Biochemical and Biophysical Research Communications 417 (2012) 771–776

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

MiR-19b-1 inhibits angiogenesis by blocking cell cycle progression of endothelial cells

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ARTICLE INFO

Article history:
Received 29 November 2011
Available online 16 December 2011

Keywords:
MicroRNAs
MiR-19b-1
Angiogenesis
Cell cycle
FGFR2
S phase

ABSTRACT

MicroRNAs are endogenously expressed small, non-coding RNAs that modulate biological processes by recognizing specific gene transcripts, leading to translational repression or degradation. Previous work showed that the miR-17-92 cluster is highly expressed in human endothelial cells that participate in angiogenesis. In this study we showed that miR-19b-1, a component of this cluster, controls the intrinsic angiogenic activity of human umbilical vein endothelial cells (HUVECs) *in vitro*. *In silico* and *in vitro* analyses have suggested that miR-19b-1 targets mRNA corresponding to the pro-angiogenic protein, FGFR2, and blocks the cell cycle from the S phase to the G_2/M phase transition by controlling the expression of cyclin D1. Thus, miR-19b-1 may serve as a valuable therapeutic agent in the context of tumor angiogenesis.

1. Introduction

MicroRNAs (miRNAs), an important class of 22 nucleotide-long, non-coding RNAs that are crucial regulators of gene expression at the post-transcriptional level, have been shown to be involved in various biological processes including proliferation, differentiation and apoptosis, as well as maintenance of stemness; therefore, their dysregulation contributes to many human diseases [1–3]. It is well known that angiogenesis is a fundamental step in human physiological and pathological processes, such as development and tumor growth. First evidence proving the essential roles of miRNAs in angiogenesis was revealed by Dicer depletion in Zebra fish [4]. Furthermore, knockdown of Dicer expression has been shown to cause profound dysregulation of angiogenesis related genes *in vitro* and *in vivo* [5]. Other studies demonstrated that miR-221 and miR-222 depress the angiogenesis properties of human umbilical vein endothelial cells (HUVECs) [6]. In addition, miR-15b, miR-16 and miR-20a/b have been shown to control the expression of VEGF, a key pro-angiogenic factor involved in tumor angiogenesis [7]. Lastly, research suggests that let-7f and miR-27b could promote angiogenesis by targeting the anti-angiogenic gene, thrombospondin-1 (Tsp-1) [5].

Likewise, the polycistronic miRNA-17-92 cluster, induced by c-MYC/MYCN [8], was first identified as an essential oncogene [9], and was proved to promote tumor angiogenesis in a paracrine manner [10–12]. Previous work has confirmed that both anti-angiogenic Tsp-1 and connective tissue growth factor (CTGF), which are down-regulated correlated with enhanced revascularization, were predicted targets for repression by the miR-17-92 cluster [13]. The miRNA-17-92 cluster comprises seven miRNAs, including miR-17-5p, miR-19b-1, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a [14]. Recent bioinformatics predictions and several experimental validations suggest that each component is likely to regulate hundreds of mRNA targets, and that the unique gene structure of miR-17-92 may underlie the molecular basis for its pleiotropic functions in a cell type- and context-dependent manner [15]. Continuing reports have attributed the mechanism behind the pro-angiogenic functions of the cluster to miR-17-5p [16], miR-18a, and miR-19a [10], which promote tumor angiogenesis by suppressing the release of soluble anti-angiogenic factors by tumor cells or up-regulating expression of the key factors involved in cells proliferation, survival or motility [8,10,17–19]. Meanwhile, some findings showed that over expression of miR-92a in endothelial cells blocks angiogenesis *in vitro* and *in vivo* by targeting integrin α5 [20].

It is reported that miR-19 (miR-19a/19b-1) was necessary and sufficient to promote *c-myc*-induced B lymphomagenesis. Enforced miR-19 expression dampens the expression of the tumor suppressor Pten, thus activating the Akt–mTOR signaling pathway and promoting cell survival [21]. So far, the impact of miR-19b-1 on endothelial cell biology is unclear. In view of this, the function of

Abbreviations: cDNA, complementary DNA; HUVEC, human umbilical vein endothelial cell; FGFR2, fibroblast growth factor receptor 2; Flk-1, fetal liver kinase-1; GAPDH, glyceraldehyde phosphate dehydrogenase; ITGB8, integrin β8; miR-19b-1, microRNA-19b-1; miRNA, microRNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide.

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0006-291X/$ - see front matter © 2011 Published by Elsevier Inc.
doi:10.1016/j.bbrc.2011.12.032

an individual member of the miR-17-92 cluster, miR-19b-1, on angiogenesis *in vitro* was investigated in this study. Our results demonstrated that miR-19b-1 can strongly suppress the intrinsic angiogenesis properties of endothelial cells by blocking cell cycle progression.

2. Materials and methods

2.1. Cell culture

HUVECs were isolated from freshly delivered cords, as reported previously [22], and grown on cell culture flask (Corning, USA) in endothelial cell medium (ECM, ScienCell, USA) supplemented with 5% fetal bovine serum , 100 U/ml penicillin, 100 mg/ml streptomycin and 1% endothelial cell growth supplement (ECGS) (ScienCell, USA). Cells between passages 3 and 6 were used for all experiments in order to maintain the primary characteristics of endothelial cells. HUVECs were transfected with miRNA by lipofectamine according to the manufacturer's protocol (invitrogen, USA). After 48 h post transfection, cells were used in the next assays. The level of miRNA, which was transfected into HUVECs, was checked by real-time quantitative RT-PCR. Transfection of HUVEC was performed with stability enhanced mature microRNA (miR-19b-1) (5′-UGUGCAAAUCCAUGCAAAACUGA-3′) and the control (miR-NC) double-stranded RNA oligonucleotides (Biomics).

2.2. MTT viability assay

Assessment of cell viability was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Firstly, HUVECs were transfected with miR-19b-1 (HUVEC-19b-1), miR-19b-1 inhibitor (HUVEC-19bI) or miR-NC (HUVEC-NC) at 12.5, 25 and 50 nM. Untreated cells were seeded onto 96-well tissue culture plates at a density of 6×10^3 cells per well in ECM containing 5% FBS and maintained at 37 °C. 24 h after transfection, cells were continually cultured for 48 h. Subsequently, HUVECs were seeded onto 96-well tissue culture plates at a density of 3×10^3 cells per well, then transfected with miRs at 10 nM. Afterwards, cells were continually cultured for 24, 48, 72 and 96 h. Then, 0.5 mg/mL MTT was added to each well and incubated for 4 h at 37 °C, then formazan was dissolved in 150 μL DMSO. Absorbance was measured at 490 nm.

2.3. Migration assay

Diluted matrigel (purchased from BD Biosciences) 1:3 using serum-free medium and mixed sufficiently, was coated evenly on the surface of the transwell bottom. HUVEC-19b-1 and HUVEC-NC were detached with 0.2% EDTA and resuspended in serum-free ECM at a density of 1×10^5 cells/mL, and then added to the matrigel-coated transwell (1×10^4 cells/well). An appropriate amount of ECM, which contained 5% FBS and 1% ECGS, was added to a 24-well flat-bottom polyviny1 chloride plate (COSTAR). The transwells were then attached to the 24-well plate, and cells were incubated at 37 °C in 5% CO_2 in the humidified incubator for 24 h. After that, the medium was aspirated, and the migrated cells were fixed with ethanol for 30 min and then stained with 0.1% crystal violet for 10 min. Cells not migrated were cleared by cotton swab. Photos were taken under a microscope.

2.4. Tube formation assay

The tube formation assay was performed as previously described with modifications [23]. Fifty micro liters melting matrigel was applied to 96-well cell plates and incubated with synthetic rubber and polyethylene at 37 °C for 1 h. 1×10^4 transfected cells per well were seeded in the gel. Cells were incubated at 37 °C, with 5% CO_2 for 12 h. The effects of miR-19b-1 on the differentiation of HUVECs stimulated by ECGS were observed with microscope (Leica) and four representative fields were taken. Total tube length was quantified using image analysis software, Image-Pro Plus (version 6.0).

2.5. Cell cycle measurement

For the cell cycle study, 1×10^6 HUVECs were seeded in 6-well plate and incubated at 37 °C in 5% CO_2 overnight. The next day cells were transfected by miR-19b-1 for 12 h at the dose of 10 nM. Successively, cells were harvested with trypsin and washed twice with PBS. After that, cells were fixed in cold 70% ethanol and stored at 4 °C. On the day of analysis, ethanol was removed by centrifugation and cells were washed twice with PBS, then treated with RNase (75 kU/mL) for 30 min at 37 °C. Propidium iodide (PI) was finally added (50 μg/mL) to stain the cellular DNA, and samples were processed by a flow cytometer (FAC Scan, Becton Dickenson, USA). The 1×10^4 cells were acquired for each sample using the CELLQuest software and recording propidium iodide (PI) in FL2 channel. The cell cycle analysis was performed with ModFit software.

2.6. Reverse-transcription PCR (RT-PCR)

To assess the differential miRNA expression of Flk-1, FGFR2, caspase 8 (CAPS8), integrin β8 (ITGB8) and GAPDH, mRNA was extracted from 1×10^6 transfected HUVECs using TRIzol Reagent (Biouniquer) according to the manufacturer's protocol. The first-strand cDNA was synthesized using the reverse transcription system (Promega), amplified by easy-Taq DNA polymerase (Tran), in a 25 μL reaction mixture. Polymerase chain reaction was performed using a Mastercycler (eppendorf). The primers used in the assay are listed in Table 1. The level of miRNA of transfected HUVECs was determined by real-time quantitative RT-PCR (RT^2-PCR). RT^2-PCR was performed by EzOmics miRNA qPCR Detection Primer Set (miR-19b-1) using 2 μL cDNA as a template (Biomics) and a BioRad real-time PCR detection system (BioRad, USA). A set of primers and a TaqMan probe for each gene were purchased from Biomics.

2.7. Western blotting analysis

HUVECs were seeded at 3×10^5 per well in 6-well culture plates and incubated for 24 h, transfected by miRNAs, harvested with 0.05% trysin (Hyclone, UT, USA)/0.53 mM EDTA (Hyclone, UT, USA), washed with PBS, and resuspended in 100 μL of Mammalian Protein Extraction Reagent (Shanghai Generay Biotech Co. Ltd., Shanghai, China). Concentrated proteins were separated on 12% SDS–polyacrylamide gel (SDS–PAGE) for cyclin D1, Akt, and

Table 1
A list of all primers used in this study. Primers used for RT-PCR to detect the miR-19b-1 target at mRNA level.

Primer name	Primer sequence (5′-3′)
FGFR2-F	TCGCTGGTGAGGATAACAACACG
FGFR2-R	TGGAAGTTCATACTCGGAGACCC
Flk-1-F	TCTCTCTGCCTACCTCACCTGTTTC
Flk-1-R	CTGACTGATTCCTGCTGTGTTGTC
CAPS8-F	TTTCTGCCTACAGGTTCCACT
CAPS8-R	CCTCAATTCTGATCTGCTCACTT
ITGB8-F	ATGCACAATAATATAGAAAAA
ITGB8-R	TCCTTGTACCAATGAAACTG
GAPDH-F	AAGGTCGGAGTCACCGGATT
GAPDH-R	CTGGAAGATGGTGATGGGATT