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MicroRNAs as potential novel therapeutic targets and tools for regulating paracrine function of endothelial progenitor cells

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Endothelial progenitor cells (EPCs) play a protective role in the cardiovascular system by enhancing the maintenance of endothelium homeostasis and the process of new vessel formation. Recent studies show that EPCs may induce vascular regeneration and neovascularization mainly through paracrine signaling, that is, through the secretion of growth factors and pro-angiogenic cytokines [1]. However, multiple factors might function synergistically and therefore make it difficult to manipulate EPC paracrine effects. MicroRNAs, a family of small, non-coding RNAs, are characterized by post-transcriptionally regulating multiple functionally related genes, which renders them potentially powerful therapeutic targets or tools. In this paper we propose the hypothesis that microRNAs can be utilized as a novel therapeutic strategy for regulating EPC paracrine secretion.

key words: **endothelial progenitor cells • paracrine function • microRNAs**

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Background

Identified by Asahara in 1997, endothelial progenitor cells (EPCs) are defined as circulating CD34+ cells that differentiate *ex vivo* into endothelial cells and incorporate into sites of active angiogenesis [1]. To date, great progress has been made in understanding the significance of EPCs for vascular regenerative medicine, such as neovascularization [2], vascular homeostasis and repair [3], and therapy of various vascular diseases [4,5].

However, EPC beneficial properties after transplantation or mobilization of EPCs are significantly limited due to insufficient cell number, low survival rate after transplantation and impaired function of EPCs in the patients with cardiovascular risk factors and cardiovascular diseases [6] and our group obtained similar results [7]. Furthermore, mounting evidence reveals that EPCs also participate in endothelial cell regeneration and neovascularization in an indirect way – EPCs secrete a variety of growth factors and cytokines and thus improve proliferation and migration of pre-existing endothelial cells to enhance reendothelialization and angiogenesis [8,9]. In a recent study, exosomes from human EPCs, a component of paracrine secretion, have independent angiogenic activity both *in vitro* and *in vivo* [10]. In addition, the paracrine function of EPCs provides other benefits, such as improvements of vasodilatation [11], myocardial remodeling [12] and wound healing [13]. The therapeutic capacity of EPC-conditioned medium (EPC-CM) is in general comparable to EPC transplantation, while the number of cells necessary to generate an equivalent therapeutic dose is much lower than the quantity of cells employed for EPC transplantation [14]. Therefore, the paracrine function of EPCs may hold promise in their further application in clinical therapy.

However, therapeutic utilization of EPC secretome has been partially limited by questions regarding mechanisms that are still unclear, since multitudinous cytokines secreted by EPCs probably work synergistically on vascular cells. There are as many as 174 cytokines detected in EPC-CM using a large-scale cytokine array [15]. Multiple studies have suggested that main growth factors and cytokines contributing to the EPC-mediated paracrine protection include vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), platelet-derived growth factor (PDGF), thymosin beta 4 (Tβ4), transforming growth factorβ1 (TGFβ1), hepatocyte growth factor (HGF), and insulin-like growth factor (IGF) [8]. Despite being needed in desired collateral growth and vascular regeneration, many of the factors secreted by EPCs, especially IL-1, IL-6 and MCP-1, are also involved in the growth of atherosclerotic plaque or other pathological changes. Thus the regulation of EPC-secreted cytokines remains a great challenge for researchers and few studies have been carried out to investigate how EPCs regulate their secretome and mediate favorable paracrine effects [16]. Consequently, a reasonable strategy for manipulating the paracrine pattern of EPCs seems to be essential to meet the challenges of this complicated but promising field.

MicroRNAs (miRNAs) are small noncoding single-strand RNAs, on average only 22 nucleotides long. miRNAs are initially transcribed as primary transcripts (precursor pri-miRNAs) from the introns of protein-coding genes or the exons of non-coding genes by the RNA polymerase II or RNA polymerase III [17]. These are processed into shorter precursor transcripts in the nucleus by a complex comprised of the RNase III enzyme Drosha, and a protein called Pasha in *Drosophila* or DGCR8 in mammals. The precursor transcripts are further processed by the cytoplasmic RNase III endonuclease Dicer complex. The functional miRNA strand dissociates from its complementary nonfunctional strand and locates within the RNA-induced-silencing-complex (RISC) and other RNA binding proteins such as Argonaute [17]. To induce the process of translational repression, the mature miRNAs are guided by the specific miRNA-RISC complex to the 3 prime untranslated regions (3'UTR) of protein-coding target mRNA sequences. The complementary degree between the target mRNA 3'UTR and the miRNA seed region (its nucleotides 2–7) at the 5' end of miRNAs determines the mechanism of miRNA-mediated respective degradation or translational inhibition of the target protein-coding mRNA [18]. If the miRNAs match their targets perfectly, they induce direct cleavage and degradation of their target mRNAs. However, the complementarities between miRNAs and their targets are always imperfect, and miRNAs repress their targets mainly through translational repression or/and mRNA decay [19].

Therefore, microRNAs play a powerful gene regulatory role at the posttranscriptional level by negative regulation (transcript degradation and sequestering, translational suppression) and possible positive regulation (transcriptional and translational activation).

So far, more than ten thousands of miRNA genes have been identified by either molecular cloning methods or computational approaches after the first discovered miRNAs lin-4 in *C. elegans* in 1993 [20]. In the latest miRBase database (Release 17: April 2011), 1424 human miRNAs have been identified. It is estimated that a single miRNA can regulate hundreds to thousands of target genes, and therefore about 30% to 92% of human genes are likely regulated by miRNA [21,22].

Since miRNAs function as important regulators of gene expression and play a role in multiple cellular processes, dysregulation of miRNA has been associated with diseases such as cardiac disease, cancer and neurologic disease. Accordingly, manipulation of miRNA functions, either by mimicking or inhibiting them, appears to be a highly promising therapeutic strategy. The current understanding of miRNAs as potential new therapeutic targets or tools for vascular disease and cancer therapy have been reviewed by Jamaluddin et al. [23] and Gandellini et al. [24], respectively.

Hypothesis

Since miRNAs do not require perfect complementarity for target recognition, an individual miRNA is able to regulate multiple mRNAs, and each mRNA can also be targeted by more than 1 miRNA. Regarding the complicated paracrine system involving numerous growth factors and cytokines, the features of miRNAs make it possible to simultaneously manipulate various cytokine expressions and produce measurable results.

Here, we hypothesize that miRNAs may play a pivotal role in EPC paracrine function and serve as novel targets in

Table 1. Candidate miRNAs regulating cytokine secretion by EPCs.

miRNA	Target/cytokine	Ref
miR-424	cullin 2 (stabilizing HIF1α)	[28]
miR-519c and-199a	HIF1α (affecting VEGF, HGF, MCP-1 secretion)	[29,30]
miR-15b, -16, -20a, -20b and -126	VEGF	[31]
miR-297 and -299	VEGF	[32]
miR-146a	IL-6, IL-8, IL-1β and TNF-α	[33,34]
miR-155	IL-6, IL-1β,TNF-α, KGF and G-CSF	[36–38]
miR-10a	IL-6, IL-8 and MCP-1	[35]
miR-26a	IL-6	[40]

HIF1α – hypoxia-inducible transcription factor-1α; VEGF – vascular endothelial growth factor; HGF – hepatocyte growth factor; MCP-1 – monocyte chemotactic protein-1; IL-6 – interleukin-6; IL-8 – interleukin-8; IL-1β – interleukin-1β; TNF-α – tumor necrosis factor-α; KGF – keratinocyte growth factor; G-CSF – granulocyte colony-stimulating factor.

promoting therapeutic effects of EPCs by upregulating beneficial cytokines and inhibiting chemokines and factors causing adverse effects.

Based on recent experimental results, this theory has great significance. In general, increasing secretion of factors is a natural response for many cells including EPCs to relieve the hypoxia. The mechanisms by which hypoxia increases cytokine expression include increased transcription, translation, and mRNA stabilization. HIF-1α (hypoxia-inducible transcription factor-1α) induced the transcription of multiple pro-angiogenic proteins of EPCs, for instance VEGF [25], HGF [26] and MCP-1 [27]. Several recent attempts have been made to demonstrate the mechanism of regulating HIF-1α levels by miRNAs. Ghosh et al. showed that miR-424 (microRNA-424) stabilized HIF-1α by targeting cullin 2 (CUL2), a scaffolding protein critical to the assembly of the ubiquitin ligase system, and thereby played an important role in post-ischemic vascular remodeling and angiogenesis [28]. In contrast, miR-519c and -199a were reported to reduce HIF-1α and angiogenesis [29,30].

Furthermore, since several studies have revealed that the VEGF 3'UTR is targeted by multiple miRNAs, endogenous miRNAs that direct target and repress the key factor VEGF may have physiological and pathophysiological implications. For instance, miR-15b, -16, -20a, -20b and -126 downregulate VEGF expression, but their expressions are decreased during hypoxia, thus increasing VEGF level [31]. Recently, Jafarifar F et al reported that inhibitory endogenous miR-297 and -299 restricted VEGFA expression during normoxia, but this miRNA silencing activity was prevented by hnRNP L (hypoxia-activated heterogeneous nuclear ribonucleoprotein L), which also bound the VEGFA 3'UTR CA-rich element (CARE) [32].

Unlike traditional strategies, miRNA may synchronously regulate post-transcription of different genes that encode growth factors and cytokines. miR-146a has been identified as a major miRNA contributing to negative regulation of innate immune, inflammatory response and antiviral pathway. Surveys have shown that miR-146a could inhibit NF-κB activity and suppress the expression of NF-κB target genes such as IL-6, IL-8, IL-1β and TNF-α [33,34]. miR-10a is also a post-transcriptional regulator of the NF-κB signaling pathway and down-regulates MCP-1, IL-6, IL-8 and vascular cell adhesion molecule 1 (VCAM-1)[35]. In addition, miR-155 modulates expressions of IL-6, IL-1β and TNF-α [36] as well as keratinocyte growth factor (KGF) [37] and granulocyte colony-stimulating factor (G-CSF) [38]. Potential miRNAs regulating cytokine secretion of EPCs are summarized in Table.1.

Taken together, miRNAs may have 2 unique functionalities in control of EPC secretome: precise regulation of post-transcription of a single gene, and synchronous post-transcriptional inhibition of a number of genes that are functionally interdependent. Moreover, miRNAs may contribute to manipulation of cytokine level within the optimal range by offering a negative regulatory effect that partially counterbalances positive transcriptional and post-transcriptional regulatory mechanisms.

The processing of miRNA biogenesis, function and degradation, which involves a range of mechanisms, has an important role in miRNA-mediated therapeutic effects on EPC paracrine function. Numerous protein-protein and protein-RNA interactions would be involved in the regulation of miRNA transcription, processing and stability [39]. Many transcription factors (TFs) and other proteins regulate miRNA expression and processing positively or negatively by interacting with Drosha, Dicer or miRNA precursors. For example, Zcchc11, a ribonucleotidyltransferase, regulated IL-6 production by uridylating miR-26a [40]. Drosha and Dicer generally operate in complexes with double-stranded RNA binding proteins (dsRBPs), such as DGCR8 and TRBP. Arsenite resistance protein 2 (ARS2), a component of the nuclear cap-binding complex, interacts with Drosha and is required for pri-miRNA stability and processing. Argonaute (AGO) proteins and GW182 proteins are key factors in the assembly and function of miRISCs. All these proteins could regulate miRNAs and affect the significance of miRNAs as therapeutic targets and tools for manipulating EPC secretome [39,41].

To apply miRNAs for therapeutic intervention in EPCs, 3 strategies may be involved: The first strategy aims to inhibit

miRNAs by using miRNA antagonists, such as anti-miRs, locked-nucleic acids (LNA), or antagomiRs. These miRNA antagonists are chemically engineered oligonucleotides with sequences complementary to the mature target miRNA which then are trapped in a configuration that is unable to be processed by RISC, or lead to degradation [29]. The second strategy involves the introduction of a miRNA mimic to restore a loss of function or enhance the function by over-expression of a miRNA of interest. miRNA mimics are chemically synthesized, double-stranded RNAs that act as functional equivalents to endogenous functional miRNAs [24]. The third strategy is to use DNA-based vectors (viruses, plasmids) expressing miRNA genes [32,42]. Transfection methods of miRNAs are the same as small interfering RNAs (siRNAs). miRNAs including miRNA mimics, inhibitors and pre-miR miRNA precursors can be delivered to cells by a variety of means including lipid-mediated transfection [32], electroporation and microinjection. In order to evaluate the possible effects of miRNAs on EPC paracrine activity, cytokine secretion and production can be analyzed by ELISA, quantitative PCR and protein immunoblot.

Conclusions

Mounting evidence suggests that EPCs contribute to cardiovascular protection not only by direct participation in tissue homeostasis, but mainly via paracrine mechanisms, but the application based on EPC paracrine effects is limited because multiple factors, including some pathogenic factors, might be functioning synergistically and because regulation of therapeutic concentration remains to be investigated and represents a difficult task. Although few studies have been conducted to examine the potential effects of miRNAs in EPCs, miRNAs may function as post-transcriptional regulators of gene expression and thereby provide a novel and promising strategy to promote paracrine function of EPCs while minimizing its adverse consequences.

Conflict of interest

None declared.

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