from 18.61% to 13.87% (SHR-VSMCs, $P<0.01$), 14.58% to 2.27% (WKY-VSMCs, $P<0.01$) and 28.43% to 18.21% (HCASMCs, $P<0.05$) and the population of cells at the G2 phase decreased from 27.14% to 14.31% (SHR-VSMCs, $P<0.01$), 14.19% to 6.51% (WKY-VSMCs, $P<0.01$) and 27.44% to 24.97% (HCASMCs, $P<0.01$) (Figure 4). These results indicated that overexpression of let-7d induced a G1 cell cycle arrest in VSMCs, and the percentage of the S and G2/M phases were reduced significantly.

Discussion

miRNAs play an important and indispensable role in regulating cardiovascular biological and pathological processes.¹³ Various miRNAs have been found to be associated with VSMC proliferation and necrosis. Cheng et al found that miR-145 was a novel VSMC phenotypic marker and modulator that controlled vascular neointimal lesion formation.¹⁴ miR-221 and miR-222 were also identified to regulate VSMC proliferation and neointimal hyperplasia.⁷ Lin et al confirmed that miR-21 participated in H_2O_2 -mediated gene regulation and injury response via its target gene Programmed Cell Death 4 (PDCD4) and AP-1 pathway with a protective effect against VSMC necrosis.¹⁵ Since proliferation activity was much higher in SHR-VSMCs, the aberrantly expressed miRNAs in SHR-VSMCs might be involved in VSMC proliferation regulation. Therefore, we made a thorough comparative screen using microarray analysis to identify the differentially expressed miRNAs that differed between SHR-VSMCs and WKY-VSMCs, and pinpointed let-7d as a potential miRNA candidate responsible for the differing vascular phenotype.

The let-7 miRNA is among the founding members of the miRNA family and is highly conserved in invertebrates and vertebrates. Several studies have shown that let-7 regulates cell proliferation in cancer cells by inhibiting post-translation of RAS protein.^{11,16,17} However, its role in VSMC proliferation are currently unknown. In the present study, several miRNAs were expressed aberrantly in SHR-VSMCs compared with WKY-VSMCs, with supporting results also obtained from real-time quantitative PCR. These data may allow us to identify the miRNAs involved in VSMC proliferation and in the induction of vascular remodeling, atherosclerosis and hypertension. Our miRNA microarray data suggest that the expression levels of let-7d are significantly reduced in SHR-VSMCs, which exhibits higher growth rates compared with WKY-VSMCs. The results also indicate that let-7d might play an important role in the process of VSMC growth. As it has been confirmed that the KRAS gene is associated with the let-7 miRNA family in mammalian cells,¹¹ we postulated that let-7d might regulate VSMC proliferation by inhibiting KRAS. In SHR-VSMCs, WKY-VSMCs and HCASMCs transfected with let-7d mimics, both the expression of KRAS and cell proliferation rate significantly decreased, as demonstrated by the CCK-8 assay, flow cytometric analysis and western blot analysis. Therefore, these results strongly indicate that let-7d may be an important regulator of VSMC proliferation and that KRAS might be the key gene. Hu et al found that RAS expression in the artery of SHR was higher than that of WKY.¹⁸ However, in the present study, there was no significant difference between the RAS level in SHR-VSMCs and WKY-VSMCs, which could be the result of subculturing of the primary cells.¹⁹

Cells used for the microarray were at passage 3, while cells of passage 5–6 were used to make the further functional study. Because the purpose of the present study was to investigate whether RAS expression and VSMC proliferation was regulated by let-7d, the difference in RAS expression between SHR and WKY-VSMCs was beyond the scope of the study.

VSMCs play a key role in fibrous cap formation and plaque stability in advanced atherosclerosis.²⁰ They are the major producers of extracellular matrix within the vessel wall with the ability to modulate the type of matrix proteins produced in response to atherogenic stimuli.²¹ Increased proliferation and migration of VSMCs from media to intima of vessels resulted in VSMC accumulation and vascular remodeling. Thus, VSMC proliferation is a key event in the process of vascular diseases, such as hypertension and restenosis after balloon catheterization,²² which might be reversed by inhibiting VSMC proliferation.²³ KRAS proteins are prototypical members of the superfamily of small GTPases. They transmit signals from cell surface receptors to a variety of effectors and thereby regulate pathways governing cell proliferation, differentiation and programmed cell death. Increased Ras/MEK/ERK signaling pathways in primary VSMC is implicated in the diagnosis and therapy of a variety of proliferative vascular diseases. Many efforts have been made in an attempt to develop Ras inhibitors as a therapeutic measure against vasculoproliferative diseases. In the present study, let-7d transfection resulted in a decrease of KRAS expression and an inhibition of VSMC proliferation; therefore, let-7d might be a potential therapeutic target of vasculoproliferative diseases.

Conclusion

In summary, the present study revealed that let-7d is significantly reduced in the VSMCs of the vascular disease strain, SHR, compared with the relatively healthy wild-type, WKY. Overexpression of let-7d led to a decrease in KRAS expression and VSMC proliferation. These results implicate let-7d as a miRNA regulator of VSMC proliferation via KRAS.

Disclosure

None declared.

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Supplemental Files

Supplemental File 1

Figure S1. The entire miRNA expression profiles in SHR-VSMC.

Please find supplemental file(s);
<http://dx.doi.org/10.1253/circj.CJ-10-0393>