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Overexpression of miR-133 decrease primary endothelial cells proliferation and migration via FGFR1 targeting

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ABSTRACT

Angiogenesis is one of the essential hallmarks of cancer that is controlled by the balance between positive and negative regulators. FGFR1 signaling is crucial for the execution of bFGF-induced proliferation, migration, and tube formation of endothelial cells (ECs) and onset of angiogenesis on tumors. The purpose of this study is to identify whether or not miR-133 regulates FGFR1 expression and accordingly hypothesize if it plays a crucial role in modulating bFGF/FGFR1 activity in ECs and blocking tumor angiogenesis through targeting FGFR1. The influences of miR-133 overexpression on bFGF stimulated endothelial cells were assessed by cell growth curve, MTT assaying, tube formation, and migration assays. Forced expression of miR-133 caused significant reductions in bFGF-induced proliferation and migratory ability of ECs. MiR-133 Expression was negatively correlated with both mRNA and protein levels of FGFR1 in the transfected ECs isolated from peripheral blood. Moreover, overexpression of miR-133 drastically reduced the rate of cell division and disturbed capillary network formation of transfected ECs. These findings suggest that miR-133 plays an important function in bFGF-induced angiogenesis processes in ECs and provides a rationale for new therapeutic approaches to suppress tumor angiogenesis and cancer.

1. Introduction

Angiogenesis is one of the crucial hallmarks of cancer that is critical to the growth, invasion, and metastasis of human tumors [1,2]. The key to this process is the switch from the normal quiescent vasculature to an activated state in which the proper balance between pro- and anti-angiogenic factors destructs towards a pro-angiogenic function resulting in endothelial cells (ECs) acquiring a proliferative, migratory and morphogenic phenotype [3,4]. Tumor cells upscale secret pro-angiogenesis factors (such as VEGF and bFGF) at high levels, which upsets the angiogenesis balance and leads to endothelial cells recruitment and proliferation. Among various angiogenic inducers, basic fibroblast growth factor (bFGF, also termed as FGF2) is probably one of the most important factors that interact with FGF receptor 1 (FGFR1). This interaction is necessary for the in vitro the execution of FGF-induced proliferation, migration, and ECs tube formation, as well as the in vivo onset of angiogenesis in tumors [5–8]. However, comparatively less is known about the post-transcriptional regulation of FGFR1 in ECs via miRNAs.

MicroRNAs are short non-coding RNAs that regulate gene expression through binding to identical or similar complementary sequences in the 3′-UTRs of the target genes, resulting in the translation inhibition or cleavage of the mRNA target. miRNAs play a central role in a broad range of biological processes such as angiogenesis and cell cycle control [9–15]. The global inhibition of Dicer and Drosha, two crucial enzymes for miRNAs biogenesis, has revealed the importance of endothelial miRNAs in angiogenesis [14,16]. Some miRNAs such as miR-15 and miR-16 reduce angiogenesis via inhibiting vascular endothelial cell growth factor (VEGF) expression [17]. Similarly, miR-221 and miR-222 inhibit angiogenesis by targeting human proto-oncogene c-Kit receptors in endothelial cells [18]. Conversely, miR-17-92 cluster components have been shown to participate in EC-mediated angiogenic and oncogenic functions via targeting the anti-angiogenic proteins such as Tsp1, connective tissue growth factor, and SPARC containing thrombospondin type 1 repeats [19]. Here, we focus our attention to miR-133, which has been validated as a tumor suppressor in various cancers such

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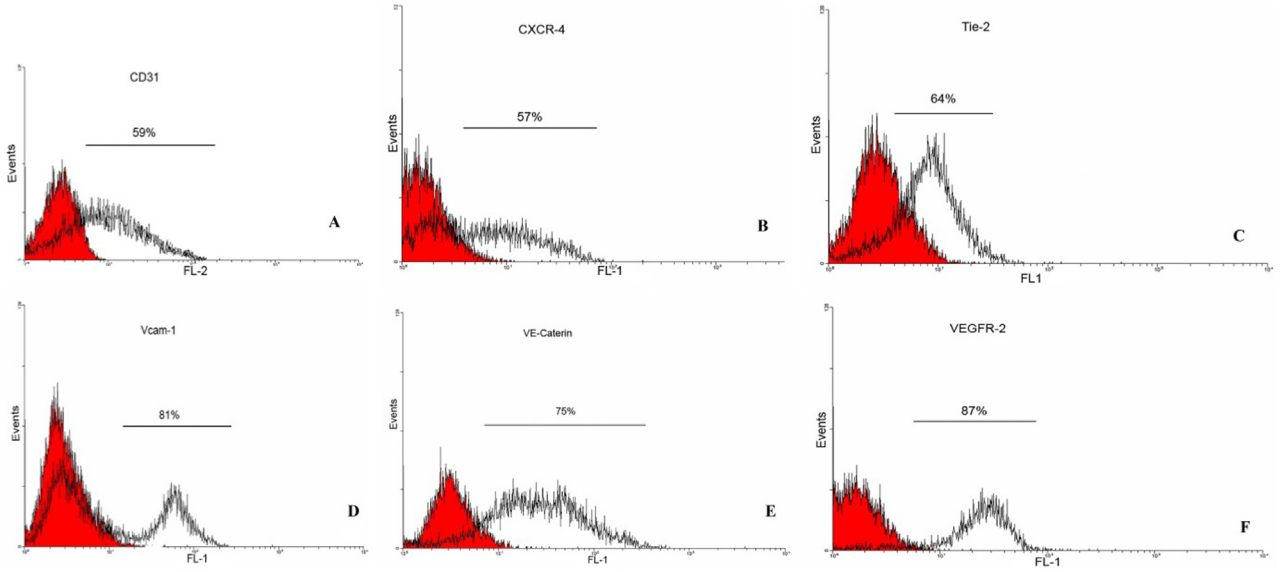

Fig. 1. EPCs specific markers were investigated by FACS in ECs isolated from cord blood: **A)** CD31 marker, **B)** CXCR-4 marker, **C)** Tie-2 marker, **D)** Vcam marker, **E)** V-caterin marker, and **F)** VEGFR-2 marker. (2 column).

as ovarian [20], colorectal [21], bladder [22,23], breast [24], and prostate cancers [25] through declining the proliferation, migration, invasion and cell cycle progression of the tumor cells. However, the roles of miR-133 in ECs and angiogenesis remain unclear. `Herein, we report a new role of miR-133 in the regulation of FGFR1 in ECs. We demonstrate that elevated miR-133 expression in ECs is negatively correlated with the angiogenesis level, in part, through the regulation of FGFR1 involved in tumor progression. These findings can open promising approaches for personalizing miR-133-based therapy.

2. Material and methods

2.1. Cells and cell culture

Peripheral blood samples (30 ml) from 3 healthy individuals (2 men and 1 woman) were collected in a Heparin solution. Informed consent was obtained from each donor. Blood samples were diluted 1:1 with Hanks Balanced Salt Solution (HBSS, Invitrogen), placed into Histopaque 1.077 (Sigma), and centrifuged at 740 × g for 30 min. Buffy coat mononuclear cells were collected and then washed three times with complete EC growth medium, comprising 8% (v/v) fetal bovine serum (FBS). Mononuclear cells were then seeded into a 12-well plate coated with collagen I (BD Biosciences) in complete EC growth medium (Cambrex) containing Endothelial Growth Media-2 (SingleQuots), final 2% FBS concentration and growth factors (Cambrex), and 1 × antibiotic/antimycotic solution (Invitrogen). The medium was changed every 24 h for the first week to remove non-adherent cells. Colonies of Endothelial progenitor cells appeared 7–10 days after the initial isolation. The ECs grew to confluence at 80% and were serially passaged onto collagen I-coated plates. The EPC-derived peripheral blood was cultivated in complete EC growth medium, and used in passages 4–9 for all experiments.

2.2. Immunophenotyping of endothelial cells

Early-passage (1-2) Endothelial colony cells-derived from peripheral blood MNCs (5×10^4) were incubated at 4 °C for 30–60 min with the primary or isotype control antibodies prepared in 100 µL phosphate buffered saline (PBS) and 2% fetal bovine serum (FBS), followed by washing 3 times, and analyzing by flow cytometry (Becton Dickinson, San Diego, CA). Primary murine monoclonal antibodies against human CD31 conjugated to fluorescein isothiocyanate (PE), human CXCR conjugated to Fluorescein isothiocyanate (FITC), human KDR conjugated to FITC, human Tie-2 conjugated to PE, human Vcam-1 conjugated to PE, and human VE-Caterin conjugated to FITC (all BD Phar Mingen, San Diego, CA, unless otherwise indicated) were used.

2.3. Transfection with synthetic miRNA

The synthetic miR-133 and scrambled oligonucleotide were purchased from Ambion. Transfections were performed using lipofectamine 3000 transfection reagent (Invitrogen) according to the manufacturer instruction. In a typical experiment, 1 µL and 1 µg of transfection reagent and DNA were mixed, and cells were exposed to the reagent–DNA complexes for 4–6 h, followed by 48–72 h of incubation depending on the experiment, and finally harvested for further studies. Studies were carried out in three different groups: the blank control group (the group that was untreated), the test groups (the group transfected by miR-133 mimic), and the negative control groups (the group transfected with FITC-conjugated scrambled oligonucleotide). Transfection efficiency was measured using optimization with scrambled which was found to be between 60% and 70%.

2.4. cDNA synthesis and Real-Time PCR for miR-133 and FGFR1

Total RNA was purified using Trizol (Invitrogen) 48 h after transfection according to the manufacturer's protocol. For FGFR1, total RNA was converted to complementary DNA (cDNA) using Fermentas Reverse Transcriptase System (Fermentas) and used for Real-Time PCR according to the manufacturer's instruction. The expression level of FGFR1 as the target of miR-133 was analyzed using real-time PCR (ABI) by SYBER premix ExTaq kit (Takara). The expression of mRNA targets relative to GAPDH was determined using the $2^{-\Delta\Delta CT}$ method. QRT-PCR primer sequences were: FGFR1 FW: 5′-CGGGACATTCACCACATC-3′; FGFR1 RW: 5′-CCGAAAGACCACACATCAC-3′; GAPDH FW: 5′-GACAA GCTTCCCGTTCTCAG-3′; GAPDH RW: 5′-GAGTCAACGGATTTGGTCGT-3.

For miR-133, total RNA reverse was transcribed to cDNA using stem-loop RT specific primers (for miR-133 and SNORD47) and M-MLV Reverse Transcriptase (Promega, USA). Subsequently, the expression level was measured by the universal reverse primer and specific miR-133 forward primer using SYBR Green PCR Master Mix (Takara). The

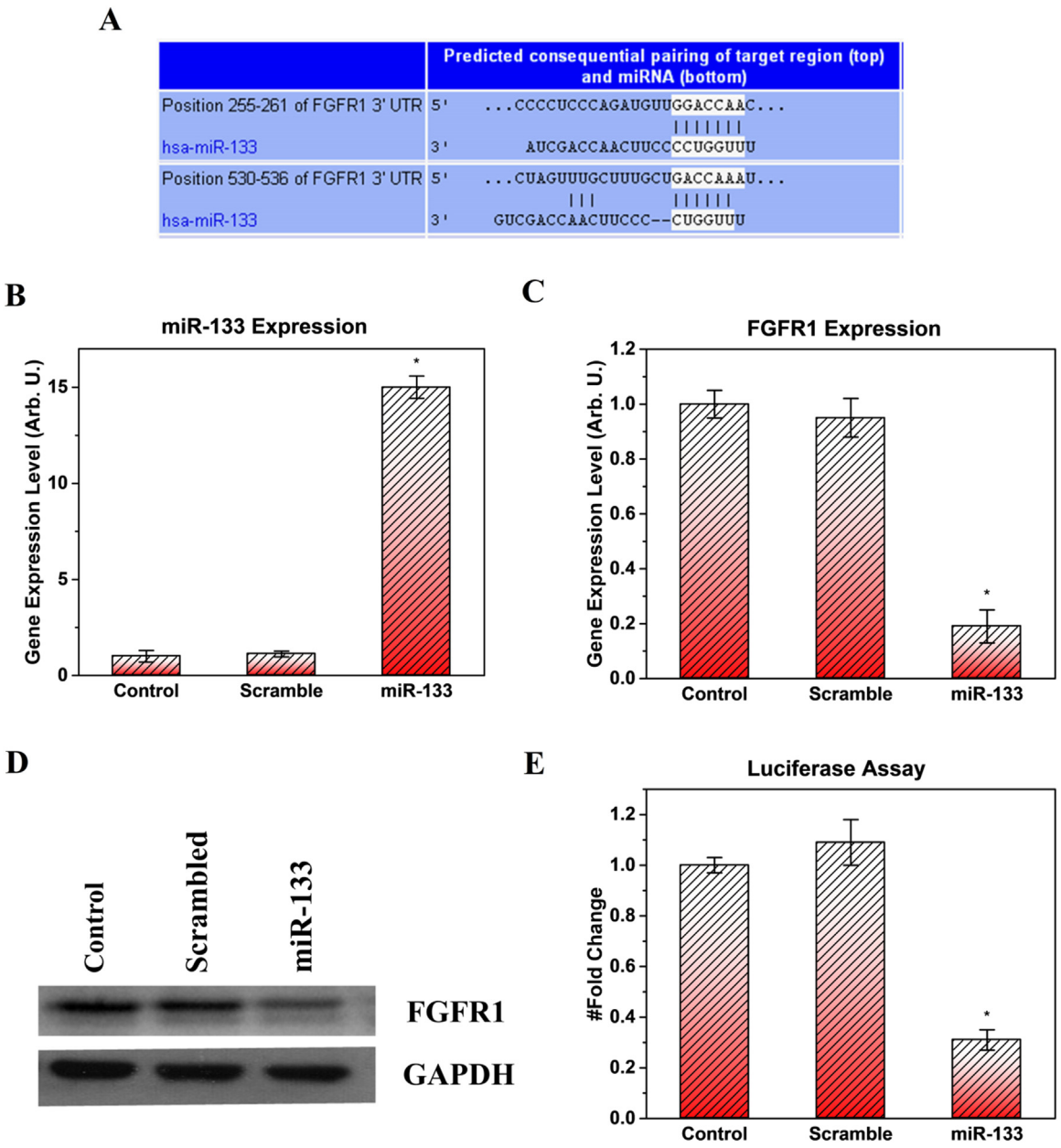

Fig. 2. MiR-133 targets FGFR1 in ECs. **A)** The interaction between miR-133 and 3′-UTR binding site of FGFR1 according to TargetScan prediction. **B)** miR-133 expression evaluated by QRT-PCR in ECs, 3 days after transfection with miR-133 mimic and scrambled relative to SNORD47 as an endogenous control. Values and bars are means and SD's of three replicates. **C–D**) Endogenous FGFR1 mRNA and protein expression measured 72 h after the post-transduction of miR-133 in ECs. β-Actin was used as an internal control in QRT-PCR. **E)** MiR-133 expression significantly inhibited the luciferase activity of FGFR1 3′-UTR. Luciferase activity was detected at 72 h post-transfection. Scrambled oligo was used as a negative control. Data shown are mean values ± SD of the ratio of luciferase activity to that of the control, obtained from at least three independent experiments; ($*p < 0.001$). (1.5 column).

relative expression level of miRNA was analyzed and normalized to endogenous expression of SNORD47 RNA as an internal control using the $2^{-\Delta\Delta CT}$ method. The following primers were used in the QRT-PCR method: SNORD47 FW, 5′-ATCACTGTAAAACCGTTCCA-3′; SNORD47 RW, 5′-GAGCAGGGTCCGAGGT −3′; miR-133 FW: 5′TTTGGTCCCCTT CAACC-3′; miR-133 RW, 5′-GAGCAGGGTCCGAGGT-3′.

2.5. Western blot

Whole-cell proteins were separated by 10% sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membrane (PDVF, pore size 0.45 mm; Millipore, Billerica, MA) using a semi-dry transfer cell (Model 755, Bio-Rad, Hercules, CA). The membrane was subsequently incubated with the primary antibodies including anti-FGFR1 and anti-GAPDH antibodies. The membrane was further probed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ig antibody (Sigma, St. Louis, MO). The protein bands were visualized using (ECL®) detection kit (Pierce. Rockford, IL). Finally, chemiluminescence was captured using a Kodak X-film (Tokyo, Japan).

2.6. Construction of 3′-UTR-luciferase plasmid and luciferase assay

The partial lengths of FGFR1 3′-UTR containing potential miR-133 target sites were amplified and cloned at the XhoI and NotI sites, downstream of the luciferase gene in the pSICHECK2 vector (Promega). The sequences of the primers are as follows:

FW: 5′-CGCTCGAGGAGGTGCAAAGAGGCAGATC-3′;

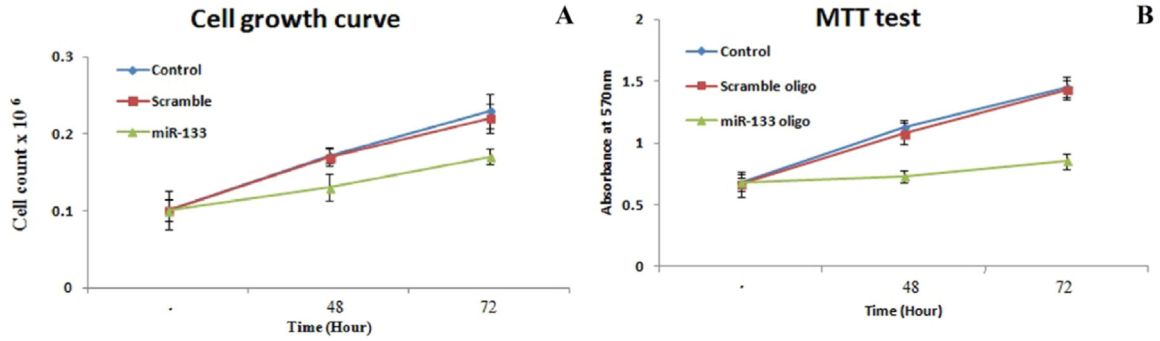

Fig. 3. MiR-133 decreased cell proliferation of bFGF-induced ECs: **A)** Cell growth curves of ECs transfected with miR-133 mimic and scrambled. **B)** Measurement of cell absorbance by MTT assay. MiR-133 influence on cell proliferation was evaluated up to 48 h after stimulation with bFGF. Each time point was expressed as total absorbance at 570 nm after background subtraction (Y-axis). Points, mean of three experiments; bars, SD. (1.5 column).

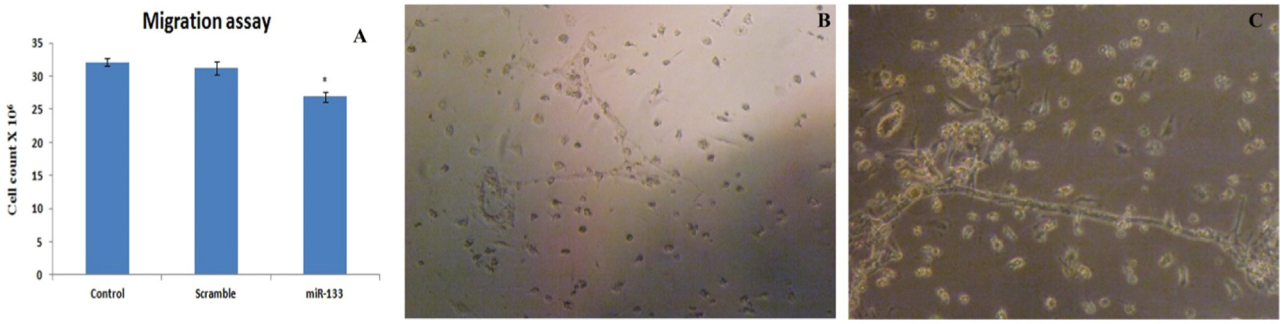

Fig. 4. MiR-133 suppressed bFGF-induced migration and tube formation of ECs. **A)** ECs migration in response to bFGF was investigated through counting the average number of ECs from five randomly chosen fields on the lower side of the membrane of each well. Data are expressed as migrated cells and correspond to mean ± SEM of three experiments performed in duplicate. **B–C)** Transfected ECs were counted and seeded on a Growth Factor Reduced Matrigel in the presence of 0.1% FBS + bFGF 6 h post-transfection: Scrambled: left panels, relatively zoomed out to show the absence of any tube formation; miR-133 mimics: right panels, relatively zoomed in to show the formed tube with more details. Cumulative sprout length of capillary-like structures was measured by light microscopy after 18 h. Representative micrographs are shown. $*p \leq 0.01$. (1.5 column).

RW: 5′-TAGCGGCCGCCATGGATACAGGAAGGACGT-3′.

For luciferase analysis, HEK293 cells were seeded in 96 well plates and cotransfected with FGFR1 3′UTR-pSICHECK2 vector and miR-133 mimic using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The empty vector of pSICHEK2 and scrambled oligo were used in each test as blank and negative controls, respectively. Luciferase activity was analyzed 72 h after transfection using the Dual-Luciferase Reporter Assay System (Promega). Renilla luciferase signal was normalized to the Firefly luciferase signal activity for the control of transfection efficiency. All experiments were performed in triplicates.

2.7. Cell proliferation assays

Endothelial cells were seeded in 24-well plate and transfected with miR-133 and scrambled oligonucleotides. After six hours, the cultivated medium was replaced with fresh serum-free medium supplemented with 20 ng/ml bFGF. Cells were harvested and counted after 48 and 72 h using a ViCell counter (Beckman Coulter). For MTT assay, ECs were seeded at 5×10^3 cells/well in 96-well plates and transfected with miR-133 and scrambled oligonucleotides. Cells were stimulated with serum-free medium containing 20 ng/ml bFGF 6 h after the post-transfection, and the proliferation rate was evaluated using MTT assay at 48 and 72 h. Briefly, 100 µL of 0.5 mg/ml of MTT solution was added to each well and incubated for 4 h at 37 °C. The medium was then removed and 100 µL of dimethyl sulfoxide (DMSO, Sigma) was added to dissolve the content. Finally, absorbance was recorded at 570 nm with a reference filter at 630 nm. All tests were carried out in triplicates. Inhibitory rate of miR-133 overexpression on ECs proliferation was determined using the *inhibitory rate of cells* equation

2.8. Migration assay

Twenty-four hours after miR-133 oligonucleotides transfection, transfected cells (2×10^5 cells) were suspended in 1 ml medium containing 20 ng/ml of FGF and added to the upper chamber. Transwell model (8 µm pore size) was applied to perform the migration assay. The upper side of transwell was coated with Matrigel, while the lower compartment was coated with type I collagen. The pre-coated transwell was then placed into a well of a 24-well plate containing 1 ml of complete EBM medium. After incubation at 37 °C for 24 h, the non-migrated cells in the upper surface of the membrane were removed with a PBS-soaked cotton swab. The membranes were subsequently fixed by incubating with 4% PFA for 30 min. Cells that migrated to the lower side of the membranes were visualized and photographed using a microscope video system.

2.9. Tube formation assay

The tube formation assay was performed using Matrigel-coated 12-well plates. Transfected ECs in EBM medium containing 0.1% FBS and bFGF (20 ng/ml) were plated on the matrigel at a density of 2×10^5 cells/well. After 18 h, the Matrigel-induced morphological changes of ECs and their tube networks were observed under microscope and photographed at 100 × magnification.

2.10. Statistical analysis

All tests were performed at least three times unless stated otherwise, and all data were presented as means ± SEM. Statistical differences were measured by either Student's *t*-test or one-way ANOVA. The P

values of ≤ 0.05 were considered statistically significant.

3. Results

3.1. Characterizing of endothelial cells isolated from cord blood

Mono-nuclear cells were successfully isolated from peripheral blood samples. Initially, spindle-shaped attached cells were appeared within 48 h after culturing. On day 7 , EPCs' specific markers were analyzed using FACS. The flow cytometric analysis showed that 59 ± 3.7% and 87.5% ± 5.0% of the cells expressed CD31 and VEGFR-2 respectively, while 57 ± 4.4%, 64 ± 1.5%, 81 ± 3.8%, and 75 ± 2.6% of the cells were positive to CXCR-4, Tie-2, Vcam-1, and V-caterin respectively (Fig. 1, A–F).

3.2. MiR-133 targets FGFR1 which regulates angiogenic activity of ECs

To investigate possible effects of miR-133, its vital targets that are important in the angiogenic activity of ECs were detected first. Among miR-133 has functional roles in angiogenesis and targets, FGFR1 was selected for further analysis as it contains two highly conserved target sites for 7mer-m8 and 7mer-1A of miR-133 located at positions 255–261 and 530–536 of the 3′-UTR respectively (Fig. 2A). To assess whether or not miR-133 could regulate FGFR1 production in ECs, the cells were transfected with miR-133 oligonucleotides. Unlike the control groups, the MiR-133 expression level was significantly increased by 15 fold ($P < 0.05$) in transfected group (Fig. 2B). Next, mRNA and protein expression level of FGFR1 were evaluated using QRT-PCR and western blot assays, respectively. It was indicated that miR-133 overexpression decreased FGFR1 and mRNA production by 5.1 and 3.2 fold after 72 h (Fig. 2C–D).

To validate the predicted miR-133/FGFR1 interactions, the FGFR1 3′-UTR was sub-cloned in a psiCHECK2 vector. The resultant constructs were co-transfected into HEK cells along with miR-133 or scrambled oligonucleotides. Transfection with the control empty psiCHECK2 without any 3′UTR did not affect the luciferase activity. Interestingly, the relative luciferase activity was significantly reduced (≈ 69%) when cells were co-transfected with FGFR1 3′UTR and miR-133 but not with scrambled (Fig. 2E).

3.3. MiR-133 inhibited proliferation of ECs induced by FGFR1

To examine the anti-angiogenic upregulation effects of miR-133 in vitro, the proliferation of bFGF-induced ECs was analyzed using cell counting and MTT assays. As shown in Fig. 3A, the proliferation of endothelial cells stimulated by 20 ng/ml bFGF was markedly decreased in miR-133 transfected ECs in comparison with the control groups. The results showed that miR-133 significantly inhibited endothelial cell proliferation.

MTT assay results demonstrated a significant statistical difference between the absorbance value of the blank control cells and the cells transfected with miR-133 oligonucleotide after 48 h (1.13 vs. 0.728) and 72 h (1.45 vs. 0.85) when stimulated with 20 ng/ml bFGF. However, no obvious changes were observed between blank control cells and cells transfected with the scrambled oligonucleotide in the same intervals ($P < 0.05$; Fig. 3B). As a result, inhibitory rate of miR-133 on the proliferation of stimulated ECs transfected with miR-133 were 34% and 41% after 48 and 72 h.

3.4. MiR-133 upregulation suppressed bFGF-induced migration and tube formation of ECs

Next, the potential role of miR-133 upregulation in blocking a series of angiogenesis-associated processes on bFGF-stimulated ECs was investigated. Migration ability is a critical parameter required for angiogenesis. Thus, the influence of miR-133 upregulation in the bFGF-induced migratory response of endothelial cells got subject to evaluation using transwell model (Fig. 4A). The lower chamber (containing bFGF as an attractant) was separated from the upper compartment (containing ECs) using a pre-coated membrane filter with pores (8 μm pore size) small enough to allow only active ECs to passage. The invasion of cells to the lower chamber significantly decreased in the miR-133 transfected group compared to the control groups, demonstrating that miR-133 could decrease the migratory potent of endothelial cells.

Finally, tube-formation assay was performed as one of the simplest, yet well-established in vitro angiogenesis assays based on the ability of ECs to form three-dimensional capillary-like tubular structures. Endothelial cells exhibited well-formed tubular structures in the absence of forced expression of miR-133 in the control groups. However, miR-133 overexpression resulted in a significant impairment of cord formation in varying degrees following stimulation with bFGF (Fig. 4B–C).

Altogether, miR-133 has the ability of suppressing the migratory process and tube formation activities of endothelial cells in the presence of bFGF.

4. Discussion

Cancer is a highly angiogenesis-dependent disease which is required to provide an adequate supply of oxygen and nutrients. Considering the predominant role of angiogenesis, different angiogenesis-suppressors and receptor-inhibitors have emerged that prohibit the neovascularization of cancer tissues as well as the growth of the tumor which might, therefore, be beneficial to the treatment of cancer [26,27]. A number of studies have indicated that miRNAs play a crucial role in diverse steps of carcinogenesis and cancer progression [13,28–30]. It is therefore important to discover cancer-related miRNAs and their target genes to understand their functions in tumorigenesis and angiogenesis as they play vital factors in cancer development and metastasis. Our focus in this study was on miR-133 as there have been several reports suggesting that miR-133 functions as a tumor suppressor in different steps of various cancers. For instance, miR-133 was found to target insulin-like growth factor 1 receptor (IGF1R) and subsequently suppressing ovarian cancer cell proliferation [20]. It has also been shown to bind to the 3′UTR domain of the ring finger and FYVE-like domain containing E3-ubiquitin protein ligase (RFFL) protein, eventually affecting the development of colorectal cancer through regulating p53 protein [21]. Other targets for miR-133, which are effective in different cancers, have been reported by other groups as well [22–24]. Our lab has recently detailed the role of miR-133 in the angiogenesis of human umbilical vain endothelial cells (HUVEC) through targeting FGFR1 [15]. However, to the best of our knowledge, the role of miR-133 in angiogenesis on primary cells, which are more similar to their human source, is yet to be elucidated. Thus, in this study, we focused our attention on whether or not miR-133 could play anti-angiogenesis roles in ECs as a model. More appealing to us was the identification of target gene(s) linked to angiogenesis and their related functions (proliferation, cell migration and capillary formation potential) using bioinformatics analyses. Several oncogenes have been predicted to be the targets for miR-133. However, because FGFR1 plays key roles in angiogenesis while little is known about its posttranscriptional regulation by miR-133, we selected FGFR1 as an important predicted target for miR-133 involvement in angiogenesis for further analysis. The FGFR1 protein interacts with basic fibroblast growth factor (bFGF, also termed FGF2), outside the cell and sends signals that help the cell respond to its environment. When a fibroblast growth factor (bFGF) attaches to the FGFR1 protein, the receptor triggers a cascade of chemical reactions inside the cell that instruct the cell to undergo certain changes important in angiogenesis including migration, proliferation, differentiation and survival [7,8]. We provide evidence that miR-133 directly targets FGFR1 since both mRNA and protein expression level of FGFR1 were decreased in the miR-133 transfected group. In agreement with these results, our

luciferase studies also indicate that miR-133 significantly reduced FGFR1 3′UTR activity and therefore confirmed FGFR1 to be a miR-133 target. The miRNAs-FGFR1 regulatory network plays an important role in carcinogenesis and angiogenesis. Chamorro-Jorganes et al. [17] reported that miR-16 and miR-424 can inhibit ECs proliferation, migration and invasion in vitro and in vivo by targeting FGFR1, VEGFR2, and VEGF. In another study, they indicated that FGFR1 was also targeted by miR-149 [31].

We next explored the impact of miR-133 overexpression on bFGF-induced ECs functions. Cell cycle growth curve analysis indicated that enforced expression of miR-133 declined the bFGF-stimulated proliferation of ECs compared with the blank control groups. MTT results also proved that miR-133 overexpression in ECs could reduce bFGF-induced cell proliferation dramatically after 48 and 72 h upon transfection. Transwell-based investigations on the migration response of ECs to bFGF demonstrated that the number of migrated ECs in the miR-133 group decreased more significantly compared to that of the control groups reflecting that miR-133 could also weaken the migratory capability of endothelial cells. Finally, the effects of miR-133 on the tube-forming ability of ECs were tested following stimulation with bFGF. According to obtained results, great destruction of tube network was observed when ECs were transfected with miR-133.

As demonstrated in this study, signaling from FGFR1 is suppressed by miR-133 overexpression through the execution of bFGF-induced proliferation, migration, and tube formation of cultured endothelial cells in vitro. Taken together, it appears likely that miR-133 contribute in the regulation of angiogenesis in ECs, at least in part by the modulation of FGFR1 signaling.

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Conflicts of interest

None.

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