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Improving stem cell therapy in cardiovascular diseases: the potential role of microRNA

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1Laboratory of Cellular and Molecular Cardiology, Department of Cardiology, Antwerp University Hospital, Antwerp, Belgium; 2Cardiovascular Diseases, Department of Translational Pathophysiological Research, University of Antwerp, Antwerp, Belgium; 3Department of Nephrology, Antwerp University Hospital, Antwerp, Belgium; 4Laboratory of Experimental Medicine and Pediatrics, University of Antwerp, Antwerp, Belgium; and 5Department of Cardiology, Antwerp University Hospital, Antwerp, Belgium

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Nollet E, Hoymans VY, Van Craenenbroeck AH, Vrints CJ, Van Craenenbroeck EM. Improving stem cell therapy in cardiovascular diseases: the potential role of microRNA. Am J Physiol Heart Circ Physiol 311: H207–H218, 2016. First published May 20, 2016; doi:10.1152/ajpheart.00239.2016.—The initial promising prospect of autologous bone marrow-derived stem cell therapy in the setting of cardiovascular diseases has been overshadowed by functional shortcomings of the stem cell product. As powerful epigenetic regulators of (stem) cell function, microRNAs are valuable targets for novel therapeutic strategies. Indeed, modulation of specific miRNA expression could contribute to improved therapeutic efficacy of stem cell therapy. First, this review elaborates on the functional relevance of miRNA dysregulation in bone marrow-derived progenitor cells in different cardiovascular diseases. Next, we provide a comprehensive overview of the current evidence on the effect of specific miRNA modulation in several types of progenitor cells on cardiac and/or vascular regeneration. By elaborating on the cardioprotective regulation of progenitor cells on cardiac miRNAs, more insight in the underlying mechanisms of stem cell therapy is provided. Finally, some considerations are made regarding the potential of circulating miRNAs as regulators of the miRNA signature of progenitor cells in cardiovascular diseases.

cardiovascular diseases; microRNA; progenitor cells; stem cell therapy

DESPITE THE INITIAL promising prospect of autologous bone marrow-derived cells in the treatment of cardiovascular diseases, stem cell therapy has yielded modest to even absent clinical efficacy in large phase II randomized controlled trials. These disappointing results could be attributed, at least in part, to a poor intrinsic quality of the transplanted autologous bone marrow cells (2, 36, 43, 83). Therefore, strategies leading to an increase in the therapeutic efficacy of the stem cell product are critical before its implementation in clinical practice can even be considered. It is against this background that microRNAs, as epigenetic regulators controlling cell function, have gained substantial attention for improving therapeutic efficacy of stem cell therapy for cardiac and endothelial repair.

MicroRNAs or miRNAs are small, endogenous, noncoding RNAs that negatively regulate gene expression at the posttranscriptional level (21, 60). They are involved in virtually all biological pathways and act as powerful modulators of diverse (patho)physiological processes, including cardiovascular embryogenesis and disease (18, 55, 63, 74). Hence, modulation of the miRNA machinery can be of great interest in the development of therapeutic modalities for cardiac regeneration (55). The possibility of modulating intracellular miRNA expression to improve intrinsic stem cell function, and thus efficiency of autologous stem cell therapy, is widely explored. Up to now, different progenitor cell types have been studied, including the total unselected bone marrow-derived mononuclear cell (BM-MNC) fraction, CD34+ BM-MNC, mesenchymal stem cells (MSC) and endothelial progenitor cells (EPC).

The aim of this review is to provide a comprehensive overview of the current evidence supporting the therapeutic potential of microRNA modulation for improving stem cell therapy in cardiovascular disease (CVD). First, we discuss the functional relevance of miRNA dysregulation in progenitor cells in the setting of different types of CVD. Second, an overview is given on the effects of modulating miRNA expression in progenitor cells on cardiac and/or vascular regeneration. Next, to increase insight into the underlying mechanisms of stem cell therapy, the influence of progenitor cells on the
miRNA expression profile of cardiomyocytes is addressed. To end, we present some general considerations regarding the potential of circulating miRNAs as regulators of the miRNA signature in progenitor cells from patients with CVD.

Noncoding RNAs: Epigenetic Fine Tuners

In addition to chromatin remodeling, posttranslational histone modification and DNA methylation, also regulatory noncoding RNAs are currently widely explored as epigenetic regulators of cellular and molecular functions. Noncoding RNA refers to a group of endogenous RNA that does not encode for proteins, but is involved in the regulation of gene expression. They can be categorized according to their nucleotide length into long (>200 nt) vs. small (<200 nt) noncoding RNA. Small noncoding RNA further comprises microRNA (miRNA), small interfering RNA (siRNA), and piwi-interfering RNA (piRNA) (29). miRNAs represent the leading class of small noncoding RNAs, and to date, most studies discussing the role of noncoding RNA have focused on microRNAs (6). Therefore, the regulating role of microRNAs will be the focus of this current review.

miRNAs negatively regulate gene expression at the posttranscriptional level, either by degradation of target mRNA by targeting its 3’-untranslated region and/or by inhibition of protein translation (21, 63). They are discovered in almost all human biological tissues and body fluids. One miRNA can regulate multiple target miRNAs, and vice versa, one mRNA can be regulated by multiple miRNA molecules (74). Hence, miRNAs can easily interfere in a wide set of molecular pathways. In this regard, it is not surprising that cardiac-specific knockout of the miRNA-processing enzyme Dicer in mice resulted in rapidly progressive cardiac failure and postnatal lethality, which emphasizes the importance of miRNA in cardiovascular development and disease (13). By mapping the miRNA expression patterns and identifying their downstream targets, more insight into the underlying mechanisms of multiple (patho)physiological processes might be obtained.

Stem Cell Therapy in Cardiovascular Disease: A Potpourri of Progenitor Cells

In the majority of the stem cell trials in CVD, the unselected heterogeneous pool of bone marrow-derived mononuclear cells (BM-MNC) has been administered intramyocardially or intracoronarily (2). However, the BM-MNC, including the CD34+ hematopoietic progenitor cells, were shown to be functionally impaired in patients with ischemic cardiomyopathy (43, 77). The methods used for isolating EPC, as well as EPC nomenclature, have been heterogeneous. Based on specific phenotypic and functional characteristics of the cultured cells in vitro, 3 distinct EPC-subtypes are now recognized (73): circulating angiogenic cells (CAC; also called circulating early angiogenic outgrowth cells, EOC; or circulating angiogenic progenitor cells, APC), derived from the adherent peripheral blood MNC fraction after 4–7 days of culture on fibronectin-coated plates in endothelial cell growth medium; second, colony forming units endothelial cells (CFU-EC), which are the nonadherent MNC fraction that give rise to colonies after 7 days; and last, late EPC, grown from the adherent MNC fraction after 6–21 days of cell culture (73).

Dysregulation of miRNAs in Progenitor Cells in Cardiovascular Diseases

Targeting specific miRNAs that are involved in stem cell dysfunction in CVD reveals opportunities for optimizing cell therapy by ex vivo modulation of these miRNAs. Several dysregulated miRNAs are identified in human bone marrow- and peripheral blood-derived progenitor cells in the setting of stable coronary artery disease (CAD), acute coronary syndrome (ACS), and heart failure (HF) (Table 1). Figure 1 outlines the most important intracellular dysregulated miRNAs and corresponding circulating dysregulated miRNAs.

Stable coronary artery disease. In patients with stable coronary artery disease (CAD), numbers and migratory function of circulating endothelial progenitor cells (EPC), defined as CAC and CFU-EC, are significantly reduced, which is related to increased cardiovascular risk and adverse clinical outcome (7, 77). Disruption of at least four processes, orchestrated by specific miRNAs, could be identified so far.

Vascular endothelial growth factor (VEGF) stimulates EPC mitogenesis, growth, mobilization and pro-angiogenic activity (35). Apart from lower serum levels of VEGF in patients with CAD, the VEGF-pathway in these patients is inhibited, as a consequence of the upregulation of several miRNAs (38). Wang et al. (80) analyzed the expression of 18 candidate VEGF-regulating miRNAs in circulating late EPC of 15 patients with CAD. The 18 anti-VEGF miRNA candidates were selected by 2 bioinformatics algorithms out of a database of 204 miRNAs that were previously found to be more abundant in healthy peripheral blood EPC compared with high proliferating cord blood EPC. Among these 18 anti-VEGF miRNAs, the levels of miR-361-5p and miR-484, but also miR-342-3p, miR-125a/b-5, miR-34a-5p, miR-103a-3p and miR-140-5p, were significantly higher in EPC of CAD patients compared with healthy controls. Of these miRNAs, only miR-361-5p and miR-484 were also found to be more abundant in the plasma of CAD patients. Transfection of synthetic miR-361-5p in EPC from healthy controls caused a decrease in intracellular VEGF transcripts, and consequently resulted in EPC with reduced cell proliferative and angiogenic capacities. Moreover, transplantation of EPC isolated from peripheral blood of CAD patients and transfected with anti-miR-361-5p angiomiRNA significantly improved the blood flow recovery after 2 wk in a limb ischemia-induced mouse model (80).
In a small pilot study by Zhang et al. (89), another panel of dysregulated miRNAs modulating EPC (i.e., CAC subtype) function was investigated in 4 patients with CAD compared with 4 healthy controls. The pro-angiogenic miR-126 appeared to be downregulated in EPC from patients with CAD, while the expression levels of miR-221/222 and miR-92a, all negative regulators of angiogenesis, were significantly augmented in CAD-EPC (89). MiR-126 suppresses the angiogenic inhibitors Spred-1 and phosphoinositol 3 kinase regulatory subunit 2 (PIK3R2) (22, 36). In particular, Spred-1 and PIK3R2 are respectively intracellular inhibitors of the Ras/ERK and PI3K/Akt cascades, both pivotal pathways in a wide range of cellular processes such as survival, motility, differentiation and cell cycle (22, 50). In addition, miR-126 increases the release of Table 1. Dysregulated miRNAs in progenitor cells in the setting of CAD, after ACS, and in the setting of HF

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Expression</th>
<th>Cell Type</th>
<th>BM or PB</th>
<th>Associated CVD</th>
<th>Target Gene</th>
<th>Influence on Cell Function</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-361-5p</td>
<td>↓</td>
<td>EPC (i.e., Late EPC)</td>
<td>PB</td>
<td>CAD</td>
<td>VEGF</td>
<td>Angiogenic capacity</td>
<td>80</td>
</tr>
<tr>
<td>miR-484</td>
<td>↑</td>
<td>EPC (i.e., Late EPC)</td>
<td>PB</td>
<td>CAD</td>
<td>VEGF</td>
<td>Angiogenic capacity</td>
<td>80</td>
</tr>
<tr>
<td>miR-342-3p</td>
<td>↑</td>
<td>EPC (i.e., Late EPC)</td>
<td>PB</td>
<td>CAD</td>
<td>VEGF</td>
<td>Angiogenic capacity</td>
<td>80</td>
</tr>
<tr>
<td>miR-125a/b-5</td>
<td>↑</td>
<td>EPC (i.e., Late EPC)</td>
<td>PB</td>
<td>CAD</td>
<td>VEGF</td>
<td>Angiogenic capacity</td>
<td>80</td>
</tr>
<tr>
<td>miR-34a-5p</td>
<td>↑</td>
<td>EPC (i.e., Late EPC)</td>
<td>PB</td>
<td>CAD</td>
<td>VEGF</td>
<td>Angiogenic capacity</td>
<td>80</td>
</tr>
<tr>
<td>miR-103-3p</td>
<td>↑</td>
<td>EPC (i.e., Late EPC)</td>
<td>PB</td>
<td>CAD</td>
<td>VEGF</td>
<td>Angiogenic capacity</td>
<td>80</td>
</tr>
<tr>
<td>miR-140-5p</td>
<td>↑</td>
<td>EPC (i.e., Late EPC)</td>
<td>PB</td>
<td>CAD</td>
<td>VEGF</td>
<td>Angiogenic capacity</td>
<td>80</td>
</tr>
<tr>
<td>miR-126</td>
<td>↓</td>
<td>EPC ((i.e., CAC)</td>
<td>PB</td>
<td>CAD</td>
<td>Spred1-PIK3R2</td>
<td>Angiogenic capacity-</td>
<td>89</td>
</tr>
<tr>
<td>miR-221/222</td>
<td>↑</td>
<td>EPC (i.e., CAC)</td>
<td>PB</td>
<td>CAD</td>
<td>c-kit-DII-4</td>
<td>VEGF and bFGF release</td>
<td>89</td>
</tr>
<tr>
<td>miR-92a</td>
<td>↑</td>
<td>EPC (i.e., CAC)</td>
<td>PB</td>
<td>CAD</td>
<td>eNOS-ITGA5</td>
<td>Angiogenic capacity</td>
<td>89</td>
</tr>
<tr>
<td>miR-21</td>
<td>↑</td>
<td>EPC (i.e., CAC)</td>
<td>PB</td>
<td>CAD</td>
<td>SOD2-SPRY2</td>
<td>Migratory capacity</td>
<td>23</td>
</tr>
<tr>
<td>miR-31</td>
<td>↓</td>
<td>EPC</td>
<td>PB</td>
<td>CAD</td>
<td>FAT atypical cad-4</td>
<td>Angiogenic capacity-</td>
<td>79</td>
</tr>
<tr>
<td>miR-720</td>
<td>↓</td>
<td>EPC</td>
<td>PB</td>
<td>CAD</td>
<td>Vasohibin 1</td>
<td>Angiogenic capacity</td>
<td>79</td>
</tr>
<tr>
<td>miR-150</td>
<td>↓</td>
<td>BM-MNC</td>
<td>BM</td>
<td>ACS</td>
<td>CXCR4</td>
<td>Mobilization Migration</td>
<td>70</td>
</tr>
<tr>
<td>miR-29c</td>
<td>↓</td>
<td>BM-MNC</td>
<td>BM</td>
<td>ACS</td>
<td>SDF-1/H9251</td>
<td>SDF-1a-induced migration</td>
<td>85</td>
</tr>
<tr>
<td>miR-494</td>
<td>↓</td>
<td>BM-MNC</td>
<td>BM</td>
<td>ACS</td>
<td>ISCU1/2</td>
<td>Cell migration</td>
<td>85</td>
</tr>
<tr>
<td>miR-34a</td>
<td>↑</td>
<td>BM-MNC</td>
<td>BM</td>
<td>ACS</td>
<td>Sirt1/Bcl-2</td>
<td>Cyclins/Cyclin-dependent kinases</td>
<td>85</td>
</tr>
<tr>
<td>miR-210</td>
<td>↑</td>
<td>BM-MNC</td>
<td>BM</td>
<td>ACS</td>
<td>ISC1U2/Casp8ap2-</td>
<td>Cell migration</td>
<td>85</td>
</tr>
<tr>
<td>miR-Let 7b/c</td>
<td>↑</td>
<td>BM-MNC</td>
<td>BM</td>
<td>ACS</td>
<td>HMGA2</td>
<td>Self-renewal</td>
<td>85</td>
</tr>
<tr>
<td>miR-1274b</td>
<td>↑</td>
<td>BM-MNC</td>
<td>BM</td>
<td>ACS</td>
<td>Sirt1/Bcl-2</td>
<td>Cyclins/Cyclin-dependent kinases</td>
<td>85</td>
</tr>
<tr>
<td>miR-34a</td>
<td>↑</td>
<td>BM-MNC</td>
<td>BM</td>
<td>ICMP/DCMP</td>
<td>SDF-1a-induced migration</td>
<td>85</td>
<td></td>
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<tr>
<td>miR-1274b</td>
<td>↑</td>
<td>BM-MNC</td>
<td>BM</td>
<td>ICMP/DCMP</td>
<td>Cyclins/Cyclin-dependent kinases</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>miR-126</td>
<td>↓</td>
<td>CD34+/EPC (i.e., CAC)</td>
<td>PB</td>
<td>ICMP</td>
<td>Spred1-PIK3R2</td>
<td>Angiogenic capacity</td>
<td>85</td>
</tr>
<tr>
<td>miR-130a</td>
<td>↓</td>
<td>CD34+/EPC (i.e., CAC)</td>
<td>PB</td>
<td>ICMP</td>
<td>HOXA5/GAX</td>
<td>Angiogenic capacity</td>
<td>36</td>
</tr>
<tr>
<td>miR-20a</td>
<td>↑</td>
<td>EPC (i.e., CAC)</td>
<td>PB</td>
<td>ICMP</td>
<td>VEGF-induced migration</td>
<td>36</td>
<td></td>
</tr>
</tbody>
</table>

This table summarizes the observed dysregulated miRNAs in progenitor cells in bone marrow (BM) and/or peripheral blood (PB) in stable coronary artery disease (CAD), acute coronary syndrome (ACS) and in heart failure (HF), as well as how this miRNA dysregulation affects the cell function. ACS, acute coronary syndrome; Bcl-2, B-cell lymphoma 2; bFGF, basic fibroblast growth factor; BM-MNC, bone marrow-derived mononuclear cell; CAC, circulating angiogenic cell; CAD, coronary artery disease; CHF, chronic heart failure; CVD, cardiovascular disease; eNOS, endothelial nitric oxide synthase; EPC, endothelial progenitor cell; Casp8ap2, Caspase-8-associated protein-2; DCMP, dilated cardiomyopathy; DLL-4, delta like ligand 4; CXCR4, chemokine (CXC motif) receptor 4; GAX, growth arrest-specific homeobox gene; HMGA2, high mobility group AT-hook 2; H9251, high mobility group protein Hox-A5; ICMP, ischemic cardiomyopathy; IL-6, interleukin-6; ISC1U2, iron-sulfur cluster assembly enzyme 1/2; PB, ITGA5, integrin alpha-5; peripheral blood; PIK3R2, phosphoinositide-3-kinase regulatory subunit 2; SDF-1/2, stromal cell-derived factor 1/2; Sirt1, Sir21, Siruin 1; Spred1, Sprouty-related EVH1 domain-containing protein 1; SOD2, superoxide dismutase 2; SPRY2, Sprouty RTK signaling antagonist 2; VEGF, vascular endothelial growth factor.

In a small pilot study by Zhang et al. (89), another panel of dysregulated miRNAs modulating EPC (i.e., CAC subtype) function was investigated in 4 patients with CAD compared with 4 healthy controls. The pro-angiogenic miR-126 appeared to be downregulated in EPC from patients with CAD, while the expression levels of miR-221/222 and miR-92a, all negative regulators of angiogenesis, were significantly augmented in CAD-EPC (89). MiR-126 suppresses the angiogenic inhibitors Spred-1 and phosphoinositid 3 kinase regulatory subunit 2 (PIK3R2) (22, 36). In particular, Spred-1 and PIK3R2 are respectively intracellular inhibitors of the Ras/ERK and PI3K/Akt cascades, both pivotal pathways in a wide range of cellular processes such as survival, motility, differentiation and cell cycle (22, 50). In addition, miR-126 increases the release of

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pro-angiogenic growth factors, such as VEGF and bFGF, and stimulates tubulogenesis by targeting Notch ligand delta-like 4 (DLL-4). DLL-4 has been identified as a negative regulator of VEGF-mediated angiogenic sprouting (67). MiR-221 and -222 control common targets, including stem cell factor (SCF) receptor tyrosine kinase c-kit that mediates VEGF expression and is crucial for cell survival, migration, and capillary tube formation. By targeting eNOS, these miRNAs play a pivotal role in vessel tone regulation and angiogenesis (66, 89). Likewise, miR-92a, a component of the angiogenic miR-17-92 cluster and a known inhibitor of pro-angiogenic proteins such as integrin subunit alpha 5 (ITGA5), also directly targets eNOS (3, 89).

Furthermore, patients with CAD have elevated plasma levels of asymmetrical dimethylarginine (ADMA), an endogenous inhibitor of eNOS and a negative regulator of EPC (i.e., CAC subtype) activity (71). Fleissner et al. (23) reported that incubation of CAC with ADMA (24 h, 1 μmol/l) results in a significant 3-fold upregulation of miR-21. Moreover, ADMA reduced the migratory response towards VEGF/SDF-1 of CAC, by directly targeting superoxide dismutase 2 (SOD2) and Sprouty-2 (SPRY2). SOD2 is involved in oxidative stress defense. SPRY2 inhibits the activation of the ERK1/2 phosphorylation in the ERK/MAPK pathway, which also leads to oxidative stress (23). Oxidative stress is known to cause severe impaired systemic bioavailability of NO, which inhibits the migration capacity of cells. In addition, miR-21 also represses HMGA2 in EPC, which will eventually induce more EPC senescence and reduced self-renewal (93). These in vitro findings mirror the in vivo situation: EPC of patients with CAD have high ADMA levels, significantly increased miR-21 expression, and an impaired CAC migratory response (23).

Finally, the miR-31-miR-720 cluster, essential in the late EPC angiogenic function, appears to be downregulated in patients with CAD, both in plasma and within late EPC (79). Downregulation of miR-31 in healthy EPC hinders the migratory capacity of EPC and their ability to form microvascular tubes, while overexpression of miR-31 in CAD-EPC to a level comparable to healthy EPC rescued this impaired angiogenic activity both in vitro and in vivo. MiR-31 regulates cell function by suppressing FAT atypical cadherin 4 and thromboxane A2 receptor, which are both involved in endothelial cell migration. MiR-720 acts downstream of FAT atypical cadherin 4: four-jointed box 1 (FJX1), necessary for the expression of miR-720 through a HIF-1α subunit-dependent mechanism, is inhibited by FAT atypical cadherin 4 (79). In turn, miR-720 represses vanoshibin 1, an intrinsic and specific negative feedback regulator of angiogenesis identified in endothelial cells (81).

Acute coronary syndrome. In response to an acute coronary syndrome (ACS), bone marrow-derived stem cells are mobilized into the circulation and recruited to ischemic tissue in an attempt to reduce infarct size and improve cardiac function. Interaction between stromal cell-derived factor-1 (SDF-1) and its receptor CXCR4 plays a key role in the migration and mobilization of bone marrow-derived stem cells into the circulation (70). For example, intravenous administration of cardiac stem cells overexpressing CXCR4 (through hypoxic preconditioning) results in enhanced recruitment of these cells into the ischemic heart tissue in a murine model of myocardial infarction (MI), thereby improving left ventricular function, capillary density and reducing infarct size (69). This mobilization is, at least partially, regulated by alterations in the miRNA profile of progenitor cells in ACS: Tano et al. demonstrated a downregulation of miR-150 in BM-MNC in response to LAD ligation in mice, which regulates CXCR4 expression on BM-MNC. Knockdown of miR-150 by treatment with lentiviral miR-150 inhibitor in irradiated mice significantly enhanced the CXCR4 expression on BM-MNC in vivo, resulting in improved mobilization and migration of these cells to the infarcted tissue (70). In addition to miR-150, the
miRNA microarray analysis performed by Tano et al. also revealed downregulated (>4-fold) expression levels of miR-29c, miR-98, miR-195 and miR-494 in BM-MNC at 5 days after MI in mice, whereas the expression of miR-1945, miR-714 and miR-574-5p was upregulated (>4-fold) in BM-MNC compared with BM-MNC from control mice (70). However, the functional impact of these aforementioned miRNAs was not discussed and merits further investigation.

Moreover, some alterations in proapoptotic and senescence-associated miRNA expression levels are reported in progenitor cells of patients with ACS compared with healthy subjects (85). Yet, no functional deficit of progenitor cells has been documented in patients after acute coronary syndrome (62).

Xu et al. performed a microarray to compare the miRNA expression levels in BM-MNC of patients with ACS compared with healthy controls and confirmed their results with RT-PCR. First, pro-apoptotic miR-34a appeared to be significantly increased in BM-MNC of patients with ACS (85). MiR-34a modulates the expression of genes involved in cell cycle and apoptosis. For example, miR-34a inhibits Sirt1 expression, which leads to increased p53 acetylation and expression of p21 and PUMA, transcriptional targets of p53 that regulate cell cycle arrest and apoptosis, respectively, as shown in human cell lines in vitro. In addition, p53 targets miR-34a, which initiates the vicious circle (85, 86). Xu et al. also reported elevated levels of miR-1274b in ACS patients compared with healthy controls, although currently not much is known about its specific impact on cell function (85). In addition, increased expression profiles of Let-7b and let-7c, both members of the let-7 family, were observed in BM-MNC of patients after ACS. Let-7 suppresses the oncogenes HMGA2 and RAS in human adult stem cells, which are both essential in self-renewal, proliferation and stemness maintenance (53, 78, 87). Moreover, increased let-7 expression reduces the release of VEGF and IL-6 (34, 68). In this regard, alterations in let-7 expression may influence stem cell paracrine activity.

Lastly, Xu et al. showed a tendency toward increased expression of miR-210 in BM-MNC of ACS patients (85). MiR-210 is induced by HIF-1α during hypoxia, but is also involved in a positive feedback loop towards HIF-1α via its suppression of glycerol-3-phosphate dehydrogenase 1-like, a negative regulator of HIF-1α stability (11). In endothelial cells, miR-210 disrupts the activity of iron-sulfur cluster assembly proteins (ISCU1/2), which are key players in the mitochondrial metabolism (10). Optimal mitochondrial ATP production is essential for AMPK-dependent migration of endothelial cells, and contributes to re-endothelialization as was shown in a mouse model of endothelial denudation (65). On the other hand, induction of miR-210 positively affects the survival of bone marrow-derived MSC by direct suppression of caspase-8-associated protein-2 (Casp8ap2) (11, 39). However, Xu et al. reported that blocking of miR-210 neither affected BM-MNC survival nor BM-MNC functions in vitro (85).

Chronic heart failure. In the setting of heart failure (HF), and in particular in patients with ischemic cardiomyopathy, bone marrow-derived progenitor cells showed a reduced migration capacity, as well as a diminished potential to differentiate into myeloid and erythroid cell lineages (30, 83). Moreover, in vivo neovascularization capacity in a hindlimb ischemia model was inferior of BM-MNC obtained from patients with heart failure compared with healthy BM-MNC (30). In analogy with CAD, multiple miRNA-based mechanisms could be at play in the functional impairment of progenitor cells in the setting of HF.

Jakob et al. (36) observed a functional relevant loss of angiomiRs miR-126 and miR-130a in circulating EPC (i.e., CAC subtype) and CD34+ cells of patients with ischemic cardiomyopathy. Transfection with anti-miR-126, as well as anti-miR-130a, in healthy EPC impaired their stimulating effects on tube formation and microvessel outgrowth in the aortic ring in vitro, as well as their capacity to improve cardiac function (as assessed by hemodynamic analysis) and cardiac neovascularization (infarct border zone) in a MI mouse model (36). MiR-126 targets SpreD-1 and phosphoinositole 3 kinase regulatory subunit 2 (PIK3R2) as discussed above, while miR-130a targets homeobox genes HOX5 and GAX (22, 50, 57). GAX is responsible for the upregulation of p21, which leads to G0 cell cycle arrest. HOX5 induces p53-dependent apoptosis, downregulates several proangiogenic genes such as VEGFR2, Ephrin A1, HIF-1α and COX2, and stimulates the expression of the anti-angiogenic gene thrombospondin-2 (15, 57).

In contrast to angiomiR-126 and -130a, miR-20a is overexpressed in CACs of patients with ischemic cardiomyopathy (36). MiR-20a, a component of the miR-17-92 cluster, exerts an anti-angiogenic function in endothelial cells (19). In particular, miR-20a targets the pro-apoptotic protein Bim, and Dicer and Jak1, members of the JAK/STAT pathway, which plays an important role in vascular homeostasis. MiR-20a negatively regulates the p38 pathway-mediated VEGF-induced migration of endothelial cells (54).

The earlier discussed miR-34a and miR-1274b were increased in BM-MNC of patients with ACS and appeared to remain dysregulated after the onset of ventricular remodeling. Pro-apoptotic miR-34a levels were increased in both patients with ischemic and dilated cardiomyopathy, whereas miR-1274b was only elevated in BM-MNC of patients with ischemic cardiomyopathy compared with healthy BM-MNC (85).

Interestingly, miR-34a, members of the let-7 family and miR-1274b were highly associated with the age of the patients. The age dependency of let-7bc and miR-34a was also observed in young vs. old mice BM-MNC. These findings highlight the importance of including age in the interpretation of miRNA data (85).

MicroRNA Modulation as Therapeutic Entity

The discovery of miRNAs can be seen as a brand-new lantern that lights the way of novel therapeutic approaches. Selective modulation of miRNA expression profiles, either ex vivo or in vivo, may impact specific cellular pathways, thereby enhancing the efficacy of established cell-based therapies as well as creating new innovative drug discovery platforms (48). Ex vivo manipulation of intracellular miRNA expression is usually accomplished by transfection of miR-mimics/pre-miRs or anti-miRs to cell cultures, thereby obtaining gain-of-function or loss-of-function of the targeted endogenous miRNA, respectively. MiR-mimics are synthetic RNA duplexes designed to mimic the endogenous function of the miRNA of interest, while anti-miRs are chemically modified antisense oligonucleotides harboring the complementary sequence of the target miRNA (76). The strategies for intracellular delivery of
miR mimic and anti-miR compounds vary from direct transfection of the synthetic nucleotides to virus- or plasmid-based delivery (90). Several classes of anti-miRs can be distinguished, based on the variety of chemical modifications used to improve the nucleotide stability, ranging from cholesterol conjugated antago-miRs to the locked nucleic acid (LNA) phosphorothioate chemistry or MOE (2′-O-methoxylumphosphorothioate) modification of oligonucleotides (21, 75). Also ex vivo preconditioning of stem cells by ischemia or reconvoluted growth factors can significantly alter the intracellular miRNA expression signature, resulting in enhanced therapeutic effectiveness after administration of the manipulated stem cell product (27).

In vivo modification of miRNA expression, either with antisense RNA molecules or miR-mimics/pre-miRs, is one of the most widely explored therapeutic approaches of the last decade. Especially the anti-miR therapeutics advanced over time, more than the miR-mimics, with main focus on specificity and stability (48, 76). Based upon convincing animal evidence, several miRNA-targeting drugs entered clinical trial testing. The first proper clinical efficacy in a phase II trial was documented with LNA-modified anti-miR-122, known as Miravirsen, in patients with chronic hepatitis C virus infection. Weekly subcutaneous injections of Miravirsen resulted in long-term suppression of viremia, without any observed adverse events, which advocates Miravirsen as the first available off-the-shelf anti-miR therapeutic drug (46). The greatest challenge will be to predict the exact effects of miRNA modulation, since miRNAs have the ability to modulate entire functional networks, which could sometimes lead to unexpected consequences (20).

miRNAs as Therapeutic Targets for Ex Vivo Stem Cell Modification

Approaches that reverse miRNA dysregulations linked to impaired stem cell function may exert a direct beneficial impact on cardiac and/or vascular regeneration. By modulating the expression of specific miRNAs in progenitor cells ex vivo, it might become possible to augment progenitor cell survival or decelerate its senescence, as well as to enhance paracrine function and differentiation capacity to a cardiomyogenic lineage. Figure 2 illustrates how modulation of specific miRNAs regulates progenitor cell function, and how this could contribute to improved cardiovascular outcomes after autologous stem cell therapy.

Effect on cell survival. The observed low rate of stem/progenitor cell homing and survival is a major limitation for autologous stem cell therapy in patients with CVD (12). In addition, the ischemic region, with local upregulation of inflammatory mediators, creates a hostile environment toward stem cell survival (32). Pretreatment of the stem/progenitor cells by targeting specific survival-associated miRNAs can significantly augment cell survival, and therefore improve clinical outcome.

As earlier discussed, Xu et al. demonstrated an increased expression pattern of several pro-apoptotic and senescence associated miRNAs, such as miR-34a, let-7 family members and miR-210 in BM-MNC from patients after myocardial infarction and/or with heart failure compared with controls (85). Ex vivo knockdown of miR-34a using LNA-34a blocked hydrogen peroxide-induced cell death and increased the SDF-1α-induced migration of treated PB-MNC in vitro (85). Manipulation of miR-34a expression also affected angiogenesis: overexpression of miR-34a by mimic transfection in rat EPC (i.e., CAC subtype) impaired the in vitro tube formation and induced senescence via suppression of Sirt 1 (91). In vivo intramyocardial injection of LNA-34a treated BM-MNC in a MI mouse model by permanent LAD-ligation resulted in improved cardiac function by means of increased wall motion, fractional shortening and ejection fraction after 2 wk follow-up compared with nontreated control BM-MNC (85).

In contrast, upregulation of miR-210 in rat BM-MSC by ischemic preconditioning or nanoparticle-based plasmid transfection induced higher in vitro survival rate under anoxia through HIF-1α-mediated inhibition of Casp8ap2 (40). MiR-210 transfection also promotes the survival of MSC under oxidative stress conditions. In a study by Xu et al. overexpression of miR-210 in rat MSC exposed to H2O2 was evaluated. Transfection with miR-210 suppressed apoptosis and ROS production and led to an increase in SOD activity. The mechan-ism involved activation of c-Met, an upstream factor of the PI3K/Akt pathway, which is pivotal for cell survival (84).

In an in vivo MI rat model, the enhanced survival posten-graftment of these pretreated MSC with miR-210 after intramyocardial administration at multiple sites in and around the infarcted zone prevented infarct size expansion and successfully preserved the left ventricular function after 4 wk follow up. This cardioprotective effect was, at least partially, accomplished by the direct transfer of miR-210 from pretreated MSC to cardiomyocytes through gap junctions, which enhanced the cell survival of cardiomyocytes (40).

In addition to miR-210, overexpression of miR-21 and miR-23a benefits survival of hypoxia-exposed rat MSC in vitro (52). MiR-23a inhibits TNF-α induced apoptosis of MSC in vivo by targeting caspase-7, which has a direct effect on cardiac regeneration by means of left ventricular function and infarct size at 4 wk after injection of these TNF-α-insensitive MSC in the border and center of the infarcted tissue of a MI rat model (49).

Moreover, let-7b, miR-133a and miR-cluster 106b-25 can also be listed as survival-improving targets in MSC to increase the efficacy of stem cell transplantation on cardiac and vascular regeneration (17, 28, 61). MiR-133a directly targets Apaf-1, which is accompanied with downregulation of the pro-apoptotic genes caspase-9 and caspase-3. The higher survival rate of miR-133a mimic transfected MSC enables better cell engraftment in the infarcted zone after intramyocardial injection in rats after LAD ligation, which benefits LVEF and reduces cardiac fibrosis by means of infarct size and LV wall thickness (17). In turn, transfection of synthesized Let-7b in human MSC, which also regulates caspase-3, not only has a positive effect on left ventricular function, but also stimulates neovascularization as assessed by microvessel density in infarcted rats compared with nontreated MSC after intramyocardial administration (28).

Lastly, also miR-126 overexpression prolongs MSC survival by targeting the Akt/ERK-related pathway. Six weeks after intramyocardial transplantation in infarcted mice, miR-126 pretreated MSC improved angiogenesis, as well as cardiac contractile function (14).
Effect on paracrine activity. The exact mechanism by which autologous stem cells improve left ventricular function and perfusion is unclear, but growing evidence is bending toward a paracrine effect of stem cells in ischemic tissue by stimulation of angiogenesis, inhibition of endothelial cell and cardiomyocyte apoptosis, activation of cardiac stem cells and recruitment of additional stem/progenitor cells (72).

The possibility of modulating specific miRNAs to further enhance the stem/progenitor cell secretome has been addressed in several studies. Huang et al. demonstrated that by transfecting MSC with miR-126, the release of angiogenic factors VEGF and bFGF is enhanced in vivo, thereby improving postinfarction angiogenesis, tubulogenesis, and left ventricular function in MI mice (31).

In analogy, transfection of human CD34+ cells with miR-377 suppresses the production of proangiogenic proteins, while more antiangiogenic proteins are secreted. Based on this observation, the group of Joladarashi et al. transplanted miR-377 silenced hCD34+ cells in a myocardial ischemia-reperfused mouse model and observed an increase in angiogenesis and contractile function (37). MiR-377 directly targets serine/threonine kinase 35 and suppresses VEGF, which negatively impacts the angiogenic capacities of stem cells (82).

The 106b-25-miR cluster in bone marrow-derived MSC is an important regulator of apoptosis, proliferation, tube formation, and migration towards VEGF, as well as of autocrine release of proangiogenic cytokines under hypoxic conditions. In this regard, overexpression of the 106b-25 cluster in bone marrow-derived stromal cells stimulates cellular paracrine activity, and thereby promotes neovascularization of ischemic tissue in a hindlimb ischemia mice model (61).

Moreover, inhibition of senescence-associated let-7 in bone marrow-derived MSC upregulates IL-6 secretion in vitro (68).
The cardioprotective effect of short-term IL-6 treatment of cardiomyocytes has been shown in vitro: IL-6 induces NO-dependent protection and preserves mitochondrial function in cardiomyocytes (64). Yet, IL-6 is a pleiotropic cytokine and can also cause chronic inflammation and fibrotic disorders at long-term exposure (24).

As mentioned earlier, transfection of healthy late EPC with miR-361-5p reduces VEGF production, proliferation, and microvasculature formation ability. In contrast, knockdown of miR-361-5p in EPC of CAD patients restores these hampered functions, thereby stimulating blood flow recovery in ischemic limbs in mice, which highlights miR-361-5p also as an interesting target to improve the efficacy of autologous stem cell therapy in ischemia (80).

**Induction of cardiomyogenic differentiation.** The paradigm that stem cells can transdifferentiate into cardiomyocytes when injected into the ischemic infarct zone is more and more abandoned. Recent data suggest that regenerated cardiomyocytes are derived through division of the residing cardiomyocytes themselves under hypoxic conditions, rather than true transdifferentiation of stem or progenitor cells (41). Postnatal cell division of adult cardiomyocytes could also be further stimulated by downregulation of the miR-15 family: in vivo inhibition of miR-15 family in neonatal mice using LNA-modified antimiRs induces cardiomyocyte mitotic entry (56).

Despite this paradigm shift, evidence has been accumulating that MSC, but not hematopoietic bone marrow cells, are able to differentiate into multiple cardiac cell lineages in vitro and in vivo (25, 51). Yet, specific requirements have to be met regarding 1) the use of the impure heterogeneous cell population obtained through plastic adherence isolation; 2) ex vivo expansion in medium containing xenogeneic factors; and 3) adequate cell engraftment and cell survival after intramyocardial administration by the current delivery techniques. In addition, the appropriate cardiac environment, as well as the direct stimulus for induction of cardiomyogenic differentiation of MSC, are essential (16). Modulation of miRNA expression could be a valuable tool to stimulate this differentiation process.

In a study by Zhang et al., overexpression of miR-499 in vitro induced differentiation of rat BM-MSC into cardiac myocyte-like cells through activation of the wnt/β-catenin signaling pathway (88). Moreover, inhibition of miR-124 in rat BM-MSC regulated STAT3 expression, which in turn affected the expression of cardiac-specific markers ANP, TNT, α-MHC and GATA-4 in vitro. Upregulation of miR-16 in human MSC promoted G1 phase arrest and expression of GATA4, NKK2-5, MEF2c, which have essential roles in cardiac muscle development and morphogenesis of the heart (9, 47). Differentiation of proliferating cells into specific phenotypes always involves G1 cell cycle arrest. In this regard, Liu and colleagues speculated that G1 phase arrest of human MSC by miR-16 upregulation contributes to their differentiation toward cardiac cells (47).

In addition, overexpression of miR-1a in mouse BM-MSC, cultured in cardiomyogenic differentiation medium, further enhanced the differentiation efficiency and induction time of myocardial cells from MSC in vitro (92).

Finally, transfection of human MSC with muscle-specific miR-133a or pretreatment of MSC with compound 56, which upregulates endogenous miR-133a, both lead to the inhibition of EGFR, thereby promoting cardiogenic differentiation of MSC. Injection of compound 56 pretreated MSC at three sites of the injured myocardial region after myocardial infarction in rats showed increased beneficial effects on cardiac function by means of LVEF, end-systolic volume, and cardiac output after 1 wk compared with the Sham group (45).

**Cardiac miRNAs Influenced by Progenitor Cells**

A cardiac event leads to a change in the miRNA expression profile of progenitor cells, but also of the cardiomyocytes [for review, see (4)]. Release of paracrine factors by the stem cell product has been shown to have a direct impact on cardiac miRNAs, thereby positively influencing the cardiomyocyte survival and function.

In this respect, Ikeshi et al. reported that the MI-induced increase of profibrotic miR-21 in the infarct border zone, as well as of proapoptotic miR-34a in cardiomyocytes in infarcted mice, is abolished after intramyocardial BM-MNC administration in a paracrine manner. Transplanted BM-MNCs release IGF-1, which inhibits cardiac miR-34a processing and consequently blocks cardiomyocyte apoptosis. By inhibiting the IGF-1 signaling in vivo using an IGF-1 antagonist, the beneficial effect of the administered BM-MNCs on wall motion score index and ejection fraction 7 days post-MI was reversed (33). BM-MNC-derived IGF-1 further mediates the upregulation of endogenous IGF-1 in cardiomyocytes in vivo, which also inhibits miR-378 and thereby initiates a feedback loop: high abundance of miR-378 promotes cardiomyocyte apoptosis by directly targeting IGF-1R and consequently inhibiting IGF-1-mediated activation of the downstream Akt signaling cascade (44). Furthermore, intramyocardial delivery of bone marrow-derived progenitor cells in infarcted (diabetic) mice also blocks MI-induced miR-155-mediated profibrosis signaling in cardiac fibroblasts 3 days post-MI through paracrine release of hepatocyte growth factor (HGF). In analogy with IGF-1, administration of neutralizing antibodies against HGF also abolished the beneficial effect of BM-MNC treatment on LVEF (42). Figure 3 illustrates the aforementioned cardiac miRNAs that are modulated by bone marrow-derived progenitor cells and how these affect cardiac function.

Taken together, paracrine regulation of cardiac miRNAs by transplanted bone marrow-derived progenitor cells results in a better cardiac outcome. Hence, by targeting specific endogenous miRNAs in progenitor cells, we can engineer progenitor cells with enhanced secretome (cfr. Supra) that will simultaneously influence the miRNA expression profile of cardiomyocytes after transplantation, which will ultimately benefit therapeutic efficiency.

Next to a paracrine effect, administered BM-MNC may also regulate miRNA expression of cardiomyocytes via gap junctions. Kim et al., for example demonstrated the transfer of miR-210 via gap junctions in cardiomyocytes when cocultured with miR-210 transfected MSC in vitro. Pretreatment of the MSC with heptanol, an inhibitor of the gap junction protein Connexin-26, blocked the miR-210 transfer towards cardiomyocytes (40).
Circulating miRNAs as Potential Regulators of the Progenitor Cell miRNA Signature

Extracellular miRNAs are identified as important signaling molecules in intercellular crosstalk and are transported by so-called miRNA-carriers, including membrane-derived vesicles (exosomes, microparticles and apoptotic bodies), lipoproteins (HDL and LDL), argonaut 2 (AGO2) protein and other ribonucleoprotein complexes (5, 59). Due to their high stability in plasma, circulating miRNAs show much promise as prognostic and diagnostic biomarkers among a wide range of diseases and conditions (8). As such, circulating miRNAs are also implicated in the pathogenesis of a wide range of CVD. Recently, Romaine et al. published a comprehensive overview of circulating miRNAs that are known to be dysregulated in the setting of ACS, CAD, and HF (58). All these altered miRNA levels may affect the progenitor cells in the bone marrow niche, perhaps hampering the progenitor cell function; yet, no evidence regarding this matter has been collected.

Some studies studied miRNA expression simultaneously in plasma and progenitor cells. For example, Wang et al. showed an upregulation of the anti-VEGF miR-361-5p and miR-484 and downregulation of miR-31 in circulating late EPC of patients with CAD, all compromising the angiogenic capacity of EPC. A mirror image was seen in plasma (79, 80). However, whether circulating miRNAs are able to regulate intracellular targets has not been completely elucidated and is still under investigation (18).

Outlook

Autologous stem cell therapy in patients with ischemic heart disease leads to a rather modest improvement of cardiac outcome. Counseling of cell and gene therapy may result into a more successful marriage for the treatment of CVD. miRNAs are powerful regulators of progenitor cell function, and therefore attractive targets for the development of novel cell-based therapeutic modalities. By modulation of specific miRNA expression profiles in progenitor cells, we are able to create high profile “second-generation” cells with increased survival and functional properties, and by that improve the efficiency of autologous stem cell therapy for cardiac and/or vascular regeneration. Still, the ideal miRNA-cocktail has not reached the bar yet. Different combinations of miRNA targets should be tested in different cell types in different cardiovascular settings, with attention for both efficacy and safety.

Encouraging animal studies for improved cell-based therapies by ex vivo miRNA modulation in CVD could set the stage for clinical translation. Several promising miRNAs have been identified and could be valuable candidates for first clinical testing. For example, overexpression of intracellular miR-126 benefits both survival as well as pro-angiogenic function of progenitor cells, thereby enhancing cardiac regeneration and neovascularization after transplantation in mice. Moreover, multiple studies demonstrated the improvement in in vivo therapeutic outcome of progenitor cells in which hypoxia-induced miR-210 expression was blocked, which contributed to higher cell survival. In analogy, ex vivo downregulation of

Fig. 3. Intramyocardial or intracoronary administered bone marrow-derived progenitor cells can influence the cardiac miRNA signature by paracrine secretion or gap junctions, thereby improving cell survival and/or inhibiting fibrosis in the myocardium. HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor 1.
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proapoptotic miR-34a in the stem cell product has proven its therapeutic benefits. Modulation of miR-34 is very up-and-coming and already under clinical investigation. Indeed, the effect of intravenous injection of MRX-34, a first-in-class liposomal miR-34 mimic, for the treatment of primary liver disease needs for further testing and fine-tuning of these new approaches in a clinical setting.

Additionally, the study of intracellular miRNA signatures in both progenitor cells and cardiomyocytes will not only contribute to the improvement of stem cell therapy in CVD, but will also provide more insight in the underlying mechanisms.

In conclusion, the ex vivo modulation of endogenous miRNA expression seems to hold a significant share in the “Wall Street” of new therapeutic modalities for improving the efficacy of autologous stem cell therapy in CVD. However, future research is needed for further testing and fine-tuning of these new approaches in a clinical setting.

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