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Research Article

Knockdown of Microrna-1260 Promotes Up-Regulation of Both Collagen Type 1 Alpha 1 and Monocyte Chemoattractant Protein-1 in Vascular Smooth Muscle Cells from Human Abdominal Aortic Tissues

Crystal Yin Tung CHAN, Bernice Lai Yee CHEUK*, and Stephen Wing Keung CHENG

Department of Surgery, University of Hong Kong Medical Centre, Hong Kong

***Corresponding author**
Bernice Lai Yee CHEUK, Division of Vascular Surgery, Department of Surgery, University of Hong Kong Medical Centre, South Wing, 14th Floor K Block, Queen Mary Hospital, Pokfulam, Hong Kong, Tel: 852-2255-4970; Fax: 852-2255-4961; Email: bernice@hku.hk

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Abstract

Objectives: MicroRNA (miR)-1260 was identified to be down-regulated in vascular smooth muscle cells (VSMCs) from human abdominal aortic aneurysm (AAA) tissues in our previous microarray profiling assay. MiR-1260 was predicted to target and down-regulate collagen type 1 alpha 1 (COL1A1), which is closely related to AAA formation, from a bioinformatics analysis. However, the role of miR-1260 in VSMCs for AAA formation still remains uncertain. This study aims to investigate the role of miR-1260 in human VSMCs.

Methods: Stable overexpression and knockdown of miR-1260 using lentivirus were performed in VSMCs cultured from human abdominal aortic tissues. Expression of COL1A1 protein was investigated, followed by investigating the expressions of several key components involved in AAA pathogenic features: monocyte chemoattractant protein-1 (MCP-1) for inflammation; matrix metalloproteinase (MMP)-2, MMP-9, tissue inhibitor of matrix metalloproteinase (TIMP)-1 and TIMP-2 for elastin fragmentation. Apoptosis of VSMCs was also examined.

Results: Both COL1A1 and MCP-1 were significantly down-regulated upon miR-1260 overexpression, whereas they were significantly up-regulated upon miR-1260 knockdown. Neither protein expressions of MMP-2, MMP-9, TIMP-1 and TIMP-2 nor apoptosis were significantly different between miR-1260 overexpression/knockdown and corresponding controls.

Conclusions: Our findings suggested suppression of miR-1260, which was previously found associated with AAA VSMCs, may promote up-regulation of COL1A1 and MCP-1 in human VSMCs, possibly promoting compensatory collagen synthesis and inflammation for AAA formation.

INTRODUCTION

The formation of abdominal aortic aneurysm (AAA) is believed to be multifactorial as several key pathogenic features have been prominently identified in AAA, such as elastin fragmentation, compensatory collagen synthesis, inflammation, and apoptosis of vascular smooth muscle cells (VSMCs) [1]. Complex regulation of the key pathogenic features is believed to determine the formation and progression of AAA. MicroRNA (miR/miRNA), a short non-coding RNA of 21-25 nucleotides in length, is a type of gene expression regulators promoting down-regulation of specific messenger RNAs (mRNAs) in cells [2]. It has been discovered to participate in regulating expressions of various disease-related genes, recently suggested to play a role in AAA pathogenesis [3]. In search of possible involvement of miRNAs in AAA, VSMCs, the major cell type in the aortic wall, were harvested from human AAA and non-aneurysmal tissues for a miRNA microarray profiling assay [1,4]. MiR-1260 was identified to be significantly down-regulated in AAA compared with non-aneurysmal VSMCs, with validation from another set of human samples [4]. It was thus suggested that a down-regulation of miR-1260 in VSMCs may be associated with the formation of AAA.

MiR-1260 has not been widely reported in the literature except a few studies reporting its down-regulation in several human tissues, such as inflamed gingival, glioblastoma and

gastric cancer tissues [5-7]. There are no reports on the expression or function of miR-1260 in AAA. To understand the role of miR-1260 in AAA, prediction of its target from a bioinformatic tool was done [4]. Several aortic diseases-related targets were shortlisted, and collagen type 1 alpha 1 (COL1A1), which is a crucial component in the aortic wall for vascular wall integrity, was identified [4]. The function of collagen is to provide tensile strength to the aortic wall to resist against the high blood pressure from pulsation of heart [1]. However, a dysregulation of such key component is believed to disrupt the vascular wall integrity, hence resulting in AAA. Collagen synthesis was found to be increased during aneurysm formation and progression. This increment is suggested to act as compensatory repair, aiming to increase as much tensile strength and resistance as possible in response to elastin fragmentation [8,9]. The increase in collagen synthesis together with the reduction of elastin will inevitably lead to a disruption of physiological ratio of elastin to collagen, resulting in aortic stiffness and weakening of the aortic wall [1,8-11]. In the later stage of AAA, collagen degradation became prominent, leading to rupture [9]. Therefore, it is hypothesized that miR-1260 may take part in the pathogenesis of AAA through direct targeting and regulating the expression of COL1A1.

This study aims to investigate the role of miR-1260 in human VSMCs for the pathogenesis of AAA. MiR-1260 was stably overexpressed and knocked down in VSMCs explant cultured from human abdominal aortic tissues, followed by analysis of COL1A1 protein expression. Levels of several key components involved in other AAA pathogenic features will also be investigated, including monocyte chemoattractant protein-1 (MCP-1) for inflammation; matrix metalloproteinase (MMP)-2, MMP-9, tissue inhibitor of matrix metalloproteinase (TIMP)-1 and TIMP-2 for elastin degradation; and apoptosis, in order to understand more about the role of miR-1260 in AAA pathogenesis.

MATERIAL AND METHODS

Collection of human abdominal aortic tissue specimens

Infrarenal non-aneurysmal abdominal aortic tissues of full thickness were harvested from six organ donors (mean age ± standard deviation (SD) =48.50 ± 13.59; all male) without known connective tissue disorders, i.e. Marfan Syndrome and Ehlers Danlos Syndrome, or cardiovascular diseases during liver transplantation. All relevant clinical data was obtained from the clinical medical system. All experiments were performed with approval from the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster. Written informed consents were obtained.

Explant culture of VSMCs

VSMCs were cultured from human abdominal aortic tissues by explant culture [12]. A segment (~2mm x 2mm) of aortic tissue was incubated in complete cell culture medium containing Dulbecco's modified eagle medium, 10% fetal bovine serum, 2mM L-alanyl-L-glutamine dipeptide, 100units/ml penicillin, 100ug/ml streptomycin and 20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Life Technologies, Carlsbad, CA) at 37°C in a humidified 5% CO_2 atmosphere for about 1-2 weeks. The culture medium was regularly changed every 3 days. Subculturing was done by trypsinization using 0.025% trypsin/ethylenediaminetetraacetic acid (Life Technologies, Carlsbad, CA) when cells were 80% confluent. The homogenity of cultured VSMCs was confirmed by immmunocytochemical staining of smooth muscle α-actin (Cat. No.: M0851; 1:200) (DakoCytomation, Glostrup, Denmark). VSMCs of 3-5 passages were used for experiments.

Stable overexpression and knockdown of miR-1260

Stable overexpression and knockdown of miR-1260 in cultured VSMCs were performed with the use of lentivirus [12]. Overexpression (miRNA-precursor sequence; cat. no.: PMIRH1260-PA-1) and knockdown (anti-miRNA sequence; cat. no.: MZIP-1260-PA-1) lentivectors of miR-1260, together with their corresponding scramble controls, were purchased from System Biosciences (System Biosciences, Mountain View, CA). Propagation and purification of lentivectors by NucleoBond® Xtra Midi EF Endotoxin-free Plasmid DNA Purification Kit (Macherey-Nagel, Düren, Germany) were done. Co-transfection of lentivectors and lentivirus packaging plasmids in 293TN cell line using PureFection™ Transfection Reagent (System Biosciences, Mountain View, CA) was followed. The generated lentivirus in supernatant was then concentrated by PEG-*it*™ Virus Precipitation Solution (System Biosciences, Mountain View, CA) and titered using Global Ultra Rapid Lentiviral Titer Kit (System Biosciences, Mountain View, CA). Transduction of lentivirus into cultured VSMCs at a multiplicity of infection of 150 in the presence of 5ug/ml polybrene (Sigma Aldrich, St. Louis, MO) was done. The green fluorescent reporter signal could be examined 4 days after transduction under fluorescence microscope (CKX41) (Olympus, Tokyo, Japan).

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Expression of miR-1260 in the infected VSMCs was analyzed by qRT-PCR [12]. Extraction of total RNA from infected VSMCs by miRNeasy® Mini kit (QIAGEN, Venlo, Netherlands) was done, followed by the RT reaction using miScript® RT kit (QIAGEN, Venlo, Netherlands) in 2720 Thermal Cycler (Applied Biosystems, Foster City, CA). qPCR was carried out using primers specific to miR-1260 and RNU6-2 as internal control (Invitrogen, Carlsbad, CA) in 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) following the protocol of miScript SYBR® Green PCR kit (QIAGEN, Venlo, Netherlands) [13]. The experiment was run in duplicate, and the data was analyzed by the $2^{-\Delta\Delta CT}$ method [14,15].

Western blotting (WB)

Protein expressions of COL1A1, MCP-1, MMP-2, MMP-9, TIMP-1 and TIMP-2 in the infected VSMCs were analyzed by WB [12]. Proteins were first extracted from infected VSMCs by radioimmunoprecipitation lysis buffer (Cell Signaling Technology, Danvers, MA) with 1mM phenylmethanesulfonylfluoride (Sigma Aldrich, St. Louis, MO), followed by electrophoresis in a sodium dodecyl sulfate-polyacrylamide gel (Bio-Rad, Laboratories, Hercules, CA) under a reducing condition. Electroblotting to polyvinylidene difluoride membrane (GE Healthcare, Little

Chalfont, UK) was done at a voltage of 100V for 2hrs at 4˚C, followed by probing with the following primary antibodies: COL1A1 (Cat. No.: sc-28657; 1:200) (Santa Cruz Biotechnology, Dallas, TX); MCP-1 (Cat. No.: 2027; 1:1000) (Cell Signaling Technology, Danvers, MA); MMP-2 (Cat. No.: sc-13595; 1:200) (Santa Cruz Biotechnology, Dallas, TX); MMP-9 (Cat. No.: NBP1-40610; 1:5000) (Novus Biologicals, Littleton, CO); TIMP-1 (Cat. No.: sc-365905; 1:100) (Santa Cruz Biotechnology, Dallas, TX); TIMP-2 (Cat. No.: sc-5539; 1:100) (Santa Cruz Biotechnology, Dallas, TX); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cat. No.: 2118; 1:10000) (Cell Signaling Technology, Danvers, MA) as internal control. Horseradish peroxidase-conjugated goat anti-rabbit or mouse IgG (Invitrogen, Carlsbad, CA) at a dilution of 1:10000 for 1hr at room temperature was incubated. Chemiluminescent signal was captured with X-ray film (Fujifilm, Tokyo, Japan) after incubation with ECL™ Prime detection system (GE Healthcare, Little Chalfont, UK). Band intensity was evaluated by Image J software (v1.46) (National Institutes of Health, Bethesda, MD).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

The level of apoptosis of the infected VSMCs was detected by TUNEL assay [12]. The infected VSMCs were first cytospinned on polysine microscrope slides (Thermo Scientific, Waltham, MA) prior to detection of apoptotic signal using the ApopTag® Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon International Inc, Billerica, MA). The apoptotic level was evaluated by the proportion of TUNEL-positive cells in all cells.

Statistical analysis

Statistical analysis was performed in SPSS 20.0 (IBM Corp, Armonk, NY). Normalization of the expression, represented in mean±SD, in the experimental group (miR-1260 overexpression or knockdown) with the control group (corresponding scramble controls) was done prior to evaluation of the statistical difference by Wilcoxon signed-rank test. The level of significance was set at 0.05.

RESULTS

Stable overexpression and knockdown of miR-1260 in human VSMCs

The effects of miR-1260 overexpression and knockdown by means of lentiviral system in the VSMCs explant cultured from human abdominal aortic tissues were examined by quantifying the endogenous level of miR-1260 using qRT-PCR (Figure 1). VSMCs infected with lentivirus carrying miR-1260 precursor sequence (3.65 ± 0.71) expressed a significantly higher level of miR-1260 than that carrying corresponding scramble sequence (1 ± 0) (n=6, P<0.05) (Figure 1A). VSMCs infected with lentivirus carrying anti-miR-1260 sequence (0.28 ± 0.05) expressed a significantly lower level of miR-1260 than that carrying corresponding scramble sequence (1 ± 0) (n=6, P<0.05) (Figure 1B). These suggested that miR-1260 was successfully overexpressed and knocked down in VSMCs.

Up-regulation of COL1A1 protein expression upon miR-1260 knockdown

The relative protein expression of COL1A1 in VSMCs with miR-1260 overexpression and knockdown compared with corresponding controls was investigated by WB (Figure 2). A significant decrease of COL1A1 protein expression was observed in miR-1260-overexpression VSMCs (0.28 ± 0.03) compared with control VSMCs (1 ± 0) (n=6, P<0.05). A significant increase of COL1A1 protein expression was observed in miR-1260-knockdown VSMCs (3.04 ± 0.42) compared with control VSMCs (1 ± 0) (n=6, P<0.05).

Up-regulation of MCP-1 protein expression upon miR-1260 knockdown

The relative protein expression of MCP-1 in VSMCs with miR-1260 overexpression and knockdown compared with corresponding controls was investigated by WB (Figure 3). Due to the low basal expression of MCP-1 in uninduced VSMCs,

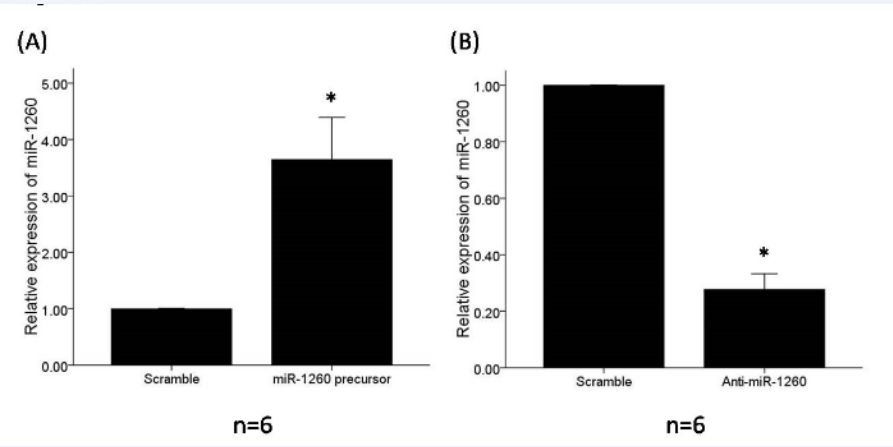

Figure 1 Detection of microRNA (miR)-1260 expression level in infected vascular smooth muscle cells (VSMCs) by real-time quantitative reverse transcription-polymerase chain reaction. (A) VSMCs infected with lentivirus carrying miR-1260 precursor sequence (3.65 ± 0.71) expressed a significantly higher level of miR-1260 than that carrying scramble sequence (1 ± 0) (n=6, P<0.05). (B) VSMCs infected with lentivirus carrying anti-miR-1260 sequence (0.28 ± 0.05) expressed a significantly lower level of miR-1260 than that carrying scramble sequence (1 ± 0) (n=6, P<0.05). Data is expressed as mean ± standard deviation (SD). The error bars show the SD. * denotes P<0.05.

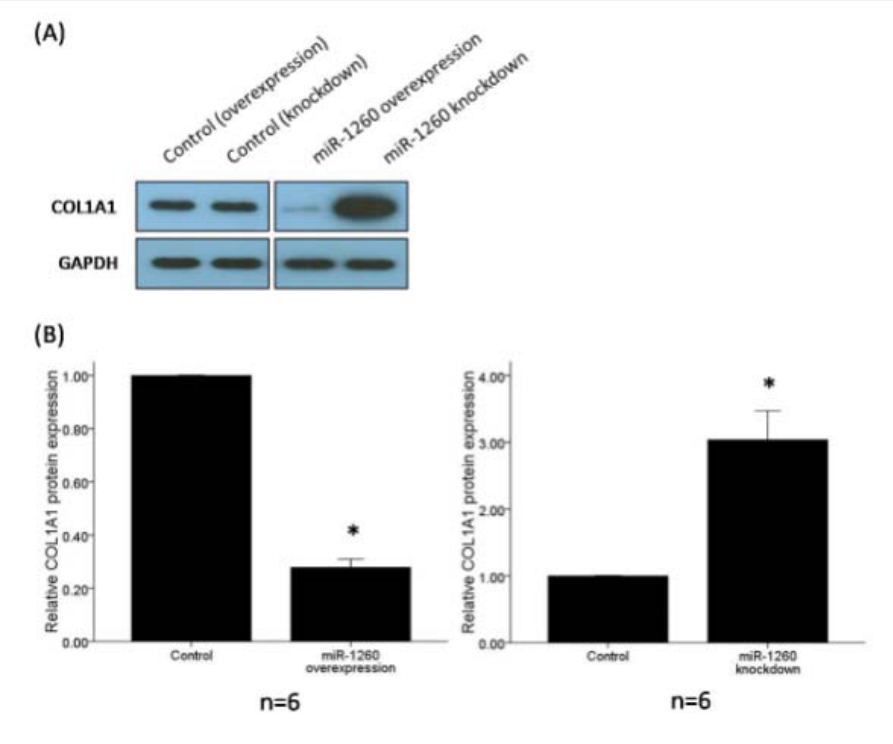

Figure 2 Analysis of protein expression of collagen type 1 alpha 1 (COL1A1) in vascular smooth muscle cells (VSMCs) with microRNA (miR)-1260 overexpression and knockdown by western blotting. (A) Representative western blot of an individual sample of VSMCs with miR-1260 overexpression and knockdown compared with corresponding controls, probing for COL1A1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control. (B) A significant decrease of COL1A1 protein expression was observed in miR-1260-overexpression VSMCs (0.28 ± 0.03) compared with control VSMCs (1 ± 0) (n=6, P<0.05) (left), while a significant increase of COL1A1 protein expression was observed in miR-1260-knockdown VSMCs (3.04 ± 0.42) compared with control VSMCs (1 ± 0) (n=6, P<0.05) (right). Data is expressed as mean ± standard deviation (SD). The error bars show the SD. * denotes P<0.05.

100ng/ml tumor necrosis factor alpha (TNF-α) was added 24hrs prior to the analysis of MCP-1 protein expression [16]. A significant decrease of MCP-1 protein expression was observed in miR-1260-overexpression VSMCs (0.27 ± 0.18) compared with control VSMCs (1 ± 0) (n=6, P<0.05), while a significant increase of MCP-1 protein expression was observed in miR-1260-knockdown VSMCs (3.13 ± 1.32) compared with control VSMCs (1 ± 0) (n=6, P<0.05).

No effect on MMP-2, MMP-9, TIMP-1 and TIMP-2 protein expressions or apoptosis upon miR-1260 overexpression or knockdown

The relative protein expressions of MMP-2, MMP-9, TIMP-1 and TIMP-2 in VSMCs with miR-1260 overexpression and knockdown compared with their corresponding controls were investigated by WB (Table 1). Protein expressions of MMP-2, TIMP-1 and TIMP-2 in miR-1260-overexpression/-knockdown VSMCs were not significantly differed from the corresponding control VSMCs (P>0.05). On the other hand, due to the low basal expression of MMP-9 in uninduced VSMCs, 100ng/ml TNF-α was added 24hrs prior to the analysis of MMP-9 protein expression [17]. Protein expression of MMP-9 in miR-1260-overexpression/-knockdown VSMCs was not significantly differed from the corresponding control VSMCs (P>0.05).

The level of apoptosis in VSMCs with miR-1260 overexpression and knockdown compared with their corresponding controls was investigated by TUNEL assay (Table 1). The level of apoptosis in miR-1260-overexpression/-knockdown VSMCs was not significantly differed from the corresponding control VSMCs (P>0.05).

DISCUSSION

The aim of this study is to investigate the role of miR-1260, which was found to be down-regulated in human AAA VSMCs from a previous miRNA microarray profiling assay, in human VSMCs for AAA pathogenesis [4]. Firstly, this study investigated the protein expression of COL1A1, which is one of the key components involved in AAA pathogenesis and, more importantly, a predicted target of miR-1260 from previous bioinformatics analysis, upon miR-1260 overexpression and knockdown in human VSMCs [4]. A significant decrease in COL1A1 protein expression was observed upon overexpression of miR-1260, while a significant increase in COL1A1 protein expression was observed upon knockdown of miR-1260 in VSMCs. These suggested that miR-1260 may play a role in targeting and down-regulating COL1A1 protein expression in VSMCs. Secondly, the expressions of several key components involved in other typical AAA pathogenic features were also investigated. Protein expression of MCP-1 upon miR-1260 overexpression and knockdown in cultured TNF-α-induced

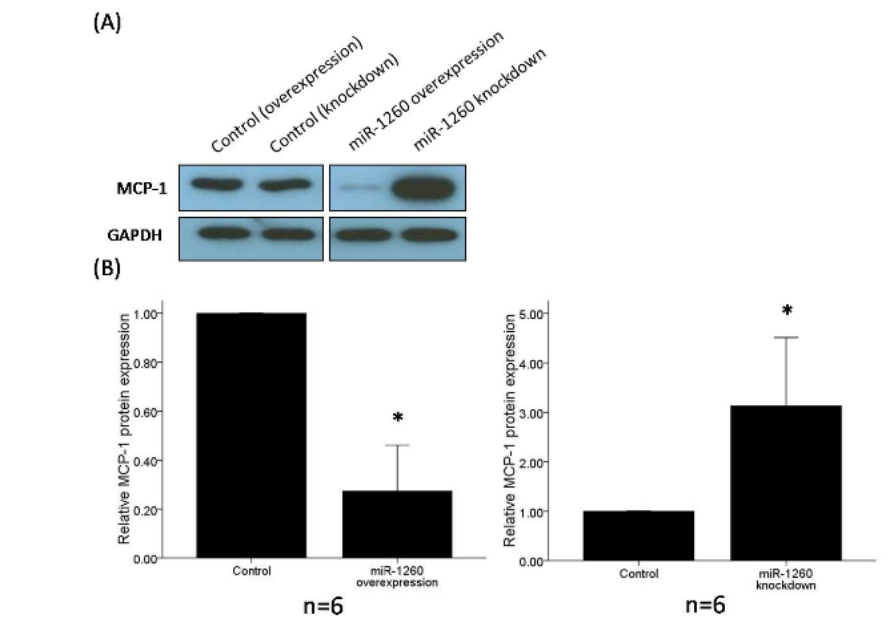

Figure 3 Analysis of protein expression of monocyte chemoattractant protein-1 (MCP-1) in 100ng/ml tumor necrosis factor alpha-induced vascular smooth muscle cells (VSMCs) with microRNA (miR)-1260 overexpression and knockdown by western blotting. (A) Representative western blot of an individual sample of VSMCs with miR-1260 overexpression and knockdown compared with corresponding controls, probing for MCP-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control. (B) A significant decrease of MCP-1 protein expression was observed in miR-1260-overexpression VSMCs (0.27 ± 0.18) compared with control VSMCs (1 ± 0) (n=6, $P<0.05$), while. (left), while a significant increase of MCP-1 protein expression was observed in miR-1260-knockdown VSMCs (3.13 ± 1.32) compared with control VSMCs (1 ± 0) (n=6, $P<0.05$) (right). Data is expressed as mean ± standard deviation (SD). The error bars show the SD. * denotes $P<0.05$.

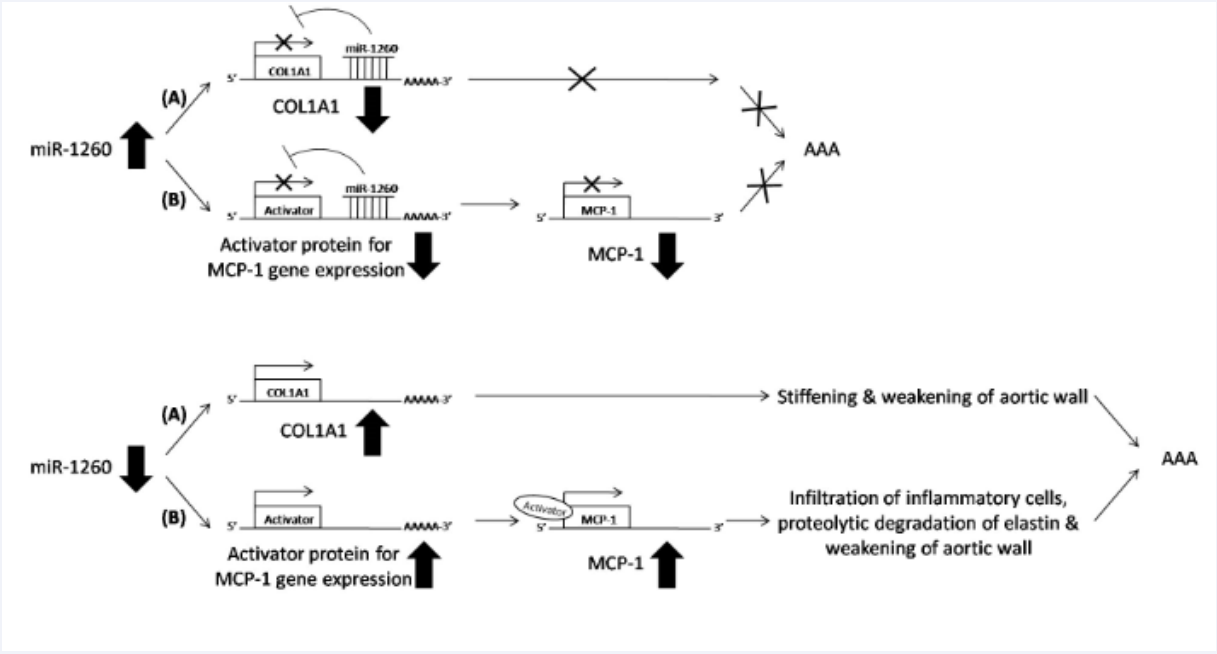

Figure 4 Hypothesis of the role of microRNA (miR)-1260 in regulations of (A) collagen type 1 alpha 1 (COL1A1) and (B) monocyte chemoattractant protein-1 (MCP-1) in human vascular smooth muscle cells for abdominal aortic aneurysm (AAA) formation. Under healthy condition, miR-1260 may be up-regulated for targeting and suppression of the expressions of both COL1A1 and the activator protein for MCP-1 expression. Neither COL1A1 nor MCP-1 is activated (top). However, a down-regulation of miR-1260 may allow up-regulation of COL1A1 expression, resulting in stiffening and weakening of aortic wall for aneurysm formation. The activator protein for MCP-1 expression may also be up-regulated upon miR-1260 down-regulation, resulting in activation of MCP-1 expression. Infiltration of inflammatory cells and proteolytic degradation of elastin may be promoted, followed by weakening of aortic wall for AAA formation (bottom).

Table 1: Summary of results from western blotting analysis of matrix metalloproteinase (MMP)-2, MMP-9, tissue inhibitor of matrix metalloproteinase (TIMP)-1 and TIMP-2 protein expressions and from terminal deoxynucleotidyl transferase dUTP nick end labeling assay of apoptosis in vascular smooth muscle cells (VSMCs) with microRNA (miR)-1260 overexpression and knockdown compared with their corresponding controls. Protein expressions of MMP-2, MMP-9, TIMP-1 and TIMP-2, or apoptosis of miR-1260-overexpression/-knockdown VSMCs was not significantly differed from the corresponding control VSMCs. Data is expressed as mean ± standard deviation.

	miR-1260 overexpression	control	*P*-value	miR-1260 knockdown	control	*P*-value
	(n=6)			(n=6)		
MMP-2	1.18±0.29	1±0	**0.25**	0.98±0.20	1±0	**0.60**
MMP-9*	1.03±0.14	1±0	**0.35**	1.00±0.07	1±0	**0.75**
TIMP-1	1.10±0.15	1±0	**0.17**	1.07±0.12	1±0	**0.25**
TIMP-2	1.11±0.22	1±0	**0.25**	1.07±0.19	1±0	**0.17**
Apoptosis	1.02±0.17	1±0	**0.92**	1.10±0.20	1±0	**0.17**

* Protein expression was investigated in VSMCs treated with 100ng/ml tumor necrosis factor alpha for 24hrs as the basal expression level was low in uninduced VSMCs.

VSMCs was found altered. A significant decrease in MCP-1 protein expression was observed upon overexpression of miR-1260, while a significant increase in MCP-1 protein expression was observed upon knockdown of miR-1260. These suggested that miR-1260 may regulate MCP-1 protein expression in VSMCs. No significant difference in the MMP-2, MMP-9, TIMP-1 and TIMP-2 protein expressions or apoptosis was observed between miR-1260-overexpression/-knockdown and the corresponding control VSMCs, suggesting that miR-1260 may not regulate MMP-2, MMP-9, TIMP-1, TIMP-2 or promote apoptosis in human VSMCs.

A down-regulation of miR-1260 was previously observed in AAA VSMCs from the miRNA microarray assay [4]. This current study showed an up-regulation of COL1A1 expression when miR-1260 was specifically knocked down in human VSMCs, which mimicked the situation observed in AAA VSMCs from the previous microarray assay [4]. This suggested that miR-1260 may play a role in the pathogenesis of AAA through compensatory collagen synthesis. Moreover, taken together with the widely accepted concept of collagen re-synthesis during aneurysm formation and collagen degradation during rupture, it is suggested miR-1260 may be responsible for the formation and progression of AAA but not rupture [9]. Therefore, a pathogenic mechanism involving miR-1260 in AAA is proposed (Figure 4A). Under healthy condition, the miR-1260 was up-regulated in VSMCs for suppression of COL1A1 expression as it may not be necessary to re-synthesize collagen. However, when there is a presence of elastin fragments, miR-1260 may be down-regulated. The expression of COL1A1 may be increased in an aim to repair the aortic wall by increasing tensile strength. Therefore, the aortic wall will become not only less elastic due to elastin fragmentation but also stiffer due to increased collagen synthesis. Weakening of the aortic wall may eventually lead to aneurysm formation. This hypothesis has to be tested in *in-vivo* model with miR-1260 knockdown, followed by investigation of whether an aneurysm is resulted accompanied by an increased collagen content at the abdominal aortic wall. In addition, it is still uncertain whether the miR-1260 down-regulation for compensatory collagen synthesis in VSMCs is stimulated by the increasing wall stress due to the loss of elasticity of aortic wall although a prior *in-vitro* study reported an increased collagen synthesis in rabbit aortic smooth muscle cells in response to mechanical stretching of elastin wall [18]. Therefore, further investigation of whether increasing mechanical stress induces miR-1260 down-regulation and COL1A1 up-regulation in human VSMCs subcultured on elastin membrane is necessary.

Apart from miR-1260, there was another miRNA reported to regulate collagen synthesis [19]. An inhibition of miR-29b by anti-miR-29b significantly increased COL1A1 mRNA expression in murine AAA, suggesting miR-29b can negatively regulate collagen synthesis [19]. This brings out a possibility that both miR-1260 and miR-29b are cooperating in regulation of compensatory collagen synthesis in AAA. Therefore, further investigation of COL1A1 expression upon miR-29b down-regulation in human VSMCs is also necessary.

Inflammation is one of the key pathogenic features in AAA, with observations indicating a significant infiltration of inflammatory cells from the circulation into the aortic wall [20-23]. More importantly, the infiltrated inflammatory cells are well-known sources of a variety of aortic wall matrix-degrading proteases, which can result in proteolytic degradation of elastin and aneurysm formation [24,25]. Therefore, inflammation, which is a key pathogenic process in promoting formation of AAA, has been widely investigated. The infiltration of inflammatory cells has been suggested to be triggered by chemokines. One example that has been reported in AAA is MCP-1, which can attract and recruit monocytes to the site [26-28]. There were findings showing an increased MCP-1 expression in AAA tissues from humans and experimental mouse model [27,28]. In agreement with these findings, our current finding showed an up-regulation of MCP-1 when miR-1260 was knocked down in VSMCs which mimicked the situation observed in AAA VSMCs from the previous microarray assay, further corroborating the involvement of MCP-1 in AAA pathogenesis possibly mediated by miR-1260 [4]. A pathogenic mechanism involving miR-1260 is thus proposed (Figure 4B). Under healthy condition, the miR-1260 may be up-regulated in VSMCs for suppression of MCP-1 expression to promote inflammation. However, the down-regulation of miR-1260 in VSMCs may allow up-regulation of an unknown intermediate component, possibly an activator protein for MCP-1 gene expression as MCP-1 is not bioinformatically predicted as target of miR-1260. An activation of MCP-1 expression may be resulted for triggering the infiltration of inflammatory cells

into the aortic wall, hence inducing proteolytic degradation of elastin and AAA formation. This hypothesis is necessary to be investigated in *in-vivo* model with miR-1260 knockdown, followed by detection of aneurysm formation, MCP-1 expression, macrophage, and elastin structure in abdominal aortic wall. Moreover, the unknown intermediate component directly regulated by miR-1260 for downstream MCP-1 regulation can be identified from gene expression profiles between miR-1260-overexpression/-knockdown and corresponding control VSMCs.

Our results also showed that miR-1260 may not regulate MMP-2, MMP-9, TIMP-1 and TIMP-2 protein expressions or promote apoptosis in VSMCs. However, these findings are still necessary to be validated in *in-vivo* model as the current study conducted in an *in-vitro* setting inevitably lacks an interaction with extracellular environment, especially elastin fibre microstructure. According to a prior study reporting elastin degradation peptides from human AAA tissues triggered inflammatory cell recruitment, there is a possibility that presence of elastin degradation peptides are the key essential for triggering other AAA pathogenic components [29]. Therefore, further study in *in-vivo* model is necessary.

MiR-1260 was previously found to be down-regulated in VSMCs from human AAA tissues [4]. However, circulating level of miR-1260 in blood of AAA patients has not been investigated. Further study of circulating miR-1260 might bring out a possible clinical implication of identifying patients at risk for AAA or predicting the severity of aneurysm through a suppressed level of miR-1260 in blood.

The molecular mechanisms of how miR-1260 functions can be further investigated, such as figuring out whether miR-1260 down-regulates its targets through mRNA cleavage or translation repression, and whether the bioinformatically identified seed regions of miR-1260 are critical for its function through mutation/deletion studies.

A limitation of this study is small sampling size due to the limited availability of human abdominal aortic tissues under the fact that the concept of organ donation is not widespread in Chinese culture. On-going collection of human VSMC samples for a large-scale study may validate the current findings. Another limitation is that the current findings, which were concluded from non-aneurysmal VSMCs, may not be translatable to AAA VSMCs. Due to the major constraint of VSMCs from AAA patients which is a shorter proliferative capacity than non-aneurysmal VSMCs, the limited amount of proliferative AAA cells would not be sufficient for such study with a series of complex experimental procedures and analyses [30]. In addition, this *in-vitro* study suggested the important role of miR-1260 at the cellular level. It is still uncertain if it reflects in *in-vivo* situation. Therefore, animal study with overexpression and knockdown of miR-1260 is necessary to validate the current findings, hence providing a possible clinical implication of therapeutic strategy by miR-1260 overexpression for preventing AAA.

CONCLUSION

This study investigated the role of miR-1260, which was found down-regulated in VSMCs from human AAA tissues previously, in human VSMCs for the pathogenesis of AAA. The current findings showed up-regulation of both COL1A1 and MCP-1 protein expressions in human VSMCs with miR-1260 knockdown, suggesting that miR-1260 may contribute to AAA through compensatory collagen synthesis and inflammation. This novel finding brings out that miR-1260 has a role in regulation of the key pathogenic events in AAA, providing an insight on the therapeutic manipulation of miR-1260 for preventing the formation and progression of AAA.

DATA AVAILABILITY

The data used to support the findings of this study are available from the corresponding author upon request.

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