J. Biomedical Science and Engineering, 2016, 9, 177-190
Published Online March 2016 in SciRes. http://www.scirp.org/journal/jbise
http://dx.doi.org/10.4236/jbise.2016.94014

Scientific Research Publishing

MicroRNAs as Modulators of Endothelial Differentiation of Stem Cells

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Received 21 September 2015; accepted 26 March 2016; published 29 March 2016

Abstract

MicroRNAs (miRs) are a class of small (~22 nucleotides), widely distributed, and highly conserved non-coding RNA molecules and play an important post-transcriptional regulatory role by targeting mRNA. Embryonic and induced pluripotent stem cells (ESCs and iPSC, respectively) hold great promise for vascular regenerative therapies. However, several limitations currently prohibit their therapeutic use. The importance of miRs in controlling the gene expression profile of a particular cell type is emerging and a multitude of miRs have been identified that play key roles in vascular development and regeneration. A combination of pluripotency transcription factors and different miRs not only enhances the pluripotency of stem cells but also has been reported to enhance their endothelial differentiation. This review will summarize the findings that focus different miR clusters in the induction, maintenance, and directed endothelial differentiation of ESCs and iPSCs.

Keywords

EPCs, ESCs, iPSC, miRs, Vascular Regeneration

1. Introduction

Endothelial cells (ECs) form the innermost lining of the blood vessels and play an active role in the maintenance of vascular integrity and homeostasis through the synthesis and release of numerous vasoactive molecules [1]. In vertebrates, the cardiovascular system is the first functional organ formed during embryonic development. During embryogenesis, first the ECs form a rudimentary vascular meshwork that undergoes a series of developmental stages forming stabilised vessels by the recruitment of mural cells [2]-[4]. During the establishment of functional vascular networks, known as vasculogenesis, a plethora of signalling pathways act to coordinate the development and maintenance of these vascular networks [4]. Loss of EC function leads to development of nu-

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How to cite this paper: Gündüz, D. and Aslam, M. (2016) MicroRNAs as Modulators of Endothelial Differentiation of Stem Cells. *J. Biomedical Science and Engineering*, **9**, 177-190. http://dx.doi.org/10.4236/jbise.2016.94014

merous chronic vascular anomalies. During the last decade, it has become clear that pluripotent stem cells can be directly differentiated toward EC lineage and may potentially be used for the repair of injured vasculature [5]. The understanding of molecular mechanisms regulating EC differentiation will greatly benefit the regenerative strategies for the treatment of various vascular diseases. Numerous methods and factors have been described to programme pluripotent cells to ECs but with limited efficiency [6]. Among these factors, micro RNAs (miRs) which are involved in regulation of at least 30% of the mammalian genes [7], are relatively new players in the field. This review gives a brief over view of various miRs involved in vasculogenesis with potential capacity to induce EC differentiation and their potential to be used in regenerative vascular medicine.

2. MicroRNAs

MiRs belong to class of small (~22 nucleotide; nt), noncoding RNAs controlling gene expression at transcriptional level [8]. miRs are first transcribed as primary miRs (pri-miRs) by RNA polymerase II either from miR genes or intronic sequences of protein coding genes which are then processed into ~70 nt precursors (pre-miRs) and finally to mature miRs by sequential action of two RNase III proteins, Drosha and Dicer, respectively [9] [10]. The mature miRs are incorporated into RNA-induced silencing complex (RISC) consisting of argonaute (AGO) and other RNA binding proteins. The RISC identifies its target mRNA sequences (usually on 3'-UTR) via specific 5'-end "seed sequence" (between nt 2 and 7 on 5'-end of miR) and thus translationally repress target proteins [11]. Beyond targeting mRNAs, some RISCs may act directly on the genome via formation of RNA-induced transcriptional silencing (RITS) complex consisting of AGO1 with an associated miR and chromodomain proteins [12]. Upon target recognition, RITS recruits histone methyl-transferases, which modify the histones associated with the target DNA locus [13]. Currently, over 1800 human miRs are annotated in the miRBASE database (www.mirbase.org) [14]. Each mRNA can be targeted by multiple miRs and each miR can target several genes and thus can regulate several signalling pathways simultaneously.

3. Vascular Regeneration

Vascular diseases which include any condition that affects the circulatory system and ranges from diseases of arteries, veins, and lymphatics to blood disorders affecting circulation, such as peripheral artery disease, renal artery disease, carotid artery disease, and peripheral venous disease, are currently the major causes of morbidity in Western world [15]. Progression of the macrovascular diseases ultimately leads to the development of microvascular complications and organ failure. The traditional surgical and non-surgical treatment strategies for vascular disease include opening-up or bypassing the heavily blocked vessel using autologous or synthetic grafts. Though, well-developed, these procedures are inefficient to prevent or reduce target organ damage.

Regenerative medicine is an emerging interdisciplinary field which aims to restore normal functions of the organ through repair, replacement, or regeneration of cells, tissues, or organs that are lost or damaged due to disease, injury, or ageing [16]. In this promising technology, new vascular network can be induced in the impaired tissues by delivering stem or progenitor cells which ultimately could promote vascular regeneration [15] via enhanced angiogenesis and augmented vasculogenesis [17]. The most studied cells for vascular regeneration include embryonic and mesenchymal stem cells (ESCs and MSCs, respectively) along with circulating endothelial progenitor cells (EPCs) [18]. Other cell sources including induced pluripotent stem cells (iPSCs), human amniotic fluid stem cells (hAFSCs) [15] [18] may also be used. Vascular repair is a complex process and requires the mobilisation, chemotaxis, adhesion, proliferation, and differentiation of ESCs or EPCs [19] to vascular cells. During the process of differentiation, ESCs/EPCs transcription factors, signalling networks, and epigenetics undergo a tremendous change [20]-[22]. Increasing evidence suggests an essential role of miRs in the self-renewal and differentiation of ESCs/EPCs, suggesting promising prospects of using miRs in regenerative medicine. Below, we will discuss the recent advances in our understanding of miRs in the induction and maintenance of pluripotent stem cells and their EC differentiation.

4. Angiogenesis and Endothelial Specific miRs

ECs play an important role in vascular development, angiogenesis, and in maintaining the vascular integrity. Recent studies have established an important role of miRs in carrying out these EC functions. Although, a true endothelial-specific miR does not exist, several miRs have been identified which are enriched in ECs and govern angiogenic capacity of ECs. These include proangiogenic (AngiomiRs) miR-126, miR-130, miR-21, and let7

family and anti-angiogenic (anti-AngiomiRs) miR-17/92 cluster and miR-221/222 family [23], which are highly expressed in ECs. The first evidence that miRs play important role in vascular development was shown by Yang *et al*. [24] using *Dicer* knockout mice. Homozygous embryos died between E12.5 and E14.5 due to deregulation of angiogenic factors/signalling leading to impaired vascular development [24]. *In vitro* knockdown of either *Dicer* or *Drosha* negatively regulated miR-let7 and miR-17/92 cluster expression and angiogenesis [25] [26]. The anti-angiogenic effect of Dicer deletion could be corrected by transfection of miR-17/92 cluster [25]. EC-specific Dicer knockdown reduced postnatal angiogenic response to multiple stimuli and impaired wound healing [27].

The first well-characterised miR in ECs is miR-126 which plays an important role in vascular development and angiogenesis during embryogenesis. EC-specific deletion of miR-126 resulted in vascular defects and haemorrhages and is embryonically lethal [28]. Knockdown of miR-126 in zebrafish embryos causes vascular leakage [29]. Selective overexpression of miR-126 in ECs enhances re-endothelisation of injured vessel and inhibited vascular stenosis [30]. Likewise, ectopic expression of miR-126 in placenta enhanced microvascular density and pub survival in rat model of pre-eclampsia [31]. In mature vessels, miR-126 enhances vessel integrity by targeting p85β and thus downstream inhibiting Ang-1 signalling [32].

The miR-17/92 cluster also known as oncomiR-1 is one of the best studied miR clusters and though dysregulation of their expression leads to a variety of abnormalities [33], their role in developmental angiogenesis is controversial and remains elusive. This cluster includes six miRs (miR-17, miR-18a, miR-19a, miR-19b, miR20a, and miR-92a) which are processed from common primary transcript [34]. Early studies reported a proangiogenic effect of miR-17/92 cluster in tumour vasculature [35], however, later studies found that over expression of individual members of the cluster exerts anti-angiogenic effects in cultured ECs [36]. Epigenetically miR-17/92 cluster is controlled by histone deacetylase 9 (HDAC9) and silencing miR-17-20a rescued angiogenic defects produced due to HDAC9 downregulation [37], suggesting an anti-angiogenic role of miR-17/92 cluster. Likewise, overexpression of miR-15a/16 lead to reduced vascularisation in hind limb ischemia model [38], and conversely their knockdown improved angiogenesis and neovascularisation [39]. MiR-130 is highly expressed in ECs and acts as pro-angiogenic miR by targeting two anti-angiogenic transcription factors, homeobox A5 (HOXA5) and growth arrest specific homeobox (GAX) [40]. MiR-21 is involved in divergent pathophysiological processes particularly related to ischemia/reperfusion injury. Short-term hypoxic conditions induce the expression of miR-21 in ECs and ESCs [41], which via VEGF signalling promotes cell survival and their angiogenic capacity. However, its long-term upregulation under certain pathological conditions promotes tissue fibrosis [42]. A schematic overview of different miRs involved in angiogenesis is presented in **Figure 1**.

5. miRs Involved Inpost-Ischemic Collateral Growth

Following a major artery occlusion two types of vascular repair responses are activated in the injured ischemic tissue: shear-stress sensitive arteriogenesis or development of collateral arteries from pre-existing arterioles and

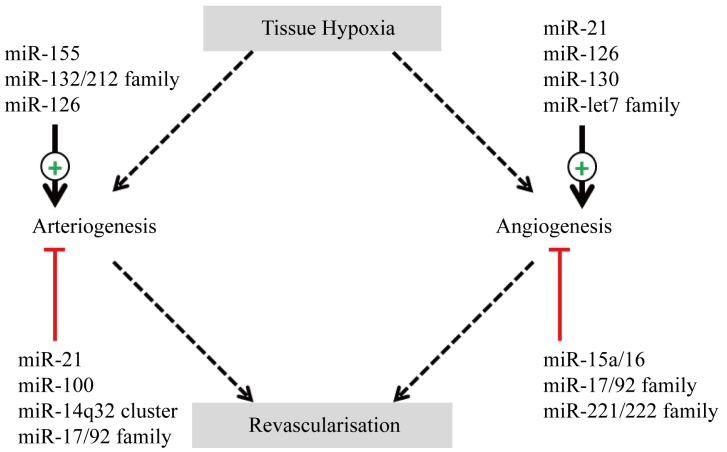

Figure 1. A graphical presentation of different miRs involved in the regulation of vasculaogenesis.

parenchymal hypoxia-driven angiogenesis or sprouting of capillaries [43]. Recent evidence suggests the importance of miRs for regulating post-ischemic arteriogenesis. Shear stress is one of the strongest inducer of arteriogenesis [43] [44] and miR-21 is upregulated in response to high shear-stress [45] which regulates smooth muscle cell proliferation and migration via upregulation of PI3K signalling [46] [47]. MiR-21 is upregulated in myocardium of metabolic syndrome JCR rats leading to enhanced smooth muscle cell (SMC) proliferation but reduced collateral growth, which could be rescued by antagomir miR-21 administration [48], suggesting miR-21 as negative regulator of arteriogenesis. Likewise, endothelial specific deletion of miR-17/92 cluster enhanced collateral growth in cardiac and hind limb ischaemia model suggesting a negative role of miR-17/92 cluster in arteriogenesis [49]. Recent data from Grundmann's group revealed a tissue-specific role of miR-155 in arteriogenesis. In hind-limb ischemia model, it has pro-arteriogenesis role and knockout mice (miR-155$^{-/-}$) presented a reduced vascularisation [50]. In contrast, hearts from miR-155$^{-/-}$ mice are protected against ischaemia/reperfusion injury [51]. Same group also identified miR-100 as a negative regulator of neovascularisation [52] [53] via suppression of mTOR expression both in ECs and SMCs [52] and can be an important target for therapeutic intervention. Likewise, a recent study has identified miR 14q32 cluster (miR-329, miR-487b, miR-494, and miR-495) as negative regulators of arteriogenesis in mouse hind limb ischaemia model [54] and knockdown of two or more of these miRs resulted in enhanced vascularisation. The miR-132/212 cluster has been identified as positive regulator of arteriogenesis which is up-regulated during early period of hind limb ischaemia and miR-132/212 knockout mice show reduced collateral growth after ligation [55]. A schematic overview of different miRs involved in arteriogenesis is presented in **Figure 1**.

6. miRs Regulating Endothelial Progenitor Cell Functions

EPCs are the circulating cells that express different cell surface markers similar to those expressed by ECs, adhere to endothelium at sites of injury, and participate in vasculogenesis [56]. Increasing body of evidence suggests an important role of miRs at different stages of EPCs differentiation to ECs and in their proper functioning.

Proliferation: The miR-221/222 family and miR-21 are reported to act as anti-proliferative miRs in EPCs. The expression of miR-221/222 is upregulated in patients with coronary artery disease (CAD) [57] which is weakly negatively correlated with proliferation of EPCs. Over expression of miR-221/222 family in healthy EPCs resulted in reduction in their proliferation rate [58] [59]. Likewise, the expression of miR-21 is upregulated in EPCs from atherosclerotic patients accompanied by their reduced proliferation and migration [60]. The downregulation of miR-21 rescues this phenotype. Unlike miR-221/222 family and miR-21, miR-126 and miR-130a have been reported as pro-proliferative miRs in EPCs. Both miR-126 and miR-130a are downregulated in EPCs from diabetic patients [61] [62]. Furthermore, overexpression of miR-126 significantly increased the proliferation and migration while knockdown of miR-126 resulted in reduced proliferation of normal EPCs [61].

Senescence: Data about the role of miRs in the regulation of EPC senescence is emerging. Expression of miR-34a was found to be upregulated in EPCs from CAD patients [63], and overexpression of miR-34a in rat EPCs resulted in reduced expression of SIRT1 accompanied by reduced angiogenesis capacity and enhanced cellular senescence of EPCs [64]. The expression of miR-10A* and miR-21 is upregulated in EPCs from aged mice and knockdown of these miRs enhanced the proliferation of aged EPCs [65]. Likewise, miR-22 is upregulated in aged compared to young EPCs and over expression of miR-22 in young EPCs induced senescence in these cells via downregulation of Akt3 [66].

Differentiation: Differentiation of EPCs into mature EC plays an important role in vascular regeneration. Accumulating data suggest the miRs play an important role in differentiation of EPCs to mature ECs during ischaemic diseases. Hypoxia, is an important factor suppressing EC differentiation of progenitor cells [67] via enhanced expression of miR-107 which targets HIF-1β [68], while knockdown of miR-107 promoted differentiation of EPCs into mature ECs. Likewise, downregulation of miR-16 in EPCs enhanced the expression of markers of mature ECs [69]. Shear stress is another important inducer of EC differentiation of EPCs [70] which modulates the expression of several genes via miR-34a. Downregulation of miR-34a resulted in loss of EPCs capacity to differentiate into mature ECs [71] but increased the SMC markers suggesting its role in transdifferentiation of EPCs. Like ECs, miR-126 is highly enriched in EPCs also and is involved in their EC differentiation at several stages. Moreover, the expression level of miR-126 is associated with prognosis of myocardial infarction (MI) patients [72]. MiR-126 controls the EC maturation via targeting PIK3R2 and ectopic expression of miR-126 enhances the angiogenic capacity [73] and vascular repair function of EPCs in deep vein thrombosis

[74]. A graphical presentation of different miRs involved in different stages of EPCs differentiation to ECs is shown in **Figure 2**.

7. ESC Specific miRs Regulating Their Commitment to ECs

Embryonic stem cells (ESCs) are the pluripotent cells that originate from inner cell mass of a mammalian blastocyst and due to their ability to self-renewal and differentiate into various cell types hold the promise of clinical applications [75]. Several studies have identified ESC-specific miRs clusters which are preferentially expressed in ESCs and are downregulated during the process of differentiation [76] [77]. The first evidence that miRs are required for self-renewal and differentiation of ESCs came from the study by Kanellopoulou *et al*. where authors demonstrated ESCs lacking *Dicer* lost the ability to proliferate or form the embryoid bodies [78]. The miR-290/295 cluster accounts for ~50% of the miR contents of mouse ESCs and its expression is downregulated during differentiation [77]. Interestingly, over expression of three miRs (miR-291a-3p, miR-294, and miR-295) from this cluster are sufficient to promote induced pluripotency in somatic cells [79]. The human homolog of the mouse miR-290/295 cluster is miR371-373, specifically expressed in hESCs and is upregulated in some tumours [80] [81]. Second ESC-specific miR cluster is miR-302/367 which is expressed both in human and mouse ESCs [82]. This miR cluster can be used to re-programme fibroblasts into induced pluripotent stem cells (iPSCs) [83]. Interestingly, all these miR clusters possess identical seed sequences (*AAGUGCU*) suggesting that they may be regulating similar pools of mRNAs [84]. During the process of differentiation certain set of miRs is upregulated which target the pluripotency genes Oct4, Sox2, and Nanog, suppressing pluripotency and thus assist ESCs towards differentiation. These include let-7 family of miRs, miR-21, miR-145, miR-134, miR-296, and miR-470 [85]-[87]. Additionally, numerous other miRs have been reported which are differentially expressed during the process of EC differentiation but their role in EC differentiation and vascular development needs to be established. Yoo *et al*., [88] reported two novel miRs, miR-6086 and miR-6087, which target vascular endothelial (VE-)cadherin and endoglin, respectively, and their expression is down regulated during EC differentiation [88]. Another study demonstrated that the expression of miR-99b, miR-181a, and miR-181b was increased in ECs during differentiation [89], which was reconfirmed by another group recently [90]. Interestingly, although over expression of these miRs promotes EC differentiation, their knockdown has no significant impact on EC differentiation of hESCs [89]. Likewise, Treguer *et al*., [91] showed miR-17/92a cluster is upregulated during EC differentiation of ESCs, however, forced knockdown of these miRs in ESCs during the process of differentiation had no effect on their EC differentiation [91]. A recent report shows an upregulation of miR-150 and miR-200c

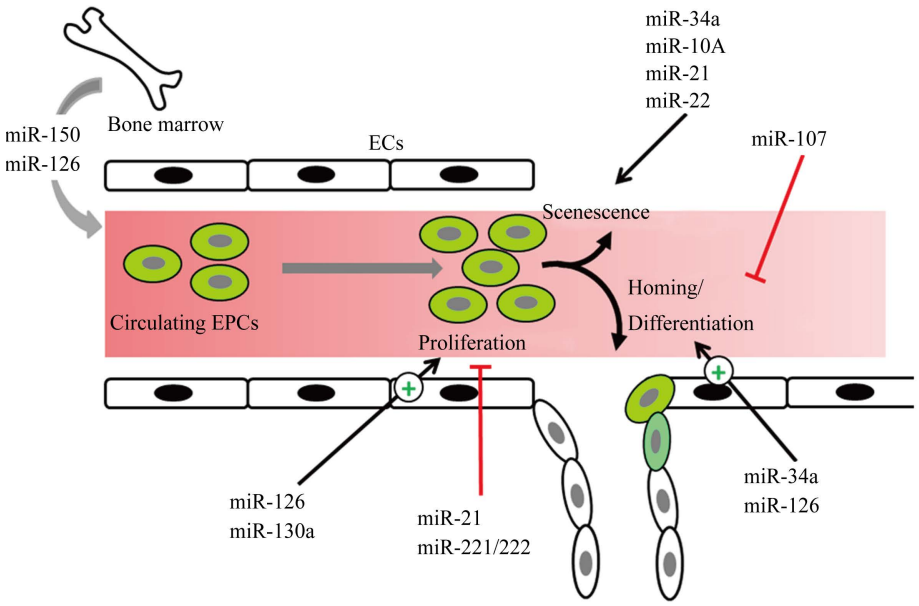

Figure 2. A graphical presentation of different miRs involved in different stages of EPCs differentiation to ECs.

during EC differentiation and antagomirs to these miRs lead to reduced vascularisation in chick embryos [92]. This finding is opposed by another report showing a downregulation of miR-200 family in mESCs during the process of EC differentiation and constitutive expression of miR-200 family members repressed EC differentiation [93]. A careful review of miRs regulating different transcription factors required for EC differentiation of ESCs may give a further insight into the miRs regulating trans-differentiation. For example, in an elegant study, Shi *et al*., [94] have demonstrated that GATA2 together with Etv2 regulates EC and haematopoietic cell differentiation of mESCs. Etv2 controls the vasculogenesis during zebrafish development [95] and its transcription is regulated by let-7 family of miRs. Ectopic expression of let-7 miRs leads to downregulation of Etv2 and vascular defects in zebrafish [96]. Likewise, Gata2 expression in murine cardiac ECs is regulated by miR-24 and its ectopic expression leads to ECs apoptosis [96]. A graphical presentation of different miRs involved in ESCs differentiation to ECs is shown in **Figure 3**.

8. Induced Pluripotent SC-Specific miRs

The clinical application of hESCs is limited by immune rejection and ethical concerns. In this regard, human induced pluripotent stem cells (hiPSCs) have recently emerged as promising alternative [97]. In this method somatic cells can be reprogrammed by forced expression of four pluripotent transcription factors (either Oct4-Sox2-Klf4-c-Myc or Oct4-Sox2-Nanog-Lin28) [98] [99]. Using Dgcr8 (miR processing protein) knockout mouse ESCs (mESCs) Wang *et al*., identified miR-290 cluster as regulators of mESC pluripotency [100]. Interestingly, forced expression of subsets of this miR cluster enhanced reprogramming efficiency of mouse embryonic fibroblasts (MEFs) in the presence of four Yamanaka factors [79] [101]. Likewise, over expression of human orthologue, miR-371 cluster, and miR-302/367 clusters enhanced reprogramming of human fibroblasts by 10 - 15 fold [102]. Later Morrisey and colleagues demonstrated that expression of miR-302/367 cluster alone could successfully induce both mouse and human somatic cells in the presence of HDAC inhibitor without the need of external Yamanaka factors [83]. Recently, Deng *et al*., [103] demonstrated that transfection of miR-302/367 cluster mimetic along with Oct4 and Sox2 could increase the pluripotency efficiency by 50-fold. On the other hand, knockdown of miR-302/367 cluster completely blocked iPSC generation [104] suggesting an important role of miR-302/367 cluster in induction of pluripotency. The increased efficiency by miR-290 (miR-371 human homologue) is mediated by promoting a mesenchymal-to-epithelial (MET) transition, affecting the cell cycle, and inhibiting the TGF-β signalling [101], while miR-302 cluster targets epigenetic regulators responsible for DNA methylation [105]. Some recent reports indicate other miRs may also be contributing to somatic cell reprogramming. Rana and colleagues identified that c-Myc regulated miRs, miR-21 and miR-29a, are highly expressed in MEFs and depletion of these miRs enhanced reprogramming efficiency of MEFs [106]. Likewise, miR-34 deficient MEFs depicted higher reprogramming efficiency [107], suggesting these miRs act as negative

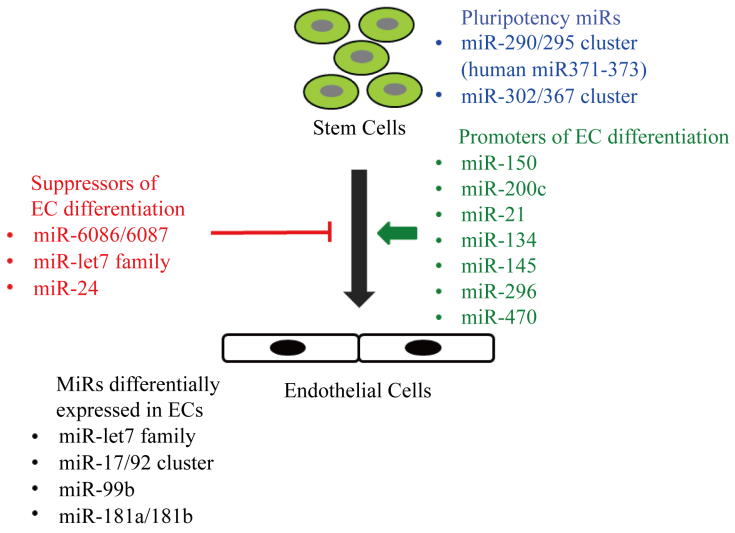

Figure 3. A graphical presentation of different miRs involved in endothelial differentiation of ESCs.

regulators of pluripotency.

The role of miRs in EC differentiation of iPSCs is emerging. As discussed earlier, Rana and colleagues demonstrated that depletion of miR-21 enhanced reprogramming efficiency of MEFs [106] and contrarily, re-expression of miR-21 in iPSCs enhanced their EC differentiation capacity [108], suggesting miR-21 an important candidate for reprogramming of somatic cells into ECs. Recently, Chen and colleagues identified miR-199a and miR-199b as differentially regulated miRs during EC differentiation [109] [110] but their role in EC differentiation needs to be verified. Likewise, Wang *et al*., [90] have performed an extensive miR profiling of hiPSCs and differentiated ECs and identified several differentially regulated miRs including miR-20a, miR-20b, miR-27b, miR-100, miR-125a-5p, miR-137, miR-149, miR-181a, miR-210, miR-222, and miR-296-5p, however, their role in EC differentiation of iPSCs is not verified yet.

9. Future Applications

Recent studies provide new strategies to improve reprogramming process for vascular regeneration. Almost all of these strategies require an over-expression of two or more pluripotent transcription factors. However, miR-induced pluripotency provides a new tool which has the potential to totally substitute for all transcription factors in reprogramming of somatic cells and their EC differentiation. Thus miRs may prove to be a promising and safe strategy to create high quality patient-specific iPSCs. Numerous miR clusters and individual miRs have been identified which have the potential to be used in the induction of pluripotency as well as their EC differentiation. In this regard the miR-290 and miR-302/367 clusters have been identified for the induction and maintenance of pluripotency and miR-21 as promotor of their EC differentiation. A potential clinical avenue is combining the miR modulation with cell-therapy strategies. For example, transplantation of EPCs or pluripotent cells into the site of injury with modulated expression of miRs may promote a faster healing and organ recovery via paracrine mechanisms.

10. Future Research Aspects

As miR-targeted therapies are being established to enter the clinical practice, a detailed understanding of their role in EC differentiation of stem/progenitor cells and their deregulation in cardiovascular disease is required. By detailed understanding of the role played by specific miRs in regulating endogenous vascular repair responses during injury/disease may lead to discovery of novel therapeutic strategies to activate regenerative potential of progenitor cells and thereby promote vascular repair. Although a large number of miRs have been identified which are differentially expressed in pluripotent and ECs, little data are available confirming their role in endothelial differentiation. Therefore, there is still a need to identify specific miRs actually involved in the process of endothelial differentiation.

A big challenge in miR-therapeutics would be the local delivery of specific miR at the site of injury. In this regard a combination of cell-therapy with miR expression can be exploited. Progenitor cells programmed to over express a specific set of miRs may be injected at the site of injury to locally release these endogenous miRs synthesized by these progenitor cells.

Funding

The study was supported by the University of Giessen Anschubsfinanzierung grant to M. Aslam.

Conflict of Interest

None declared.

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Abbreviations

AGO: argonaute
CAD: coronary artery disease
ECs: endothelial cells
ESCs: embryonic stem cells
hAHFSCs: human amniotic fluid stem cells
HDAC9: histone deacetylase 9
HOXA5: homeobox A5
iPSCs: induced pluripotent stem cells
MEFs: mouse embryonic fibroblasts
MET: mesenchymal-to-epithelial
miR: micro RNA
MSCs: mesenchymal stem cells
pri-miRs: primary micro RNA
RISC: RNA-induced silencing complex
RITS: RNA-induced transcriptional silencing