MicroRNAs for Restenosis and Thrombosis After Vascular Injury

Clarice Gareri, Salvatore De Rosa, Ciro Indolfi

Abstract: Percutaneous revascularization revolutionized the therapy of patients with coronary artery disease. Despite continuous technical advances that substantially improved patients’ outcome after percutaneous revascularization, some issues are still open. In particular, restenosis still represents a challenge, even though it was dramatically reduced with the advent of drug-eluting stents. At the same time, drug-eluting stent thrombosis emerged as a major concern because of incomplete or delayed re-endothelialization after vascular injury. The discovery of microRNAs revealed a previously unknown layer of regulation for several biological processes, increasing our knowledge on the biological mechanisms underlying restenosis and stent thrombosis, revealing novel promising targets for more efficient and selective therapies. The present review summarizes recent experimental and clinical evidence on the role of microRNAs after arterial injury, focusing on practical aspects of their potential therapeutic application for selective inhibition of smooth muscle cell proliferation, enhancement of endothelial regeneration, and inhibition of platelet activation after coronary interventions. Application of circulating microRNAs as potential biomarkers is also discussed. (Circ Res. 2016;118:1170-1184. DOI: 10.1161/CIRCRESAHA.115.308237.)

Key Words: angioplasty ■ microRNA ■ restenosis ■ stent ■ thrombosis ■ vascular remodeling

Restenosis, the development of luminal narrowing at the site of a previous percutaneous coronary intervention (PCI), usually occurs within months after the PCI procedure and represents the response of the vessel to injury. Multiple factors can contribute to increase the rate of restenosis, such as diabetes mellitus, hypertension, smoking, and hypercholesterolemia.1 In the early years, when balloon angioplasty alone (plain old balloon angioplasty) was performed, the restenosis rate was around 40%,2 whereas the introduction of bare metal stents (BMSs) brought about a reduction of the risk of restenosis to 25%,3 although the rate of restenosis may largely vary depending on several anatomic, clinical, or procedural variables. BMSs were designed to provide a mechanical support to the vessel wall, to prevent early recoil, and to cover coronary dissections that were frequently observed in the time of plain old balloon angioplasty.2 Their introduction into clinical practice led to a substantial improvement of the clinical outcome after PCI, propelling the widespread use of percutaneous coronary revascularization.1 However, the use of BMS is limited by a high rate of in-stent restenosis (ISR), mainly caused by uncontrolled neointimal proliferation with progressively lumen narrowing within the treated vessel segment.2,4 The risk for ISR was substantially reduced by the introduction of drug-eluting stents (DESs) into the clinical practice.5 DESs are characterized by the ability to release an antiproliferative drug within a given time window, usually set within the time frame when neointimal proliferation is most probable.6 The establishment of DESs significantly contributed to reduce the incidence of ISR, both in randomized studies and in a real-life context.7–16

However, despite their dramatic impact in terms of reduction of the restenosis rate, DESs also present some dark sides, which are mainly related, on the one hand, to the inflammatory properties of the permanent coating applied on stent struts to allow controlled drug release and, on the other hand, to the negative impact of nonselective antiproliferative drug released on endothelial regeneration, which increases the risk of late and very late stent thrombosis.17

The discovery of microRNAs (miRNAs) revealed a further layer of regulators,18 playing a key role in the fine regulation of several cardiovascular pathophysiologic processes.19–27 For this reason, their role was largely investigated in the last years, both for their potential exploitation as biomarkers and as therapeutic targets.19,21,23,25,28,29 In particular, several aspects of the vascular response to injury are modulated by miRNAs19,30,31 that can also mediate cell-to-cell communication between endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) within the vessel wall and with other cells’ populations, such as monocytes, pericytes, or platelets.32–36 In particular, besides their direct involvement in several aspects of thrombosis, atherosclerosis, and vascular remodeling,37 platelets contain large amounts of miRNAs and largely

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From the Department of Medicine, Duke University, Durham, NC (C.G.); Division of Cardiology, Department of Medical and Surgical Science, “Magna Graecia” University, Catanzaro, Italy (S.D.R., C.I.); and URT-CNRS, Department of Medicine, URT Consiglio Nazionale delle Ricerche, Catanzaro, Italy (C.I.).
Correspondence to Ciro Indolfi, MD, Chief, Department of Medical and Surgical Sciences, and Director, URT Consiglio Nazionale delle Ricerche (CNR), Magna Graecia University, Viale Europa, Catanzaro 88100, Italy, E-mail indolfi@unicz.it
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Nonstandard Abbreviations and Acronyms

| BMS | bare metal stent |
| DES | drug-eluting stent |
| ECs | endothelial cells |
| ISR | in-stent restenosis |
| PCI | percutaneous coronary intervention |
| VSMCs | vascular smooth muscle cells |

contribute to horizontal miRNA transfer to and from other cell types, also by means of microvesicles. The aim of the present review is to summarize laboratory and clinical evidences on the role played by miRNAs in vascular biology, with special focus on their potential clinical impact.

In particular, the role of miRNAs on (1) the mechanisms of restenosis and thrombosis after vascular injury; (2) biomarkers of restenosis; and (3) possible novel therapeutic targets to inhibit neointimal hyperplasia and thrombus formation are discussed in details.

Neointimal Formation as a Target to Prevent Restenosis After Stenting: VSMCs Phenotypic Switch

The response of balloon injury, even in normal arteries, is neointimal proliferation (Figure 1), which is proportional to the degree of injury. Therefore, the key pathophysiological phenomenon responsible of restenosis after BMS is generation of a neointima, which is essentially because of the proliferation of VSMCs and their subsequent abluminal migration. Under physiological conditions, VSMCs are a highly specialized almost quiescent cell population whose main function is to maintain vascular tone, ensuring contractility of the vessel. They present a differentiated status in adult blood vessel, characterized by a low rate of proliferation (=5%) and expression of a peculiar set of contractile proteins (ie, SMMHC [smooth muscle myosin heavy chain], α-SMA [α-smooth muscle actin], Calponin, etc). Nevertheless, VSMCs retain a remarkable plasticity and can easily undergo a phenotypic switch from the highly specialized contractile to a synthetic or proliferative status in response to specific stimuli, with a consequent increase in the rate of proliferation and migration, with a parallel reduction in the expression levels of contractile proteins. Depending on the pathophysiological milieu, VSMCs from the tunica media can assume a synthetic phenotype, hence starting to proliferate and migrate to the vessel interior, causing the lumen reduction. Given the strong pathophysiological impact, the mechanisms underlying VSMCs’ phenotypic switch were extensively studied. In particular, our group demonstrated that the rupture of internal elastic lamina is a key phenomenon in this process, which could explain why PCI is a potent trigger for neointima formation. In line with this hypothesis, uncontrolled neointima formation is not observed when the internal elastic lamina remains intact. Adventitia, the connective tissue surrounding the vessel responsible for the release of key regulators of vessel function, is also implicated in this process. It has been proposed that, under pathological conditions, adventitial progenitor cells can be activated and can undergo changes, resulting in proliferation, differentiation, and migration. In fact, a study performed in a rat model of balloon injury showed that labeled myofibroblasts added to the adventitia are able to migrate within the vessel, passing first into the media and then into the intima after 7 days. Moreover, progenitor cells from the vessel wall have a clonogenic potential similar to that of circulating progenitor cells and could differentiate into smooth muscle cells, as well as into ECs. Given the increasing amount of evidence on the importance of the mitogenic/clonogenic potential during vascular remodeling, the most common pathways gathering mitogenic signals from several transmembrane receptors in VSMCs in response to arterial injury were object of study in our and other laboratories. In particular, we previously demonstrated that the ras-raf-mitogen-activated protein kinases pathway and the cAMP-dependent signaling in activated VSMCs were found to be of key importance in this context.

miRNAs Profiling After Vascular Injury

Several studies contributed to disentangle the differential modulation in the expression levels of different miRNAs in the vessel wall in response to several endogenous or exogenous stimuli. Ji et al profiled 140 miRNAs at 7, 14, and 28 days after vascular injury, revealing that 60 of them are upregulated while 53 downregulated at 7 days. Furthermore, 63 are upregulated and 47 downregulated at 14 days, whereas 55 are upregulated and 47 downregulated at 28 days. The complex modulation observed in the levels of miRNAs suggests that they could play a relevant role in modulating vascular response to injury (Figure 2).

Figure 1. Representative case of arterial restenosis, observed by means of intravascular optical coherence tomography (OCT). The picture shows a human artery with intimal hyperplasia 2 years after PCI with implantation of a coronary scaffold (struts-remnants indicated by yellow arrows). A thick neointimal layer is evident at the adluminal side of the struts-remnants (neointimal area included between the yellow dotted line and the green line).
miRNAs Which Are Upregulated After Vascular Injury

Among the others, the miR-21 is highly expressed in both VSMCs and ECs, suggesting an active role in vascular remodeling. Its expression is increased in human atherosclerotic lesions or after vascular injury.55,56 On the contrary, differentiation of VSMCs induced by culture under serum starvation results in a decrease in its expression levels, suggesting that this miRNA has a proproliferative effect on VSMCs.55 Accordingly, downregulation of miR-21 enhances apoptosis and inhibits cell growth in VSMCs in vitro and after vascular injury in vivo. This effect seems partially mediated by PTEN,55 which is a miR-21 direct target; however, the miRNA also targets indirectly Bcl-2, suggesting that it could exert its effect through the modulation of different pathways.55 Interestingly, similar effects were observed in adventitial fibroblast and myofibroblast through the miR-21/PDCD4/JNK/e-Jun pathway.57 In addition, miR-21 modulates VSMCs proliferation also in human pulmonary arteries. In this latter setting, miR-21 targets several molecules, including bone morphogenetic protein receptor type II, WWP1, YOD1, and SATB1, finally leading to the chronic hypoxia-induced vascular remodeling.58

The involvement of miR-21 in the regulation of many different processes and in several contexts suggests the relevance of its regulatory potential. The miR-146a also raised much interest because it targets the Krüppel-like factor 4 (KLF-4), a key transcription factor implicated in VSMCs phenotypic switch.59,60 Interestingly, miR-146 promotes VSMCs proliferation by targeting KLF-4, which in turn binds miR-146 promoter, forming a feedback loop to regulate each other. In addition, KLF-5 exhibits an opposing effect on miR-146a transcription as a KLF-4 competitor.61

The miR-221/222 cluster is another interesting example because both are highly expressed in both VSMCs and ECs, and their expression is increased on injury in a rat model of angioplasty62; they are clearly involved in the modulation of proliferation, migration, and apoptosis in VSMCs, as well as of ECs.63 Their overexpression results in uncontrolled neointimal growth, whereas their downregulation leads to a 40% reduction of neointima formation.62,63 In agreement with these results, p27 and p57 are direct targets of miR-221/222 upregulated after injury in rat carotid treated with miR-222 inhibitor.62 In contrast to their effect on VSMCs, miR-221/222 upregulation inhibits proliferation of ECs by targeting c-kit.
This paradoxical effect of the overexpression of those miRNAs might be because of the different expression profile of their target genes between VSMCs and ECs.

Another miRNA, which results upregulated after vascular injury, is miR-424 and its rat ortholog miR-322. miR-424 shows a peculiar behavior compared with the other miRNAs involved in the modulation of VSMCs. In fact, despite its levels are increased after injury, it inhibits VSMCs proliferation, directly targeting cyclin D1, a cell cycle–related gene that controls G1/S transition, whereas it indirectly affects VSMCs biology, modulating the calcium-handling proteins stromal interaction molecule-1 and calumenin. Stromal interaction molecule-1 prevents VSMCs proliferation, whereas calumenin binds Ca2+, contrasting SERCA2 (sarco/endoplasmic reticulum Ca2+-ATPase) activities in cardiac myocytes.

Also, the well-known miR-17–92 cluster was recently found to be involved in vascular remodeling. In fact, all cluster members are upregulated in restenotic carotid arteries and target—miR-18 being the only exception—the bone morphogenetic protein receptor type II, finally inhibiting VSMCs proliferation. Given that several Smad-binding elements were found in the miR-17–92 promoter, Smad3 could activate miR-17–92 transcription, thus indirectly downregulating bone morphogenetic protein receptor type II, as already reported for other miRNAs, providing a cross talk between these 2 major regulatory pathways. This mechanism could explain the dual action on VSMCs and ECs described for miR-92a, which significantly prevents neointimal hyperplasia after balloon injury, maybe accelerating reendothelialization process in a KLF-4/mitogen-activated protein kinase kinase 4–dependent manner. The large number of miRNAs that were found to be upregulated both in VSMCs and in ECs after or during vascular injury represents a further confirmation of their importance in pathophysiology of restenosis and arterial thrombosis after injury.

miRNAs Which Are Downregulated After Vascular Injury

Among several miRNAs, miR-143 and miR-145 are the major modulator of smooth muscle cell phenotype. Encoded by the same cluster, their expression is driven by serum response factor, myocardin, and other myocardin-related genes, as well as by the jag-1/Notch axis. They are both necessary to maintain VSMCs differentiation, and their overexpression prevents neointimal hyperplasia. On the contrary, their levels are downregulated after vascular injury or hemodynamic stress and in atherosclerotic vessels. miR-143 and miR-145 cooperate to promote the contractile phenotype by targeting a network of transcription factors: miR-145 directly targets the KLF-5, an inhibitor of myocardin, KLF-4, calmodulin kinase IIβ, and several other factors implicated in serum response factor activity. Conversely, miR-143 exerts its function mainly by targeting Elk-1. The platelet-derived growth factor (PDGF), a key element in mediating vascular response to injury, is seemingly the most important trigger for down-regulation of miR-143 and miR-145, acting through Src and p53, with the net result of promoting VSMCs proliferation and migration. Conversely, the cytokines transforming growth factor-β and bone morphogenetic proteins promote an increase in miR-143 and miR-145 expression levels, leading to KLF-4 downregulation and to the subsequent induction of contractile genes. Similarly, we showed that miR-133 modulates the phenotypic switch. In fact, it targets the stimulating protein-1, resulting in the inhibition of VSMCs proliferation and migration through the serum response factor pathway. Through gain- and loss-of-function experiments, we showed that miR-133 overexpression reduces neointimal hyperplasia, whereas its downregulation leads to an increased cellular growth in rat model of vascular injury.

The miR-195 is another modulator of VSMCs phenotype, which prevents proliferation and migration, as well as the release from VSMCs of proinflammatory biomarkers (ie, IL-6, IL-8, IL-1β). miR-195 overexpression results in a downregulation of the Rho-GTPase Cdc42, FGF1, and cyclin D1, 3 key regulators of cell proliferation and migration. In particular, miR-195 primarily acts directly targeting Cdc42 and FGF1, whereas the effect observed on the cyclin D1 could be direct, as well indirect because cyclin D1 is a downstream effector of Cdc42. We demonstrated that these processes are also regulated by miR-23b (Figure 3), which exerts its inhibitory effect on VSMCs proliferation by targeting different modulators of the phenotypic switch, such as uPA, SMAD3 and FOXO4, thus acting on different—sometimes concurrent—pathways. Of note, other members of the same cluster, miR-24-1 and miR-27b, show the same trend after injury, suggesting that this cluster plays a pivotal role in modulating the phenotypic switch. Recently, our group also demonstrated the involvement of miR-125a-5p in the regulation of VSMCs phenotypic switch. In fact, its expression levels are downregulated in response to PDGF-BB and are inversely correlated to ETS-1 levels. Because the transcription factor ETS-1 is induced by PDGF-BB, we hypothesized that miR-125a-5p mediates, at least in part, PDGF-BB effects on ETS-1 and consequently on downstream genes involved in the phenotypic switch.

Figure 4 reports the most important miRNAs modulating VSMC proliferation and their molecular targets. Altogether, the above described regulatory mechanisms represent a further and important confirmation of the relevant regulatory role played by miRNAs in vascular remodeling.

Endothelial miRNAs That Are Modulated After Injury

One of the major drawbacks of DESs is the nonselective effect of antiproliferative drugs that prevent both VSMCs proliferation and endothelial regeneration. Experimental studies from our laboratory demonstrated that physical exercise increases endothelial regeneration via eNOS stimulation and reduces neointimal formation. However, there are no currently available pharmacological strategies to foster the regeneration of the endothelial layer after stenting. Therefore, new strategies to counteract or even avoid the deleterious effect of endothelial inhibition after DESs implantation are needed. The discovery of miRNAs opened novel perspectives in this challenging puzzle. In fact, miRNAs were shown to be important modulators of human ECs. The miRNA with the highest expression levels in ECs is miR-126. It is involved in the inflammatory
Figure 3. An example of how microRNAs (miRNAs) can modulate restenosis. In particular, this figure shows how the treatment with miR-23b inhibits neointimal hyperplasia after balloon injury. A, Haematoxylin and eosin staining in carotid arteries in normal condition (upper left) or 14 days after balloon injury without additional treatment (upper right) or treated with Ad-GFP 1010 pfu/mL (lower left) or with Ad-miR-23b (lower right). Scale bars indicate 100 mm. B, The bar graph depicts the quantitative results of the morphometric analysis of 8 arterial sections, treated as described for panel A. Injury-induced neointimal area is significantly reduced by the treatment with Ad-miR-23b, which increases intracellular levels of the miR-23b. Data are presented as mean±SD. *P, 0.05 compared with the control group. Modified from laconetti et al.24

response and endothelial dysfunction; this effect is mediated, at least in part, to the repression of Spred-1, an inhibitory signal in VEGF- and FGF-mediated angiogenesis.

Also, miR-126 is involved in the inflammatory response and endothelial dysfunction, targeting vascular cell adhesion molecule 1 and negatively modulating leukocyte adhesion to ECs. Loss-of-function studies demonstrated its essential role in maintaining vascular integrity. In fact, its depletion results in loss of vascular integrity in both zebrafish and mice. Interestingly, miR-126 mediates the production of CXCL12, an anti-apoptotic chemokine that helps maintaining vascular homeostasis after tissue damage. During apoptosis, ECs release miR-126-enriched microvesicles, which are then delivered to adjacent cells, where they exert an inhibitory effect on atherosclerosis, enhancing the endothelial repair capacity by Sca-1+ ECs progenitor cells in a mouse model of atherosclerosis. A similar phenomenon could take place in the heart after an ischemic injury because miR-126 is consumed during its passage through the infarcted myocardium, suggesting its uptake. The miR-92a is of key importance for maintenance of ECs function. It results upregulated after ischemia, whereas its downregulation leads to better functional recovery of ischemic tissue in different mouse models, acting at least in part through downregulation of the integrin α5. In line with these observations, we showed that the functional inhibition of miR-92a enhances ECs proliferation and migration. Interestingly, miR-92a is also involved in the modulation nitric oxide (NO) production in ECs. In particular, its inhibition leads to an increase in NO production by ECs. As already stated in the previous paragraph, NO production is paramount in fostering endothelial integrity and regeneration. In addition, NO plays an important role in restenosis because it inhibits VSMCs proliferation, thus preventing neointimal hyperplasia.

Other known regulators of NO production are miR-221/222, whose overexpression results in downregulation of eNOS in DICER knockdown mice. miR-221/222 also modulate c-kit expression, an important marker of cardiac stem cells, responsible for capillary tube formation of human umbilical vein endothelial cells, as well as for the promotion of ECs migration and survival. A similar mechanism, with inhibition of c-kit driven by miR-221/222, was observed in hematopoietic progenitor cells and in VSMCs. In these cells’ populations, downregulation of c-kit promotes the contractile phenotype by reducing the expression of myocardin. Hence, it seems that miR-221/222 exert different effects on VSMCs and ECs, but their trigger is still unclear. As it is known, alteration of physiological flow conditions can have a substantial impact on ECs (and to a lower extent to VSMCs). Moreover, miR-221/222 are involved in the inflammatory processes underlying atherosclerosis. By targeting Ets-1, they indirectly modulate the expression of numerous inflammatory molecules in ECs, partially contrasting the angiotensin II activation. Negative consequences of an altered flow include endothelial dysfunction, local development of a prothrombotic milieu, and increase in platelet-to-endothelium contact. Interestingly, miRNAs were reported to modulate several aspects of these biological processes. In particular, an increasing number of studies are focusing on miRNAs-mediated regulation between 2 different cell lines. It is well know that miR-143/145 are fundamental in regulating VSMC phenotype. In addition, recent evidences demonstrated the existence of a cross talk between ECs and SMCs mediated by these miRNAs. In particular, it was hypothesized that miR-143/145 could be transferred by line-like membrane tubes which directly connect cells, as well as through the secretion of extracellular vesicles. As described earlier, miRNA-mediated cell-to-cell communication was also described for miR-126, which is enriched in apoptotic bodies derived from ECs, and provides an athero-protective effect. Given the growing evidence linking extracellular vesicles to miRNA transfer between cells, this could represent a promising therapeutic target to interfere with miRNAs-modulated biological processes. Table lists the most relevant miRNAs that modulate VSMCs’ and ECs’ biological response to injury, with their main targets.
Platelets miRNAs Are Involved in Vascular Injury

Platelets are active partaker in several aspects of thrombosis, atherosclerosis, and vascular remodeling. In fact, therapeutic modulation of platelet activity has a major prognostic impact in CVD, especially in the setting of acute coronary syndromes. They contain large amounts of miRNAs, and largely contribute to horizontal miRNAs transfer to and from other cell types, also by means of microvesicles. In addition, some miRNAs are closely related to platelet function, their responsiveness to antiplatelet treatment, and, hence, to stent thrombosis. Therefore, this represents an active area of research as discussed in the next paragraphs.

Cell-to-Cell Cross Talk

Over the last years, it became increasingly evident that miRNAs can be actively exported or up-taken by several cell types. Most important, miRNAs are able to exert their regulatory function on protein expression also in recipient cells after transfer. Transfer of miRNAs from one cell to another mostly occurs by means of membrane vesicles. These can be of different type and size, ranging from exosomes (30–100 nm) to shedding microvesicles (≤1000 nm) or even apoptotic bodies (50–5000 nm). However, also nonvesicle-mediated intercellular transfer of miRNAs was described. Examples include association to macromolecular complexes, such as high-density lipoprotein; or protein complexes, including argonaute-2, or nucleophosmin 1. In recent times, several examples of intercellular transfer of miRNAs involving cells of the vessel wall were reported. For example, miRNAs can be transferred between ECs and VSMCs by means of membrane vesicles. Interestingly, ECs are able to modify intracellular signaling of other cells through apoptotic bodies-mediated transfer of miR-126. In addition, human umbilical vein endothelial cells can release a specific set of miRNAs that can be transferred to VSMCs through microvesicles. Additional reports demonstrated miR-143/145 transfer between ECs and VSMCs through different trafficking networks. A specific cell-to-cell communication network was described for miR-126 through EC-derived apoptotic bodies. Also recently, it was reported that endothelial-derived microparticles can remotely modulate intercellular adhesion molecule 1 (ICAM-1) expression through intercellular transfer of miR-222. Similarly, high-density lipoprotein-mediated transfer of miR-223 can affect ICAM-1 expression in ECs.

Cell-to-cell crosstalk was also reported between cells of the vessel wall and other cell types. In fact, monocytes can influence ECs migration through selective transfer of miR-150. It was recently reported that ECs are able to release and transfer miR-503 to pericytes through microparticle trafficking. This mechanism has important implications for vascular complications of diabetes mellitus. It was shown that platelets are enriched with a large number of miRNAs, which are continuously exchanged between platelets, platelets-derived microparticles, and other cell populations. In fact, platelet and their shedding microparticles were proposed to provide horizontal transfer of biological information through iRNA trafficking. Accordingly, specific miRNAs involved in the modulation of inflammation isolated from platelet-derived microparticles were also found in ECs and other circulating blood cells.

Interaction With Other Noncoding RNAs

Recently, another class of noncoding RNA, long noncoding RNAs, emerged as potential therapeutic targets of cardiovascular diseases. Among the various classes of long noncoding RNAs, circular RNAs are particular interesting, given their regulatory function on protein expression also in recipient cells after transfer. Transfer of miRNAs from one cell to another mostly occurs by means of membrane vesicles. These can be of different type and size, ranging from exosomes (30–100 nm) to shedding microvesicles (≤1000 nm) or even apoptotic bodies (50–5000 nm). However, also nonvesicle-mediated intercellular transfer of miRNAs was described. Examples include association to macromolecular complexes, such as high-density lipoprotein, or protein complexes, including argonaute-2, or nucleophosmin 1. In recent times, several examples of intercellular transfer of miRNAs involving cells of the vessel wall were reported. For example, miRNAs can be transferred between ECs and VSMCs by means of membrane vesicles. Interestingly, ECs are able to modify intracellular signaling of other cells through apoptotic bodies-mediated transfer of miR-126. In addition, human umbilical vein endothelial cells can release a specific set of miRNAs that can be transferred to VSMCs through microvesicles. Additional reports demonstrated miR-143/145 transfer between ECs and VSMCs through different trafficking networks. A specific cell-to-cell communication network was described for miR-126 through EC-derived apoptotic bodies. Also recently, it was reported that endothelial-derived microparticles can remotely modulate intercellular adhesion molecule 1 (ICAM-1) expression through intercellular transfer of miR-222. Similarly, high-density lipoprotein-mediated transfer of miR-223 can affect ICAM-1 expression in ECs.

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Several studies have shown that miRNAs can be found in the circulation in a surprisingly stable form. The unusual stability shown by miRNAs in the bloodstream, together with the observation that they remain stable even during extended storage, boiling, freeze-thaw cycles, and across different pH values, make circulating miRNAs interesting as new potential biomarkers. Similarly, some miRNAs were identified as potential biomarker of intrastent restenosis. A miRNA profile was performed on plasma from ISR patients, non-ISR patients, and healthy controls. Circulating miR-21, miR-143, miR-145, and miR-100 showed a dysregulation in ISR patients compared with non-ISR patients and are closely related to the occurrence of intrastent restenosis after DESs implantation.

Interestingly, a target analysis revealed that the circulating miRNAs that showed a different expression profile in ISR patients as compared with non-ISR patients were involved in the regulation of several proteins that modulate biological processes underlying ISR. These are indeed promising results that could be easily exploited in the next future to risk-stratify patients after PCI, especially after multiple stent implantation or in patients with multivessel disease treated in which a complete revascularization could not be achieved. In those cases, the availability of noninvasive biomarkers with a high resolution for specific biological processes could add a significant incremental value to the clinical armamentarium that is currently available for the short-term follow up of patients treated with PCI, thus potentially reducing the number of candidates to invasive control angiography. Also interestingly, He et al reported that plasma levels of specific miRNAs showed differential modulation in diffuse or focal ISR groups, independently to the incidence of diabetes mellitus in the 2 groups. This specific phenomenon could be of large clinical value in the currently difficult risk-stratification of diabetic patients after PCI.

Because platelets contain large amounts of miRNAs, they have a major impact on the pool of circulating miRNAs and yield a large potential regulatory role on several cellular processes. This could have a major impact on both restenosis and stent thrombosis because platelets play a key role both in triggering aggregation on stent struts and in inducing biological alterations of the vessel wall that in turn facilitate arterial remodeling and luminal thrombosis. Not surprisingly, miRNAs modulate platelet function in several ways, including activation and aggregation. The large clinical potential of platelet-derived miRNAs is well exemplified by the evidence that low plasma levels of miR-223 are a reliable marker of platelet responsiveness to antiplatelet therapy, a key issue in stent thrombosis. Besides circulating platelet-derived miRNAs, these molecules can be also easily measured from platelet pellets. Interestingly, elevated levels of miR-340 and miR-624 were found in platelets from young patients with early noninvasive identification of both restenosis and stent thrombosis before coronary angioplasty and stent implantation are performed. This would have a large clinical impact because those patients could be optimally counseled toward choice of the most appropriate revascularization strategy in the early phases of the process.

### Circulating miRNAs As Biomarkers After Coronary Angioplasty

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<tr>
<th>miRNA</th>
<th>Modulation After Injury</th>
<th>Effect of miRNA Upregulation on Cell Growth</th>
<th>Validated Targets</th>
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<tr>
<td>miR-21</td>
<td>↑</td>
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<td>PTEN,35 BMPR2,36 WWP1,31 YOD1,31 SATB1,58</td>
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<td>↑</td>
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<td>KLF-4,51</td>
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<td>p27, p57,32</td>
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<td>miR-424</td>
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<td>Cyclin D1,64,65, STIM,66; Calumenin 67</td>
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### miRNAs related to VSMCs phenotypic switch

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<td>miR-133</td>
<td>↓</td>
<td>−</td>
<td>Sp1,52</td>
</tr>
<tr>
<td>miR-195</td>
<td>↓</td>
<td>−</td>
<td>Cdc-42,29 FGFI1,29 Cyclin D1,73</td>
</tr>
<tr>
<td>miR-23b</td>
<td>↓</td>
<td>−</td>
<td>FOXO4,24 uPA,24 SMAD3,24</td>
</tr>
<tr>
<td>miR-125</td>
<td>↓</td>
<td>−</td>
<td>Ets-1,73</td>
</tr>
</tbody>
</table>

### miRNAs related to endothelium formation

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Modulation After Injury</th>
<th>Effect of miRNA Upregulation on Cell Growth</th>
<th>Validated Targets</th>
</tr>
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<tr>
<td>miR-126</td>
<td>−</td>
<td>+</td>
<td>Spry1,22 VCAM-1,144</td>
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<tr>
<td>miR-92a</td>
<td>−</td>
<td>+</td>
<td>ITGα6,35,36 KL-4,36 M KK4,36 WW P1, YOD 1,31</td>
</tr>
<tr>
<td>miR-221/222</td>
<td>−</td>
<td>−</td>
<td>C-kit,73,88</td>
</tr>
</tbody>
</table>

BMPR2 indicates bone morphogenetic protein receptor type II; Cdc-42, cell division control protein 42; FOXO4, Forkhead box protein 04; KLF-4, Kruppel-like factor 4; KLF-5, Kruppel-like factor 5; MKK4, mitogen-activated protein kinase kinase 4; PTEN, phosphatase and tensin homolog; SMAD3, small mother against decapentaplegic; STIM, stromal interaction molecule; uPA, urokinase-type plasminogen activator; VCAM-1, vascular cell adhesion molecule 1; and WWP1, NEDD4-like E3 ubiquitin-protein ligase.
strategy. Despite further advances are needed before circulating miRNAs can be effectively measured as disease biomarkers in the clinical setting, several steps forward were already made. In fact, because circulating miRNAs were recognized as promising biomarkers, many efforts were directed toward the development of fast and reliable detection methods. In particular, several diagnostic methods based on fast real-time polymerase chain reaction were developed to be used as a stand-alone technique or in conjunction with technological platforms, such as those using primers on multi-well plates or microfluidic cards exploiting nanotechnologies to prefilter the samples. These latter technologies were also proposed in association with microarrays. Other promising detection methods are those based on enzyme-linked assays or on direct hybridization. However, the most promising techniques are seemingly those developed by means of nanotechnologies. Nanosensors or nanowires, especially if coupled with microfluidic platforms, could provide in the future label-free, cheap, reliable, and fast detection kits.

Challenges and Future Perspectives for Therapeutic Application of miRNAs to Counteract Restenosis and Stent Thrombosis

Being inhibition of endothelial regeneration one major drawback of DES, miRNAs could be an efficient alternative to differentially interfere with VSMCs and ECs. In other words, the attracting possibility is the identification of specific miRNAs as therapeutic targets to contemporarily inhibit VSMCs proliferation and migration, while potentiating endothelial repair after vascular injury.

However, despite their large application potential, the development of therapeutic strategies based on the modulation of specific miRNAs in humans is still tackled by several methodological issues. Although mimicking the effect of specific miRNAs in target cells remains challenging, their inhibition is effective. In fact, specific inhibition of miR-122 was recently tested with excellent results in patients with hepatitis C virus (HCV)-related hepatitis, using a locked nucleic acid–modified antagomir named miravirsen. Because miR-122 is required for the HCV stabilization, its inhibition by means of the specific antagomir strongly counteracts the invasive capacity of the virus. Given the excellent results, miravirsen will be probably the first human therapy based on specific antagonization of a miRNA. In this regard, all miRNAs described to be upregulated in the setting of neointimal hyperplasia are interesting potential therapeutic targets because their inhibition would prevent restenosis after angioplasty. The mir-195 could potential be an effective mean to prevent restenosis because of its role in cell cycle modulation and its anti-inflammatory effects.
Other interesting examples are the miR-92a and miR-221/222, given their differential effects on VSMCs and ECs.\textsuperscript{30,63} On the contrary, novel and more efficient possibilities to mimic or foster the effect of endogenous miRNAs will be probably developed in the near future, given the continuous technical improvements in this field.

**Site-Specific Delivery**

Another major issue with miRNA-based therapeutics, as with many other biological treatments, is the target tissue–specific delivery. In fact, the above cited example with miravirsen represents the exception because injected antagonirs preferentially reach up to the liver, whereas specific delivery to the heart or the vasculature can be much more challenging. On the contrary, examples of systemic delivery of antagonirs or miR mimetics were already described by several groups, both through intravenous injection and intraperitoneal administration, that reported measurable effects on cardiac remodeling\textsuperscript{110} or neoangionesis during limb ischemia.\textsuperscript{86} More recently, McDonald et al recently demonstrated that inhibition of miR-21 attenuated neointimal formation after stenting in mice.\textsuperscript{131} In this regard, interesting approaches were recently proposed to inhibit restenosis.\textsuperscript{131,132}

However, despite the major steps forward moved within the last years, site-specific delivery still remains a major challenge for the development of therapeutic strategies based on miRNAs. Among the most promising strategies for site-specific targeting of therapeutic agents, the use of stents or coronary scaffolds as platforms for local delivery of high concentration of miRNAs-modulating molecules seems attractive. Using this approach, Wang et al used an anti-21-coated stent to reduce ISR.\textsuperscript{133} Alternative strategies for targeted delivery include conjugation to targeting molecules, such as antibodies, peptides, or other molecules, that allow achievement of site-specific delivery despite systemic administration. Using a different approach, Santulli et al, for example, showed that binding of endogenous miR-126–3p to the Ad-p27/126TS might prevent p27 overexpression in ECs but not in VSMCs.\textsuperscript{132} In this latter example, the intrinsic cell specificity of a signal pathway is exploited to warrant the selectivity of the treatment. Recent progresses in biotechnologies led to the development of a wide range of new potential delivery systems. One of the most interesting is represented by microsphere-integrated DES.\textsuperscript{141} These particular DESs are coated by microsphere containing 1 or 2 different anti-restenosis agents, which will be released locally for prolonged time after stent implantation.\textsuperscript{134} Figure 6 schematically depicts the potential use of different miRNA-based agents on vascular scaffolds.

Circulating nanoparticles could represent a different fascinating way to deliver specific miRNAs. Among these, exosomes represent the most important class. It was demonstrated that under different conditions, exosomes are released from cardiac cells, mediating cell-to-cell communication.\textsuperscript{135–138} Another promising class of nanoparticles useful for miRNAs delivery is represented by virus-like particles.\textsuperscript{139} Virus-like particles are monodisperse particles, uniform in size and shape, consisting of the virus capsid devoid of genetic material. Various virus-like particles show different characteristics and conformations, depending on the virus of origin,\textsuperscript{140} which allows optimal selection of the most appropriate virus-like particle for any use. Some recent evidences indicate their potential in miRNAs delivery.\textsuperscript{141,142}

**Potential Limitations and Caveats of the Therapeutic Use of miRNAs**

Because some of the miRNAs of potential therapeutic interest have proangiogenic properties, some concerns were raised on potential induction of tumor angiogenesis as a side effect. It has been previously reported that some miRNAs might have an ambivalent effect that is both a beneficial therapeutic effect in terms of angiogenesis in ischemic tissues and a detrimental promotion of tumor angiogenesis. Examples include the miR-16 that could be involved in the tumorigenesis of multiple myeloma through VEGF-A-mediated angiogenesis.\textsuperscript{143} In addition, downregulation of miR-133a or miR-143/145 was associated with colorectal cancer through modulation of the PI3K/Akt pathway.\textsuperscript{144} A further example is represented by miR-223, which is involved in platelet function as well as in several processes underlying coronary artery diseases, diabetes mellitus, and cerebrovascular diseases, seems to be involved in tumor growth, through inhibition of Akt phosphorylation and IGF-1R expression.\textsuperscript{144}

The miR92a, one of the key players in angiogenesis and vascular remodeling, was shown to inhibit secretion of the tumor suppressor DICKOPF-3 (DKK3) in neuroblastoma.\textsuperscript{145} Also miR-24 was associated to tumorigenesis through different mechanisms.\textsuperscript{146–148} These issues should be taken into account before designing a therapeutic strategy based on the modulation of miRNAs. In fact, specific miRNAs that are known to play a role in tumorigenesis should be avoided. In addition, site-specific targeting could avoid most of the above described side effects of specific miRNA.

It should be pointed out that the VSMCs originate from different cellular lineages, most of them are derived from mesoderm, whereas a subset originates from neural crest.\textsuperscript{149} This difference led to a specific gene expression pattern in embryonic VSMCs, which converge in a unique phenotype in adult vessels,\textsuperscript{150} although it has been proven that some genes can show peculiar expression patterns in different vascular beds.\textsuperscript{151} Moreover, it was also reported that several cell types activate a subset of embryonic genes in response to injury,\textsuperscript{152–154} this could provide new insights about the mechanism by which equal cells in different vessels (ie, aorta, carotid) respond in disparate ways to similar stimuli.

Experimental results reported and discussed in the present article should be interpreted with caution. In particular, the potential application of those findings to human therapy needs further research.

However, it should be pointed out that the previous concerns on possible side effects of miRNAs are referred to systemic high dose administration. The possible side effects could be avoided using local delivery of miR-based agents via polymers, nanotech, and catheter balloons of coated stents. Indeed, toxic agents (ie, taxol, syrolimus, etc) are already used...
in the clinical scenario to prevent restenosis in coated stents without systemic toxic effects.

Currently, late and very late stent thrombosis are among the major limitations of DESs. Although this event is infrequent, stent thrombosis results in an acute coronary syndrome or cardiac death and represents the Achilles’ heel of PCI. The mechanisms of this phenomenon are related to the nonselective antiproliferative effects of the drugs eluted by the stents (ie, taxol, evelorimus, etc). Because no pharmacological agent is available yet that would be able to selectively inhibit smooth muscle cells but not ECs, there is a large interest in alternative strategies to prevent restenosis without exposing patients to stent thrombosis, a rare complication, but often leading to catastrophic clinical events. Several additional steps are still needed before developing an actual clinical treatment.

In other words, the key issues affecting percutaneous coronary revascularization could be summarized as follows: (1) the process of restenosis is limited to a narrow time window after balloon injury (weeks to months); (2) acute stent thrombosis is usually because procedural flaws (ie, dissections, stent underexpansion, etc); late and very stent thrombosis are mainly related to negative effects of drugs on ECs. Hence, a possible approach to reduce stent thrombosis could be a strategy aimed to selectively inhibit smooth muscle cells during the first week after vascular injury (PCI), without affecting the regular growth of the ECs. Furthermore, the association of an agent able to foster re-endothelialization could favor and accelerate the formation of novel endothelium on injured segments.

Conclusions

The introduction of vascular stents improved the arterial revascularization, and more of 2 millions of procedures are performed every year. The introduction of DESs into clinical practice dramatically reduced the restenosis rate observed with BMSs, bringing about a progressive increase in late stent thrombosis. Despite several issues associated with the use of DESs were mitigated by the development of the current generation of DESs, stent thrombosis and late restenosis still remain a major challenge. Even the latest advancement in coronary angioplasty, namely the introduction of bioresorbable scaffolds, did not solve the restenosis neither the dreadful thrombotic issue that might be even increased.

Ideally, percutaneous interventions should remove the stenosis, prevent arterial recoil using a scaffold, and inhibit smooth muscle cell proliferation, while enhancing endothelial regeneration. At present time, there is no such therapeutic strategy.

MiRNAs are useful to better understand the mechanisms underlying neointimal hyperplasia and restenosis, as well as endothelial regeneration and maintenance of vascular integrity. Most important, miRNAs offer promising targets for novel more efficient and selective therapeutic strategies to inhibit smooth muscle cell proliferation and selectively enhance endothelial regeneration after coronary interventions.

Finally, the fact that miRNAs are actively released by virtually all cell types offer the interesting possibility to exploit them as smart biomarkers that can provide fine and detailed biological information on several biological processes.

Conclusions

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References


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MicroRNAs for Restenosis and Thrombosis After Vascular Injury
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