MicroRNAs in Endothelial Development and Differentiation

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Abstract

Endothelial integrity or homeostasis is not only essential for regulating arterial activity and vascular tone under physiological conditions, but also critical for triggering various cardiovascular diseases including atherosclerosis and balloon angioplasty if such a balance has been impaired. Moreover, endothelial cell development and differentiation are key steps during embryogenesis and involves co-ordinations of diverse signaling molecules and transcription factors. Therefore, characterizing the molecular mechanisms underlying endothelial differentiation and development will not only improve our understanding of pathogenesis of vascular disease, but also facilitate our ability in generation of vessels cells from pluripotent stem cells for therapeutic purpose. MicroRNAs, a class of small, non-coding RNAs, have been extensively implicated in the regulation of various aspects of biological processes such as embryonic development, tissue/organ homeostasis, and metabolism, as well as almost all the human disease, particularly cancers and cardiovascular diseases. Accumulating evidence has implicated that microRNAs play an important role in regulation of endothelial development, phenotype and function. In this review, we will summarize new findings from recent studies in this field and discuss our current understanding of how microRNAs regulate endothelial development and differentiation from stem cells.

Keywords: MicroRNA; Stem cells; Endothelial; Blood vessel; Non-coding RNA

Introduction

Vascular system forms an extensive network in a living organism to deliver oxygen and nutrients to cells/tissues throughout the body. It is one of the first organ systems to develop and is fundamental for embryonic development and adult life. The Endothelial Cells (ECs) line the internal surface of the entire vascular system and form the barrier between circulating blood and the rest of the vessel wall. Endothelial cells serves to prevent thrombosis and regulate arterial activity through synthesis and release of numerous vasoactive molecules. The endothelium is thus considered as a dynamic and heterogeneous organ with secretory, metabolic, synthetic and immunological functions [1]. Vasculatures are established in two distinct, but close associated processes: vasculogenesis and angiogenesis [2]; initial vasculogenesis generates a primitive network of vessels through de novo formation of endothelial cells from precursor cells called angioblasts. Subsequent angiogenesis then leads to vessel expansion with further EC sprouting and branching. Maturation of the blood vessel involves the recruitment of mural cells to enwrap nascent ECs tubules for stabilization and remodeling. Further specialization of the endothelial cells into arteries, veins, capillaries and lymphatic vessel ensures the proper functioning of the vasculature.

During mammalian embryonic development, the establishment of endothelial cells occurs both extraembryonically and intraembryonically. In the yolk sac, mesodermal precursors of both hematopoietic and endothelial lineage differentiate into a cluster of cells called blood islands, where the inner cells gives rise to hematopoietic cells and the outer cells differentiate into endothelial cells [3]. The subsequent coalescence of blood islands and formation of lumina lead to a primitive vascular plexus. Within the embryo, endothelial precursor cell called angioblasts migrate and differentiate to form the primordial aorta. Simultaneous migration of angioblasts from presomitic cranial mesoderm form endocardial tube in the pericardial area [4]. The commitment of EC lineage from its precursors involves co-operative interaction of many different signaling molecules and transcription factors (for reviews, see Ref [5,6]). The understanding of molecular mechanism of EC differentiation will greatly benefit regenerative medicine for treating certain vascular disease like atherosclerosis, dissections and aneurysms. Various methods have been described to generate endothelial cells from pluripotent stem cell, which include Embryonic Stem Cells (ESCs) and induced Pluripotent Stem Cells (iPSCs), but often with limited efficiency [7]. Thus, new techniques and refined protocols are still in need to produce sufficient number of desired cell type for regenerative medicine.

MicroRNAs (miRNAs) are small, non-coding RNAs that play important regulatory roles in various aspects of development, homeostasis and disease by pairing to the mRNAs of protein-coding genes as negative or positive posttranscriptional regulators [8-10]. In mammals, approximately 30% of protein-coding genes are regulated by microRNAs [11], miRNA are transcribed into primary miRNAs (pri-miRNAs), which is first processed in the nucleus into an intermediate form (pre-miRNA) by the microprocessor protein complex composed of an RNase III enzyme Drosha and its cofactor DGC8. The pre-miRNAs are then transported via export in 5 into the cytoplasm and further processed by another RNase III enzyme Dicer to form mature
miRNAs. One strand of the mature miRNAs base-pair imperfectly with the 3' Untranslated Region (UTR) of target mRNA by forming an ribonucleaseprotein known as the RNA-Induced Silencing Complex (RISC), which contains the Argonaute protein Ago2. RISC complex finally repress mRNA translation and/or induce mRNA degradation, with the latter playing a predominant role [12]. Over the past several years, there has been an increasing amount of evidence suggesting that miRNAs are implicated in regulation of EC development, phenotype and function [13,14]. It has been suggested that miRNAs play a divergent role in EC development and differentiation from stem cells: some can maintain stem cells pluripotence, while other promote their differentiation into specific lineages depending on the targets it regulates. In this review, we will discuss the emerging findings to show the functional involvements of microRNAs in regulation of endothelial development and differentiation and signal pathways involved. In particular, we will highlight the individual miRNAs which have been recently identified as the modulators of endothelial differentiation and vasculature development.

**Dicer and Endothelial Differentiation and Function**

The global effect of miRNAs on vascular development comes from knock-out studies of the miRNA processing enzyme, Dicer [15]. Zebrafish embryos lacking dicer1 undergo a relative normal morphogenesis during the first week due to maternal Dicer activity, but went into growth arrest and died afterwards [16]. Mutants for both maternal and zygotic dicer (MZdicer) undergo axis formation but display abnormal morphogenesis during gastrulation, brain formation, and heart development associated with lack blood circulation [17]. However, MZ dicer mutants still developed endothelial and hematopoietic precursor cells as their lineage markers flk-1 and scl were expressed. In mouse, deletion of dicer led to lethality at embryonic day (E)7.5 before formation of primitive streak, due to loss of pluripotent stem cells [15]. Hypomorphic dicer mutants (Dicer<sup+m12</sup>) of mouse embryo also displayed retarded phenotype and died between E12.5 and E14.5 [18]. Formation of blood vessels in embryo and yolk sacs are compromised in dicer<sup+m12</sup> mutants, with altered expression of VEGF with its receptor FLT-1 and angiopoetein receptor Tie-1. In consistence with this observation, another line of mouse mutant with hypomorphic dicer1 (Dicer<sup+m8</sup>) resulted in female infertility caused by impaired growth of new capillary vessels in the corpus luteum [19].

The generation of two EC-specific Dicer knockout mouse lines, conditional Tie2-Cre; Dicer<sup+m8cKO</sup> mice and the tamoxifen-inducible VE-Cad-Cre-ER<sup>TM2</sup>; Dicer<sup+m8cKO</sup> mice, facilitate study on endothelial-derived miRNAs in EC differentiation and function [20]. These two mouse lines were hypomorphic for Dicer expression since Dicer levels were reduced but not abolished. As a result, newborn litters were viable and overtly normal. EC-specific Dicer hypomorphs showed significant reduction in angiogenic behavior with exogenous VEGF treatment, and displayed defects in postnatal angiogenesis in response to limb ischemia and wound healing [20]. Dicer silencing in ECs increased the expression of anti-angiogenic factor Thrombospondin-1 (Tsp1), and transfection with components of the miR-17-92 cluster, rescued the defect in EC proliferation and morphogenesis. In Human Microvascular Endothelial Cells (HMECs), the knockdown of Dicer reduced capillary sprouting and tube forming by silencing of critical target miRNAs like let-7f and mir-27b [21,22]. Also, knocking down of Dicer in ECs altered the expression of several key molecules regulating endothelial functions such as Tie-2/TEK, KDR/VEGFR2, Tie-1, endothelial nitric oxide synthase and interleukin-8 [22]. However, Drosha siRNA-transfected ECs does not reduce migration or angiogenesis in the in vivo Matrigel plug model [21]. This is probably because of the alternative miRNA-processing pathway without Drosha-mediated cleavage [23].

**miRNA and Endothelial Differentiation**

miRNAs are well-established masters to control the self-renewal and differentiation program of ES cells [24,25]. Dicer or DGCIR8-deficient ESCs display a significant defective in cell differentiation [26-28], which is further supported by the finding that dicer1-knockout mice die at early stages of development [15]. Numerous miRNAs have been shown to promote ESC differentiation into various cell lineages, including cardiomyocytes [29], endothelial cells [30], smooth muscle cells [31], skeletal muscle cells [32], Neuronal Progenitor Cells (NPCs) [33] etc. It has been reported that such cell lineage specifications mediated by miRNAs involves the repressing of the stem cell self-renewal program by inhibiting core pluripotent factors including homeobox protein Nanog, Sex-Determining Region Y (SRY)-box containing gene 2 (Sox2), Octamer-Binding Protein 4 (Oct4) and Krueppel-like factor 4 (Klf4) [31,34-36], as well as the induction of lineage-specific gene expression program [29,31]. Conversely, ESC-specific cell cycle-regulating miRNAs inhibit ESC differentiation and maintain ESC pluripotency [37]. Introduction of these miRNAs like miR-291-3p, miR-294, and miR-295, together with the core pluripotent factors Oct4, Sox2 and Klf4, substantially enhanced the efficacy of reprogramming towards iPSCs [38].

Roles of several miRNAs have been carefully examined in regulating vascular development, angiogenesis and endothelial functions through fine-tuning signaling pathway like VEGF, Notch and Slit/Tie signaling, and those include miR-126 [39,40], miR-221[41], miR-132 [42], miR-218 [43-44], miR-23–27–24 clusters [45], miR-27a/b [46], miR-92 [47], etc. (Refer to Ref [14] for a thorough review). Among them, miR-126 is the most extensively studied for its role in maintaining vessel integrity and directing angiogenesis. Targeted deletion of miR-126 in mouse endothelium led to partial embryonic lethality and leaky vessels, due to loss of vascular integrity and defects in endothelial cell proliferation, migration, and angiogenesis [40]. MiR-126 enhance VEGF signaling by inhibiting negative regulators spout-related protein Sprod1 and phosphoinositol-3 kinase regulatory subunit2 (PIK3R2) [39,40]. Moreover, miR-126 in zebrafish can be induced by blood flow in the aortic arch, thereby facilitating VEGF-dependent angiogenic remodeling [48]. However, miR-126 does not control endothelial lineage commitment, as evidenced by no increase in CD31-positive endothelial cells or endothelial gene expression with miR-126 overexpression during ES cell differentiation [39]. It rather inhibits hematopoietic differentiation from the common hematopoietic and endothelial progenitors called hemat endothelial cells, and may thus tip the balance to endothelial lineage [49]. This is also supported by the partial but not full embryonic lethality with miR-126 deletion [40]. Thus, although miR-126 are enriched in endothelial cells and Flk-1+ mesodermal endothelial progenitors [50], miR-126 does not specify endothelial lineage, but rather regulates angiogenesis and EC functions like maintaining vessel integrity and controlling vascular inflammation [51].

Though many of the aforementioned miRNAs have been shown to regulate proper EC functions, little is known about their role in EC differentiation. In order to explore the potential roles of miRNAs in endothelial differentiation, researchers used different protocols to induce EC differentiation from ESCs and have identified sets of miRNAs that may share a role [30,52,53]. Among them, some are
associated with angiogenesis (let-7b, 7f, miR-126, 130a, 133a, 133b, 210, and 296), while others impaired angiogenesis (miR-20a, 20b, 221, and 222) [54]. However, many of them have not been directly tested as whether being able to direct EC differentiation. In this review, we only summarize those microRNAs with a definite role in modulating EC differentiation (Figure 1).

**miR-21**

Identified as one of the first mammalian microRNAs, microRNA-21 (miR-21) has been extensively studied and found to be dysregulated in many pathological conditions including cancer, organ fibrosis and cardiovascular disease [55]. miR-21 is highly expressed in almost all kinds of cancers and therefore considered as an oncomiR. Many of the target genes identified for miR-21 are well-known tumor suppressors, such as Phosphatase and Tensin Homolog (PTEN) [56], Programmed Cell Death 4 (PDCD4) [57], RECK [58], RhoB [59], Bcl-2 [60], sprouty 1/2 [61], Tropomyosin [62], Maspin [63] and so on. Recent studies have also revealed the critical role of miR-21 in mediating diverse pathological process in cardiovascular disease, including cardiac fibrosis [64], myocardial infarction [65,66] and ischemia/reperfusion [67], endothelial-to-Mesenchymal Transition (EndMT) [68], and in advanced peripheral arterial disease [69]. Moreover, miR-21 has been implicated in angiogenic process [70], possibility via regulating endothelial progenitor cell senescence [71] and Angiogenic Progenitor Cells (APCs) dysfunction [72].

The involvement of miR-21 in regulating stem cell self-renewal and differentiation originate from a genetic study on deletion of the neuronal repressor REST (RE1-silencing transcription factor) in and differentiation originate from a genetic study on deletion of the neuronal repressor REST (RE1-silencing transcription factor) in [72].

**Figure 1: MicroRNA-mediated EC differentiation.** Both miR200c and miR-150 promote EC differentiation by transcriptionally de-repressing ZEB1 expression, while miR-21 enhances EC differentiation through inhibition of PTEN/Akt pathway. Moreover, miR-21 also indirectly enhanced TGF-β2 production, resulting in EC gene activation in a SMAD3-dependent manner. However, miR-181a/b and miR-99b mediate EC lineage specification through yet uncharacterized mechanism.
However, the action of miR-200 family on normal stem cell self-renewal or differentiation remains controversial. Maintenance of miR-200 expression stalls differentiating ESCs at the Epiblast-Like Stem Cell (Ep ISC) stage [90], and overexpression of miR-200c in human ESC inhibited Embryonic Body (EB) formation and repressed the markers for all three blastoderms, partially through targeting transcriptional factor GATA binding protein 4 (GATA4) [91]. miR-200c, together with miR-302s, and miR-369s, can directly reprogram mouse and human somatic cells into iPSCs [92], suggesting a role of these miRNAs in stem cell pluripotency. During OSKM (Oct4, Sox2, Klf4 and c-Myc)-induced iPSCs generation, miR-200 mediate BMP-driven epithelial-to-mesenchymal transition, which is essential for iPSCs formation at early stage [93]. Furthermore, Oct4 and Sox2 can bind to the promoter regions of mir-141/200c and mir-200a/b/429 cluster, respectively, to induce the transcription activation of miR-200 with subsequent suppression of ZEB protein, thus facilitating MET in reprogramming [94].

On the other hand, our recent study has firmly demonstrated that the miR-200c-ZEB feedback loop plays an important role in endothelial differentiation from human ESCs in vitro and in vivo by targeting ZEB1 [30]. Importantly, ZEB1 has been identified as a repressor for EC-specific gene expression in these processes, and miR-200c represses such inhibitory effect by inhibiting ZEB1 transcription, and thus promotes endothelial lineage differentiation. Moreover, by using the Matrigel-CD146+ EC-committing cells mixture implantation model, we observed that blocking ZEB1 signaling could rescue the inhibitory effect of miR-200c inhibition on in vivo vasculogenesis [30]. Finally, in this study we have demonstrated for the first time that miRNA-200c-ZEB1 axis is a critical regulator for chick embryonic blood vessel formation by in vivo inhibition of miRNA-200C or -150 in developing chick embryos. In the meantime, other researchers also reported that miR-200c promotes mesodermal specification while repressing neuroectodermal differentiation from ESCs [95], suggesting that miR-200c may play a cell-autonomous role in mediating EC differentiation from stem cells. Interestingly, miR-200b displays an anti-angiogenic activity in tumor and in embryonic vascular development [96,97], partially through repressing the crucial endothelial lineage related transcription factor E26 oncogene homolog 1 (Ets-1) [98]. This may reflect the distinct functions and dynamic regulation of miR-200 family members in EC differentiation, behaviors and embryonic vascular development at different stages. Further investigations into explaining such discrepancies and uncovering functional redundancy of different miR-200 family member in EC differentiation, embryonic vascular development and postnatal vasculogenesis/angiogenesis are urgently needed.

miR-150

miR-150 has been classically implicated in hematopoiesis by regulating cell differentiation in both lymphoid and myeloid lineage [99], and is considered as tumor suppressive gatekeeper in leukemia [100] as demonstrated by its aberrant expression is critical for pathogenesis in a variety of hematopoietic malignancies. However, other studies showed miR-150 secreted via microvesicle from monocytes promoted endothelial cell migration and angiogenic ability [100,101], indicating miR-150 also plays a role in angiogenesis as well as EC differentiation. Such a notion has been nicely demonstrated in our recent study [30]. During EC differentiation from human ESCs, miR-150 expression was significantly increased. Over-expression of miR-150 promotes, while knockdown inhibits EC differentiation from human ESCs in vitro or in vivo. Interestingly, the functional involvement of miR-150 in EC differentiation seems similar to miR-200c, in that miR-150 promotes endothelial lineage specification by transcriptionally repressing ZEB1 expression [30]. However, mice deficient in miR-150 are viable, fertile, and morphologically normal [102], indicating the signaling networks by which miR-150 mediates EC differentiation may not be essential for embryonic vasculogenesis, although such discrepancies could be attributed to the compensatory effects of other molecules in the miR-150 knockout mice. Thus further studies to examine the discrepancy existed in the literature and the functional redundancy of different miRNAs or other molecules in EC differentiation and vasculogenesis/angiogenesis would be warranted to fully understand the functional importance of miR-150 in in vivo vasculogenesis or blood vessel formation.

miR-181

The miR-181 family is composed of six members: miR-181a1/2, miR-181b1/2, miR-181c, and miR-181d. They are expressed in a numbers of tissues like muscle, eye, brain, lung and the hematopoietic compartment [103]. miR-181 family members play critical roles in controlling cardiovascular inflammation by regulating critical signaling pathways such as NF-κB signaling and molecules relevant to endothelial cell activation [104,105] and immune cell homeostasis [106-108].

The earliest evidence of miR-181 as a player in stem cell differentiation comes from a study showing miR-181 regulated B-cell development during hematopoietic lineage differentiation [109]. miR-181 also promotes myoblast differentiation through targeting the homeobox protein Hox-A1 [110]. In ESCs, miR-181a and miR-181b are expressed at low levels, but are sharply induced during differentiation [111]. Through downregulating expression of Cxb7, one of the polycystic repressive complex 1 (PRC1) component, miR-181 causes loss of Cxb7/PRC1-mediated repression on lineage-specific genes and guide the embryonic cell toward lineage commitment[111].

In a recent study performing microRNA microarray during defined stages of EC differentiation from human ESCs, miR-181a and -181b were found to increase in a time-dependent manner during EC differentiation and peak in mature hESC-ECs [53]. Overexpression of miR-181a and -181b enhanced the expression levels of EC-specific markers, Pecam1 and VE Cadherin, increased nitric oxide production, and numbers of tissues like muscle, eye, brain, lung and the hematopoietic compartment [103]. miR-181 family members play critical roles in controlling cardiovascular inflammation by regulating critical signaling pathways such as NF-κB signaling and molecules relevant to endothelial cell activation [104,105] and immune cell homeostasis [106-108].

miR-99 family

The miR-99 family consists of miR-99a, -99b, and -100. They predominantly act as tumor suppressors by inducing cell cycle arrest [113] and inhibiting cell proliferation [114]. The miR-99 family also
modulates injury response like post-radiation DNA damage [115] and dermal wound healing [116]. miR-99b was co-identified with miR-181 in the abovementioned study of EC differentiation from ESCs [53]. Like miR-181, augmentation of miR-99b induced EC-specific markers expression and nitric oxide generation, but its knockdown did not impact endothelial differentiation, implying that though capable to potentiate EC differentiation from pluripotent ESCs, miR-99 is not indispensable for EC differentiation.

Other miRNAs

Another set of new miRNAs that may also play a role in endothelial differentiation has been identified by Yoo and coworkers [117-119], but none of them have been functionally characterized. Among them, miR-5739 and miR-6087 modulates the expression of endoglin [117, 118], which is glycoprotein receptor of Transforming Growth Factor-β (TGF-β) expressed on endothelial cell. Authors reported that miR-6078 targets E-cadherin (Cdh) gene [118], while miR-7641 suppressed expression of CXCL1 [119], a member of the CXC chemokine family known to promoting neovascularization, during EC differentiation from ESCs.

Conclusion

Although extensive efforts have been put into deciphering the molecular mechanism of EC differentiation in the past years, a comprehensive understanding of the exact differentiation program is still far from complete. It has been well established that co-coordinative actions of diverse transcription factors and signaling molecules are required for EC differentiation and vasculature development. Emerging evidence clearly suggests that various miRNAs also play an indispensable in these processes, adding another regulatory layer to EC gene regulation network. Since tissue engineering and stem cell therapy has enormous clinical implications for treating vascular disease like atherosclerosis and artery dissections, a better understanding of EC differentiation program will greatly facilitate the generation of vessels cells from pluripotent stem cells. Particularly, with the maturation of iPSC technology, more seed cells are available for constructing bio-compatible vessels or intravascular injection of vascular progenitor cells for therapeutic purpose. Importantly, it have been recently reported that fibroblasts can be reprogrammed into endothelial cell and smooth muscle cells capable of generating tissue-engineered vessels which can, to some extent, substitute native vessels in vivo [120,121]. miRNAs play a broad role in multiple aspects of endothelial biology, and have been proven to be critical in mediating EC differentiation, as such modulating the expression levels of individual miRNAs in stem cells to generate unlimited functional endothelial cells in vitro will undoubtedly beneficial to cardiovascular regenerative medicine and have huge therapeutic implications in a variety of human diseases.

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