Free Radical Biology and Medicine 64 (2013) 52–60

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

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

Review Article

MicroRNA regulation of endothelial homeostasis and commitment—implications for vascular regeneration strategies using stem cell therapies ☆

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ARTICLE INFO

Available online 9 May 2013

Keywords:
MicroRNA
Embryonic stem cells
Induced pluripotent stem cells
Pluripotency
Vascular endothelial cells
Regenerative medicine
Free radicals

ABSTRACT

Human embryonic (hESC) and induced pluripotent (hiPSC) stem cells have broad therapeutic potential in the treatment of a range of diseases, including those of the vascular system. Both hESCs and hiPSCs have the capacity for indefinite self-renewal, in addition to their ability to differentiate into any adult cell type. These cells could provide a potentially unlimited source of cells for transplantation and, therefore, provide novel treatments, e.g. in the production of endothelial cells for vascular regeneration. MicroRNAs are short, noncoding RNAs that act posttranscriptionally to control gene expression and thereby exert influence over a wide range of cellular processes, including maintenance of pluripotency and differentiation. Expression patterns of these small RNAs are tissue specific, and changes in microRNA levels have often been associated with disease states in humans, including vascular pathologies. Here, we review the roles of microRNAs in endothelial cell function and vascular disease, as well as their role in the differentiation of pluripotent stem cells to the vascular endothelial lineage. Furthermore, we discuss the therapeutic potential of stem cells and how knowledge and manipulation of microRNAs in stem cells may enhance their capacity for vascular regeneration.

Contents

Introduction

Accounting for around 17 million deaths in 2008 (http://www.who.int/gho/ncd/mortality_morbidity/cvd/en/index.html), cardiovascular disease (CVD) is one of the leading causes of mortality worldwide. Current therapies are limited and, therefore, the derivation of new and novel treatments is of great importance. Regenerative medicine is one important area of research for the development of novel treatments, and in recent years, a great deal of focus has been on the use of pluripotent stem cells for the repair and regeneration of diseased and damaged tissues.

Abbreviations: CVD, cardiovascular disease; miRNA, microRNA; EC, endothelial cell; hESC, human embryonic stem cell; mESC, murine embryonic stem cell; iPSC, induced pluripotent stem cell; EPC, endothelial progenitor cell; VEGF, vascular endothelial growth factor; ROS, reactive oxygen species

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http://dx.doi.org/10.1016/j.freeradbiomed.2013.04.037

Pluripotent stem cells have the ability to differentiate into any cell of the three germ layers, mesoderm, endoderm, and ectoderm, thus potentially allowing for generation of any fetal or adult cell type. Human ESCs are derived from the inner cell mass of the blastocyst stage of a developing human embryo. First cultured in 1998 [1], these cells are capable of indefinite self renewal, while maintaining their pluripotency and a normal karyotype. Human ESCs are thought to have great potential as a potentially unlimited source for the derivation of functional, transplantable cells for the treatment of a wide range of diseases. However, therapeutic transplantation of these cells into a patient would be allogeneic, thus carrying the risk of rejection by the recipient, and this source of cells is also considered to be ethically challenging by some. Thus, in recent years, research has focused on identifying populations of autologous pluripotent cells, which may be used in therapeutic transplantation without the risk of rejection, and which are more ethically acceptable. Reprogramming of a patient's own somatic cells, using a combination of defined factors, including Oct3/4, Klf4, Sox2, and c-Myc, as well as Nanog and Lin28, has now been achieved, and these "personalized" pluripotent stem cells are known as induced pluripotent stem cells (iPSCs) [2,3]. The ability of stem cells to maintain pluripotency and differentiate into any adult or fetal cell type is governed by complex interactions between transcription factors, epigenetic changes, signaling pathways, and microRNAs.

Discovered in *Caenorhabditis elegans* [4,5], microRNAs (miRNAs) are noncoding RNAs approximately 22 nucleotides in length. Controlling a number of important cellular functions, miRNAs exert their effects by acting posttranscriptionally to control translation of messenger RNAs (mRNAs) [6], through a binding site in the 3′ untranslated region (UTR) of the mRNA. Before being processed into their mature form, miRNAs are transcribed as primary miRNAs in the nucleus by RNA polymerase II [7]. These primary transcripts then undergo processing within the nucleus by an RNase III-type enzyme known as Drosha [8], along with DiGeorge syndrome critical region gene 8 (DGCR8), a double-stranded DNA-binding protein. This processing results in the formation of pre-miRNAs, miRNA precursors with a stem–loop structure, ~70 nucleotides in length, which are then transported out of the nucleus and into the cytoplasm via exportin 5 [9]. Once in the cytoplasm, pre-miRNAs undergo further processing by Dicer [10], another RNase III-type enzyme, leaving short (~22 nt) double-stranded mature miRNAs. A single strand of this mature form is then incorporated into the RNA-induced silencing complex, which then binds the 3′UTR of a target mRNA, determined by the seed sequence, nucleotides 2–8 in the mature miRNA, thought to control miRNA target specificity. Here, the miRNA acts to control translation in one of two main ways, either inhibition of translation or targeting of the mRNA for degradation [11], depending on the complementarity between the seed sequence of the miRNA and the binding site in the 3′ UTR. Additionally, although unusual, it has also been shown that miRNAs may occasionally act to increase protein levels [12]. It is hypothesized that there are more than 1000 miRNAs in the human genome, with each miRNA working to control the translation of a network of genes. Patterns of miRNA expression levels are generally tissue specific, and dysregulation can lead to cellular dysfunction. Indeed, miRNAs have been implicated in a number of diseases, including cancer [13] and metabolic disease [14], as well as CVD [15].

MiRNAs and the vascular endothelium

Endothelial cells (ECs) play an important role in vascular development during embryogenesis, and are responsible for the maintenance of vascular integrity with respect to angiogenesis and wound repair after vascular injury [16]. Several miRNAs have been identified to play a role in the regulation of function, proliferation, and growth of vascular ECs [17]. These include miR-126, the proangiogenic miR-17–92 cluster, and the antiangiogenic miR-221 and -222. The roles of Dicer and Drosha, the aforementioned main miRNA processing enzymes, have been studied previously in ECs [18,19]. Combined in vitro small interfering RNA silencing of both Dicer and Drosha reduced the sprout-forming and angiogenic properties of ECs [20].

One of the most extensively characterized EC miRNAs is miR-126. Thought to play an important role during embryonic

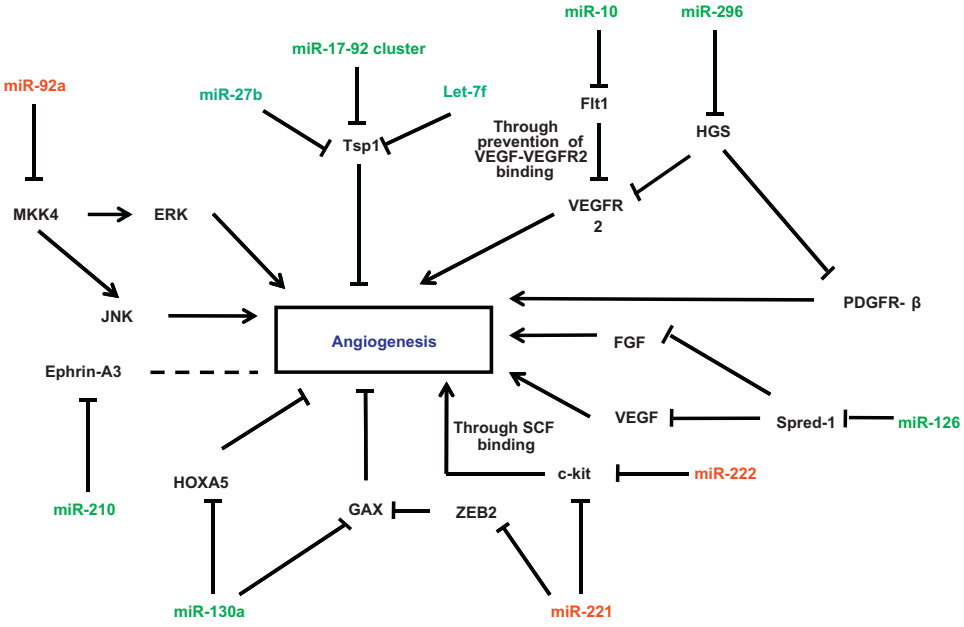

Fig. 1. MicroRNAs (miRNAs) shown to be involved in the regulation of angiogenesis. The schematic shows a number of examples, both pro- and antiangiogenic, and the pathways regulated by individual miRNAs. Antiangiogenic miRNAs are shown in red and proangiogenic miRNAs in green. It is speculated that miR-210 controls the angiogenic response to ischemia via the targeting of ephrin-A3, although more studies are needed for this to be confirmed. Citations to studies can be found in the text. Abbreviations used: VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2; FGF, fibroblast growth factor; PDGFR-β, platelet-derived growth factor receptor-β; HGS, hepatocyte growth factor-regulated tyrosine kinase substrate; MKK4, mitogen-activated protein kinase kinase 4; ERK, extracellular signal-regulated kinases; JNK, c-Jun N-terminal kinases; HOXA5, homeobox A5; GAX, growth arrest-specific homeobox; ZEB2, zinc finger E-box-binding homeobox 2.

development, miR-126 is EC specific and expressed at high levels in the vasculature of both the heart and the lungs [16]. Endothelial-specific deletion of miR-126 in mouse embryos is lethal in ~40% of cases, causing hemorrhaging and rupturing of blood vessels during development. In the surviving miR-126-null mice, EC integrity is severely hampered because of a lack of tight cell–cell interactions. It has also been demonstrated that miR-126 targets the expression of Spred-1. Spred-1 inhibits intracellular angiogenic signaling, consequently promoting the formation of blood vessels by enhancing the proangiogenic properties of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) (Fig. 1). Therefore, miR-126 expression serves to "limit" the antiangiogenic effects of Spred-1. MiR-126 also regulates the expression of vascular cell adhesion molecule 1 (VCAM-1), a critical adhesion molecule, which allows for EC:leukocyte adherence. Reduced expression of miR-126, in tumor necrosis factor-α-stimulated ECs, increases the expression of VCAM-1 and, in turn, enhances leukocyte adherence and, ultimately, vascular inflammation [21]. MiRNA-126 is also expressed in mature vessel ECs, where it targets p85β, a subunit of phosphatidylinositol 3-kinase. This inhibits the function of angiopoietin-1, which plays an important role in vessel stabilization and maturation [22].

The miR-17-92 cluster, containing miR-17, -18a, -19a/b, -20a, and -92a, is expressed in ECs and has been shown to play a role in tumor angiogenesis. p53-null mouse-derived colonic epithelial cells transformed with Kras, and then additionally transduced with c-Myc, produced large tumors, with enhanced neovascularization in vivo [23]. This correlates with a downregulation of thrombospondin-1 (Tsp1), an antiangiogenic factor, and other related proteins, such as connective tissue growth factor (CTGF), both of which are predicted targets of the miR-17-92 cluster. The miR-17-92 cluster is upregulated in these tumors, and knockdown causes partial restoration of Tsp1 and CTGF levels [23]. Tsp1 is also a predicted target of let-7f and miR-27b, two miRNAs whose expression is reduced in endothelial cells after silencing of Dicer and Drosha [20]. MiRNA-130a is also thought to play a proangiogenic role in ECs by inhibiting the expression of GAX, a homeodomain gene, also known as MEOX2, which is implicated in the inhibition of angiogenesis in vascular ECs. It has also been shown to inhibit the function of another antiangiogenic homeobox gene, HOXA5, suggesting that it could be a potential regulator of the angiogenic phenotype of vascular ECs [24]. Taken together, these findings demonstrate that proangiogenic miRNAs, such as the miR-17-92 cluster, let-7f, and miR-27b, promote angiogenesis in vascular ECs via the targeting and subsequent repression of antiangiogenic factors, for example, Tsp1 and GAX (Fig. 1).

Proangiogenic endothelial-associated miRNAs also function by targeting growth factors and their receptors, both directly and indirectly (Fig. 1). For example, as previously mentioned, the angiogenic properties of miR-126 have been well characterized, as it functions to enhance VEGF and FGF signaling via the targeting of Spred-1 [16]. Working in a similar way, via indirect targeting of growth factors, miR-296 targets hepatocyte growth factor-regulated tyrosine kinase substrate (HGS), leading to its suppression [25]. A reduction in the expression of HGS results in a decrease in HGS-mediated degradation of two growth factor receptors: vascular endothelial growth factor receptor 2 (VEGFR2) and platelet-derived growth factor receptor β (PDGFR-β). Another angiogenic miRNA, miR-10, also acts via the promotion of VEGF signaling. In human umbilical vein endothelial cells (HUVECs), it has been shown that miR-10 targets and, therefore, causes a decrease in Flt1 protein expression. Flt1 is thought to sequester VEGF, hence preventing the binding of VEGF to VEGFR2, and thus antagonizing VEGFR2-mediated signaling. Therefore, miR-10 exerts its angiogenic affects via the promotion of VEGFR2-mediated signaling, to control EC proliferation, migration, and adhesion [26]. This suggests that miR-126, -296, and -10 may represent new therapeutic targets in the selective modulation of angiogenesis.

Reduced miR-210 expression has been shown to inhibit EC growth and induce apoptosis, suggesting a proangiogenic role for this miRNA. In hypoxic ECs, it has been shown that miR-210 levels are increased compared to cells under normoxic conditions, leading to changes in cell survival, migration, and differentiation. This effect is thought to be through its direct targeting of the membrane-bound ephrin-A3 [27]. Ephrins bind to Eph receptor tyrosine kinases to exert their effects on a number of signaling pathways within the cell. Previously Eph receptors and their ephrin ligands have been shown to play an important role in the development of the cardiovascular system and vascular remodeling [28] and, therefore, it may be speculated that ephrin-A3 has an important role in the control of angiogenesis. A recent report has further elucidated the role of miR-210 in angiogenesis, by using lentiviral-mediated overexpression of miR-210 in HUVECs. This study demonstrated an induction of capillary formation, due to upregulation of Notch-1 signaling molecules, which facilitate migration of endothelial cells [29,30]. MiR-210, therefore, could also be used as a therapeutic target for the modulation of angiogenesis in the treatment of ischemic diseases. Similarly, a proangiogenic role has also been suggested for miR-378, a miRNA previously studied in a number of cancer cell lines. Like miR-210, it was shown that expression of miR-378 enhanced cell survival and decreased apoptosis, while also causing increased tumorigenesis and angiogenesis [31]. All three of these functions were thought to occur through miR-378 targeting of two different tumor suppressor genes, suppressor of fused (Sufu) [32], whose loss of function causes excessive tumor cell proliferation [33], and tumor suppressor candidate 2 (Fus-1) [34], suggesting a possible therapeutic role for miR-378 in the control and prevention of tumor angiogenesis.

A number of antiangiogenic miRNAs have also been identified and, like those identified as proangiogenic, may also be useful targets allowing for the control of angiogenesis in the treatment of cardiovascular diseases. In HUVECs, high expression of antiangiogenic miRNAs that have been shown to target receptors of a variety of angiogenic factors has been observed (Fig. 1). MiR-221 and -222 target the protein expression of c-kit, the receptor for stem cell factor (SCF), without affecting the mRNA levels. The interaction of these two miRNAs with c-kit modulates the ability of endothelial cells to form new capillaries, consequently regulating the angiogenic activity of SCF, and is also thought to inhibit endothelial cell proliferation and migration [35]. MiRNA-221 and -222 also inhibit the tube formation and wound healing ability of endothelial cells in vitro [35], confirming their antiangiogenic properties. MiRNA-221 also exerts its antiangiogenic effects via a strong upregulation of GAX, a potential master regulator of EC angiogenic phenotype [36], through downregulation of ZEB2, a zinc-finger nuclear factor that primarily acts as a transcriptional repressor [37], resulting in an inhibition of angiogenesis [38]. Like miR-221 and -222, miR-92a has also been shown to have antiangiogenic properties. Inhibition of this miR-92, transcribed as part of the miR-17-92 cluster, enhanced proliferation and migration in rat aortic ECs in vitro and also enhanced reendothelialization in injured rat carotid arteries in vivo [39].

Moreover, miR-15b and -16, along with miR-20a and -20b, are also thought to have antiangiogenic properties, acting through the targeting of VEGF [40]. All four miRNAs were downregulated in carcinoma cells during induction of hypoxia, along with the corresponding hypoxia-induced increase in VEGF expression, allowing for promotion of angiogenesis. As well as VEGF, other angiogenic factors were also found to be targets of all or some of these miRNAs, including COX2, c-MET, and uPAR. Again, these data suggest a possible role for these miRNAs in the treatment and

regulation of angiogenesis in carcinomas. Another miRNA that has been identified as an antiangiogenic modulator is miR-100 [41]. Expressed in both ECs and vascular smooth muscle cells (VSMC), miR-100 was found to be downregulated during induction of hypoxia in vivo and was shown to negatively regulate the levels of mammalian target of rapamycin (mTOR), a gene required for angiogenesis and endothelial cell proliferation in response to hypoxia [42]. Like many of the other miRNAs mentioned, control of miR-100 has therapeutic potential in the treatment of ischemic conditions via the stimulation of new blood vessel growth, as well as in the treatment of other mTOR-dependent processes.

MiR-181a is also thought to play an important role during vascular development. It targets Prox1, a homeobox gene shown to be involved in the development of the lymphatic system [43], in primary lymphatic ECs and reprograms these cells toward a blood vascular phenotype, thereby determining cell fate, a key process during development [44]. The closely related miR-181b is also widely expressed in the vascular endothelium and has been identified as a regulator of NF-κB-mediated vascular inflammation, via targeting of importin-α3. Importin-α3 is involved in the nuclear translocation of NF-κB, and targeting of importin-α3, therefore, leads to the suppression of a set of NF-κB target genes, such as VCAM-1 and E-selectin [45]. These two molecules are involved in adhesion, and suppression leads to a reduction in leukocyte adhesion and vascular inflammation in vivo. This may, therefore, suggest that miR-181b has potential as a target for the control of vascular inflammation.

Dysregulation of miRNAs in endothelial cells

Variations in miRNA expression levels have been implicated in changes in EC phenotype, potentially resulting in vascular dysfunction and ultimately the development of disease. Identification of these miRNAs and the mechanisms by which they exert their effects may lead to the discovery of new targets for the treatment of vascular disease.

Differential expression of subsets of endothelial miRNAs has been observed in athero-susceptible and athero-protected endothelial regions in vivo [46]. Fang et al. [47] hypothesized that miRNAs differentially expressed in these two distinct endothelial phenotypes may work to target Krüppel-like factors 2 and 4 (KLF2 and KLF4), factors that regulate gene networks associated with athero-protective properties. The authors demonstrated that miR-92a suppresses the expression of these two factors, therefore suggesting that miR-92a plays an important role in arterial homeostasis and could be used as a possible target in the treatment and prevention of atherosclerosis.

Caporali et al. [48] demonstrated that miRNAs also play a role in diabetes mellitus-induced endothelial dysfunction. Diabetes mellitus can induce a number of vascular-associated problems, with intracellular hyperglycemia leading to both endothelial dysfunction and microvascular rarefaction. MiR-503 was found to be upregulated in diabetic and ischemic conditions, both in vitro and in vivo. It was also found that overexpression of miR-503, through the targeting of cell cycle-related proteins, resulted in inhibition of EC proliferation and migration. This suggests that miR-503 could be used as a novel therapeutic target in the treatment of patients suffering from peripheral ischemic problems as a result of diabetes mellitus.

Another miRNA reported to play a role in the regulation of vascular disease is miR-320, which inhibits the glucose-induced expression of endothelin-1, VEGF, and fibronectin, genes associated with chronic diabetic complications, in HUVECS. In diabetic patients, therapeutic modulation of this miRNA could potentially be used to control regulation of genes induced by high levels of glucose [49]. High exposure to glucose, in diabetic patients, also reduces the expression of miR-221. Reduction of miR-221 expression results in higher c-kit expression, thus further inducing c-kit-mediated migration and homing of endothelial cells, important in vascular tissue repair and regeneration. Thus, modulation of miR-221 expression, and hence manipulation of the miR-221-c-kit pathway, may be useful in the treatment of vascular dysfunction in diabetic patients [50].

Endothelial progenitor cells (EPCs) remain a relatively poorly defined cell type. Derived from the bone marrow, they are implicated in the repair and maintenance of the vasculature via reendothelialization and neovascularization [51]. Previously, it has been shown that the number of circulating EPCs, as well as their function, decreases in response to risk factors associated with coronary artery disease (CAD), including age, diabetes, and high levels of blood cholesterol; however, the mechanisms behind this are poorly understood. Zhang et al. [52] found that isolated EPCs expressed a number of endothelial cell-specific angiogenesis-associated miRNAs (miR-126, -221, -222, -130a, and -92a), suggesting that this subset may play a role in the function of these cells, particularly in the differentiation of these cells to mature vascular endothelium. When levels of these miRNAs were compared between healthy individuals and those suffering from CAD, it was found that the expression of miR-126 was decreased, whereas there were increases in the levels of miRNAs with antiangiogenic properties, miR-221, -222, and -92a. Thus, dysregulation of endothelial-associated miRNAs in EPCs may contribute to the observed reduction in their regeneration capacity.

MiRNAs in stem cells

Along with transcription factors, signaling pathways, epigenetic changes and other noncoding RNAs, microRNAs form a complex network that acts to control the two defining functions of both hESCs and hiPSCs: their capacity for indefinite self-renewal and ability to differentiate into any adult cell type. In pluripotent cells, there exist pluripotency-regulating miRNAs, whose role it is to inhibit the expression of factors associated with differentiation to allow for continued self-renewal, and differentiation-promoting miRNAs, which target mRNAs associated with pluripotency, and as a result allow cells to differentiate.

Knockdown of Dicer [53,54] and DGCR8 or Drosha [55] in murine ES cells (mESCs) results in severe defects in the differentiation and self-renewal capacities of the cells and, therefore, highlights the importance of miRNAs in both pluripotency and differentiation of embryonic stem cells. In hESCs, knockdown of Drosha or Dicer resulted in a longer cell cycle, as well as an upregulation of a number of pluripotency-associated genes [56], observations that are consistent with miRNAs playing a role in the division and self-renewal of pluripotent stem cells. More specifically, lentivirus-mediated knockdown of Dicer has also been observed to cause significant reduction in the ability of hESCs to commit to the endothelial lineage [57], once again reinforcing the importance of miRNAs in the differentiation of pluripotent stem cells.

Maintenance of pluripotency in ES cells has been shown to be regulated by a core network of stem cell-specific transcription factors, comprising Nanog, Oct3/4, and Sox2, as well as other pluripotency factors, including KLF4, c-Myc, and Lin28 [58]. Together, these factors work to form a positive autoregulatory loop, driving self-renewal and preventing differentiation. MiRNA expression in ES cells is thought to be controlled by these key pluripotency factors, although the mechanism by which this is executed is poorly understood. However, using both mESCs and hESCs, Marson et al. [59] have shown that the core regulatory

factors Oct3/4, Nanog, and Sox2, as well as Tcf3, occupy the promoter regions of miRNAs specifically expressed in ES cells. These key regulators bind to the promoter regions of a subset of miRNAs that are silenced in pluripotent ES cells, owing to the presence of Polycomb group proteins, but become expressed at increasing levels in a tissue-specific manner as cells differentiate and become committed to specific lineages. Occupancy of promoter regions of these miRNAs may allow for cells to be "primed," ready for rapid and efficient differentiation [59].

In recent years, profiling of miRNAs has led to the identification of a subset of miRNAs that are preferentially expressed in ES cells and embryonic tissue [60–62]. A number of ES-specific miRNAs have been identified as having a significant role in the maintenance of pluripotency, including the miR-371 cluster, homologous to the murine miR-290 cluster, the most abundant miRNAs in mESCs [59]; the miR-302 cluster; and the miR-17-92 cluster [63]. These miRNAs were found to have similar seed sequences, suggesting that there exists some overlap between the mRNA targets of these miRNAs, with a number of shared targets being key cell cycle regulators.

The miR-302 cluster, a long transcript containing a number of miRNAs (miR-302b, miR-302c, miR-302a, miR-302d, and miR-367), is expressed specifically in ES cells. Four of the eight miR-302 miRNAs (that is, miR-302a, -b, -c, and -d) are highly homologous in their mature forms, suggesting the existence of a number of shared target mRNAs [64]. When tested in a number of different hESC lines, the miR-302 cluster was shown to be consistently downregulated during early differentiation [65]. Card et al. [66] demonstrated that the transcription factors Nanog, Oct4, and Sox2 bind to the promoter region of the miR-302 cluster in hESCs and that Sox2 and Oct4 were required for transcriptional regulation of miR-302a. Oct4 and miR-302 also work together to regulate the activity of NR2F2 (COUP-TFII), a member of the NR2F/COUP-TF nuclear orphan receptor family of transcription factors, which is transcriptionally activated during differentiation as Oct4 and miR-302 levels decline [67]. This suggests that miR-302 expression levels are important in pluripotency, working in a network alongside well-characterized pluripotency-regulating transcription factors to ensure cells remain in their undifferentiated state.

In ES cells, the cell cycle allows for rapid proliferation, owing to a shortened G1 phase and a higher proportion of cells in S phase [68]. As cells differentiate this profile changes, and cells begin to undergo a cell cycle more similar to that of adult cells, which incorporates a longer G1 phase. MiR-302a, when expressed in primary nonpluripotent cells, was found to decrease the number of cells in the G1 phase coupled with an increase in the number of cells in S phase. Inhibition of this miRNA in pluripotent cells, however, led to an increase in cells entering the G1 phase. The miR-302 family targets a number of cell cycle-regulating proteins, and Card et al. demonstrated that miR-302 acts posttranscriptionally to control the expression of cyclin D1 [66]. As Oct4 and Sox2 activate transcription of miR-302, and miR-302 causes repression of cell cycle-regulating proteins, it may be suggested that there exists a link between key regulators of pluripotency (Oct4/Sox2) and the control of the cell cycle in hESCs. Present in mature ECs, the miR-17-92 cluster is also expressed in pluripotent stem cells, as well as in a number of cancers, in which its expression has been shown to increase in the presence of c-Myc [69]. Smith et al. [70] showed that the miR-17-92 cluster, much like the miR-302 family, controls the cell cycle in pluripotent cells by regulating a number of cell cycle regulators, such as Rb2. The expression of miRNAs in the miR-17 family has also been shown to be upregulated during differentiation of mESCs [71]. MiRNAs, therefore, play a critical role in cell cycle regulation in pluripotent stem cells, a process that is important for their self-renewal—one of the defining features of ES and iPS cells.

A number of critical determinants of pluripotency, Oct4, KLF4, and Sox2, are targets of another miRNA, miR-145 [72]. This miRNA is expressed at low levels in hESCs and is upregulated during differentiation to repress self-renewal capacity and the expression of pluripotency-associated genes. Sox2 is also regulated by miR-126 [73], which also regulates KLF4, and its increased expression represses pluripotency and the self-renewal capacity of human ES cells [74]. These data suggest that these two miRNAs are involved in the transition of the cell from pluripotency toward specification.

Information gained from the study of hESCs has been used to help improve the process of hiPSC generation, something that may be advantageous for autologous stem cell therapies to progress to the clinic. Understanding the mechanisms of pluripotency-regulating miRNAs could be important in increasing the efficiency of hiPSC generation. It has recently been found that induction of miR-302 expression, either alone [75] or together with other ESC-associated miRNAs [76], in human and mouse somatic cells enables reprogramming to hiPSCs. This knowledge may lead to improvements in the efficiency of hiPSC generation for stem cell therapies.

MiRNAs in ESC–EC differentiation

To allow for the differentiation of pluripotent stem cells, factors and networks involved in the maintenance of pluripotency and self-renewal are downregulated, and those involved in differentiation and commitment of cells to specific lineages are upregulated. A number of miRNAs involved in the maintenance and function of vascular ECs, and those that are upregulated during hESC–EC differentiation, have been identified. However, very little is currently known about the contribution of miRNAs to the differentiation of this specialized cell type.

MiR-126, previously identified as a miRNA with a role in endothelial cell structure and function (see earlier in this review), was shown by Kane et al. [77] to be upregulated during hESC–EC differentiation, performed using a feeder- and serum-free protocol. An increase in other endothelial angiogenesis-associated miRNAs, including miR-210, -133a, -133b, -130a, and -296 and let-7b and -7f, accompanied by a decrease in antiangiogenic endothelial miRNAs, including miR-20a, -20b, -221, and -222, was also observed after cells had been differentiated for 10 days, although it was not determined whether any of these miRNAs are specifically involved in the commitment or maturation of hESCs to the endothelial lineage.

In an attempt to identify miRNAs involved in human EC development, Kane et al. [57] further analyzed miRNAs during the early stages of EC commitment from hESCs. From this study, three miRNAs were identified, miR-181a, miR-181b, and miR-99b, all of which are found in adult human ECs. MiR-99b is an intergenic miRNA, which is transcribed in a cluster with both miR-125a and let-7e, thus supporting previous work showing that members of the let-7 family were induced upon EC cell differentiation [77]. All three miRNAs were found to be upregulated in a time- and differentiation-dependent manner. Overexpression of either miR-99b or miR-181a during the differentiation resulted in a significant increase in the percentage of cells expressing both of the endothelial markers Pecam-1 and VE-cadherin on day 10 of the endothelial differentiation. This suggests that these miRNAs can increase hESC–EC differentiation capacity when their levels are manipulated and may specifically be involved in the commitment of pluripotent stem cells to the vascular lineage.

As mentioned previously, the miR-17-92 cluster has also been shown to play a role in endothelial cell function [78]. However, recent studies of mESCs and miPSCs [79] have shown that, although changes in the expression of the cluster occur, none of

the members of this cluster significantly influence the differentiation of pluripotent cells to the endothelial lineage. Knockdown of the miRNAs in the miR-17-92 cluster using antagomirs, either individually or in combination, had no effect on the expression of pluripotency genes, mesoderm-associated genes, or endothelial markers, and the morphology did not appear any different between the antagomir-treated and the untreated cells. Therefore, despite its importance in the regulation of vascular integrity and angiogenesis, this cluster seems to have no effect on ESC–EC differentiation [79] and is therefore more likely to regulate the structure and function of mature ECs, rather than their formation.

As well as ECs, there are number of other important cell types that also govern vascular integrity, including VSMCs. Differentiation from pluripotent stem cell to VSMC is also controlled by a network of miRNAs and genes. This has been previously reviewed by Howard et al. [80], and understanding of this complex process may also be important in the development of new vascular regeneration strategies.

Stem cell and miRNA therapies for vascular regeneration

Treatments to repair damaged vasculature resulting from a range of vascular conditions, including hypertension and ischemia, are currently limited. As discussed, stem cells are thought to have great potential in the field of regenerative medicine and, hence, could provide solutions to the problem of vascular repair and regeneration. It has also been speculated that miRNAs could play an important role in the development and optimization of such treatments, either as novel drug targets and as therapeutics themselves, or as biomarkers, and miRNA profiles indicative of a number of diseases, including cancer [81], coronary artery disease [82], and diabetes [83], have already been identified.

Oxidative stress, redox signaling, and miRNAs in vascular development, regeneration, and ES cells

Reactive oxygen species (ROS) and redox signaling may also play key roles in angiogenesis and vasculogenesis, as well as functioning in stem and progenitor cell differentiation, proliferation, and senescence. Generated through a variety of processes, ROS, such as superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), have previously been shown to act as secondary messengers in cell signal transduction [84]. The effects of ROS on signal transduction-mediated biological events in cells have been shown to be concentration dependent. At low levels, ROS function to mediate both angiogenesis and vasculogenesis [85–87]. Excess concentrations of ROS, however, can be toxic, leading to both EC and stem and progenitor cell senescence and apoptosis [88].

Within endothelial, stem, and progenitor cells, one of the major sources of ROS is NADPH oxidase [89]. NADPH oxidase comprises a number of different subunits, including the catalytic $gp91^{phox}$ subunit, which has several homologues [90]. In ECs the expression of four different homologues has been observed, Nox1, Nox2, Nox4, and Nox5; and in stem and progenitor cells it has been found that only two of these homologues are expressed, Nox2 and Nox4. Activated by a number of angiogenic signaling pathways and cytokines, including VEGF, angiopoetin-1, and G-protein-coupled receptor ligands, e.g. angiotensin II, NADPH oxidase and its derived ROS play key roles in EC angiogenesis [91], as well as controlling proliferation, migration, and differentiation in stem and progenitor cells.

ROS production and the oxidative state have also been shown to be regulated by miRNAs, via the targeting of NADPH oxidase and its subunits [92]. Knockdown of the Dicer processing enzyme resulted in significant impairment of the angiogenic response in human microvascular endothelial cells (HMECs), a phenomenon that could be rescued by the addition of low concentrations of H_2O_2. Dicer-knockdown HMECs also showed lower levels of inducible ROS production, attributed to lower expression of $p47^{phox}$ protein, a subunit of NADPH oxidase. Reduction in global miRNA content induced an increase in the expression of the transcription factor HBP1, which negatively regulates $p47^{phox}$ expression, resulting in reduced redox signaling. It has also been demonstrated that, during experimental diabetic nephropathy, miR-25 may target the Nox4 subunit of NADPH oxidase [93], henceforth regulating the production of $O_2^{\bullet-}$ under these conditions. As well as NADPH oxidase, superoxide dismutase (SOD), an enzyme also responsible for the production of ROS, is also regulated by a miRNA [94]. During cancer, miR-21 targets SOD to regulate ROS production and promote tumorigenesis. These studies, therefore, highlight the importance of miRNAs in the control of redox signaling and their ability to directly control the production of ROS, in both ECs and other cell types, providing us with a novel mechanism of control with great therapeutic potential.

Both stem and progenitor cells have low levels of baseline ROS and, in hESCs, it has been shown that ROS can act to enhance differentiation to the mesendoderm lineage [95]. Stimulation of intracellular ROS generation in ES cells, via the application of either an electrical field [96] or mechanical strain [97], has also been shown to stimulate cardiomyogenesis, as well as vascular sprouting in embryoid bodies, caused through an increase in NADPH oxidase expression, leading to increases in the levels of hypoxia-inducible factor-1α and VEGF, knowledge that could be useful for the application of therapeutic angiogenesis. Differentiating mESCs were also shown to display increased levels of vasculogenesis when stimulated with PDGF-BB, a factor shown to cause an increase in Ca^{2+}-mediated generation of ROS [98].

Indeed ROS may exert their effects through the targeting of miRNAs. Recently, it has been shown that miR-200c, and the cotranscribed miR-141, were upregulated when HUVECs were treated with H_2O_2 in vitro [99]. Overexpression of miR-200c and treatment with H_2O_2 caused HUVEC growth arrest, senescence, and apoptosis, corresponding to an observed downregulation in both the mRNA and the protein level of the miR-200c target ZEB1, an effect that was partially rescued by the inhibition of miR-200c. Inhibition of miR-200c may, therefore, be an important therapeutic target in preventing the negative effects of oxidative stress on cell function and survival.

MiRNA regulation of the oxidative state is a newly recognized mechanism that has great potential therapeutically. Understanding the mechanisms by which ROS act to govern both angiogenesis and postnatal vasculogenesis, via the recruitment of stem and progenitor cells, as well as their interaction with miRNAs, could also have great potential in a clinical setting. Derivation of a method of controlling the levels of ROS, or the activity of NADPH oxidase, could in theory be used in the treatment of a number of angiogenesis-dependent diseases, such as cancer, and for the promotion of angiogenesis in ischemic injury.

Concluding remarks

MicroRNAs are critical regulators of a number of important cellular pathways and processes, and in recent years their roles in governing vascular integrity and the development of vascular dysfunction and CVD have been elucidated. In endothelial cells miRNAs have important roles in both structure and function, and a number of both anti- and proangiogenic miRNAs have been identified. MiRNAs have also been found to play an important role in the self-renewal and differentiation capacities of pluripotent stem cells, a phenomenon that has been shown in a number

of studies. Through their regulation of a wide variety of targets, including transcription factors, miRNAs contribute to the control of the two defining abilities of pluripotent stem cells; however, their role in the commitment of stem cells to endothelial lineages is still relatively poorly understood. Acquiring a better understanding of how miRNAs regulate the commitment of cells to vascular endothelial lineages, as well as their role in the structure and function of mature endothelial cells, may have far-reaching implications in the development of new vascular regeneration strategies, for the repair of damage resulting from ischemic injury. Indeed, modulation of miRNAs identified as playing an important role in the differentiation of hESCs to vascular lineages, as well as those involved in governing vascular integrity, holds promise for a range of clinical applications, including the derivation of more efficient differentiation protocols for the production of large numbers of transplantable cells for vascular regeneration strategies. Despite the ever-growing hope for new treatments that accompanies each new advance in the field of stem cell biology, there are still a number of major problems, which must be overcome before therapies begin to reach the clinic, including safety, immune-specific issues, and survival of transplanted cells [100]. Current focus, therefore, is on the use of stem cells as in vitro models of development and disease, useful for the identification of new therapeutic targets. However, it is hoped that in time many of the problems associated with the use of these cells will be overcome as clinical experience develops, and that such therapies may become more widely tested in diseases with unmet clinical need.

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