This Review is in a thematic series on MicroRNA in the Cardiovascular System, which includes the following articles:


MicroRNAs in Vascular and Metabolic Disease

MicroRNAs in Vascular and Metabolic Disease

Anna Zampetaki, Manuel Mayr

Abstract: Recent findings demonstrated the importance of microRNAs (miRNAs) in the vasculature and the orchestration of lipid metabolism and glucose homeostasis. MiRNA networks represent an additional layer of regulation for gene expression that absorbs perturbations and ensures the robustness of biological systems. This function is very elegantly demonstrated in cholesterol metabolism where miRNAs reducing cellular cholesterol export are embedded in the very same genes that increase cholesterol synthesis. Often their alteration does not affect normal development but changes under stress conditions and in disease. A detailed understanding of the molecular and cellular mechanisms of miRNA-mediated effects on metabolism and vascular pathophysiology could pave the way for the development of novel diagnostic markers and therapeutic approaches. In the first part of this review, we summarize the role of miRNAs in vascular and metabolic diseases and explore potential confounding effects by platelet miRNAs in preclinical models of cardiovascular disease. In the second part, we discuss experimental strategies for miRNA target identification and the challenges in attributing miRNA effects to specific cell types and single targets. (Circ Res. 2012;110:508-522.)

Key Words: cholesterol homeostasis ■ diabetes ■ microRNA ■ vascular endothelium ■ vascular smooth muscle

MicroRNAs (miRNAs) are small, noncoding RNAs that function as posttranscriptional regulators of gene expression. They are potent modulators of diverse biological processes and pathologies comprising 1–5% of mammalian genes.1,2 Recently, distinct miRNA signatures have been reported in cardiovascular disease3,4 and miRNAs are implicated as targets for therapeutic interventions.5 Intriguingly, miRNAs emerged as key regulators of established cardiovascular risk factors, such as diabetes, hypercholesterolemia, and so forth.6 We review the role of miRNAs in vascular and metabolic diseases and the contribution of -omics technologies to further our understanding of miRNA function and their mechanisms of action.

MiRNAs as Regulators of Metabolic Pathways
Animal models and genetic studies have highlighted the prominent role of miRNAs in the regulation of lipid metabolism (Figure 1). MiRNAs also safeguard insulin expression
and secretion, thereby contributing to the maintenance of glucose homeostasis (Figure 2).

**MiRNAs in Lipid Metabolism**

**MiR-33**

Vertebrates have 2 genes for sterol regulatory element-binding proteins (SREBP): SREBP-1 activates the synthesis of fatty acids whereas SREBP-2 activates the synthesis and uptake of cholesterol.\(^7\,^8\) Both genes encode within their introns miR-33a and miR-33b that regulate cholesterol homeostasis. These miRNAs have a remarkably different evolutionary history. Whereas miR-33a is highly conserved throughout species, miR-33b sequences are only present in large mammals. However, both miRNAs share common targets. The most prominent one among vertebrates is the adenosine triphosphate-binding cassette A1 (ABCA1) cholesterol transporter (Figure 1), an important regulator of high-density lipoprotein (HDL) synthesis and reverse cholesterol transport.\(^9\) In mouse macrophages, miR-33 also targets ABCG1, reducing cholesterol efflux to nascent HDL. Inhibitors of miR-33a stimulate cholesterol efflux to apolipoprotein A1 leading to a modest but significant rise in plasma HDL.\(^7\)\(^-\)\(^9\) After injections of anti–miR-33, LDL receptor \(-/-\) mice showed increased hepatic expression of ABCA1, higher levels of circulating HDL and enhanced reverse cholesterol transport. This was accompanied by a reduction in the size and lipid content of atherosclerotic plaques, increased markers of plaque stability and decreased inflammatory gene expression, suggesting that miR-33 could be atheroprotective.\(^10\) In nonhuman primates, inhibition of miR-33 resulted in reduced plasma levels of very-low-density lipoprotein (VLDL)-associated triglycerides.\(^11\) Systemic delivery of an anti-miR oligonucleotide that targets both miR-33a and miR-33b increased hepatic expression of ABCA1 and induced a sustained increase in plasma HDL levels in African green monkeys over 12 weeks. Additional miR-33 target genes were observed by microarray analysis and validated by real-time PCR (qPCR): carnitine O-octanoyltransferase (CROT), carnitine palmitoyltransferase 1A (CPT1A), hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase \(\beta\)-subunit (HADHB) and protein kinase, AMP-activated, \(\alpha1\) catalytic subunit (PRKAA1) are all involved in fatty acid oxidation whereas sterol regulatory element binding transcription factor 1 (SREBF1), fatty acid synthase (FASN), ATP citrate lyase (ACLY) and acetyl-CoA

---

**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>adenosine triphosphate-binding cassette</td>
</tr>
<tr>
<td>APC</td>
<td>angiogenic progenitor cell</td>
</tr>
<tr>
<td>Ago</td>
<td>Argonaute</td>
</tr>
<tr>
<td>CLIP</td>
<td>cell-based cross-linking and immunoprecipitation</td>
</tr>
<tr>
<td>DIG</td>
<td>digoxigenin</td>
</tr>
<tr>
<td>ECs</td>
<td>endothelial cells</td>
</tr>
<tr>
<td>KLF</td>
<td>Krüppel-like factor</td>
</tr>
<tr>
<td>LNA</td>
<td>locked nucleic acid antisense oligonucleotide</td>
</tr>
<tr>
<td>LAMP</td>
<td>labeled miRNA pull-down</td>
</tr>
<tr>
<td>miRNAs</td>
<td>microRNAs</td>
</tr>
<tr>
<td>(\text{ob}/\text{ob})</td>
<td>obese/obese</td>
</tr>
<tr>
<td>OC2</td>
<td>One-cut-2</td>
</tr>
<tr>
<td>qPCR</td>
<td>real-time polymerase chain reaction</td>
</tr>
<tr>
<td>Rab</td>
<td>Rabphilins</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing</td>
</tr>
<tr>
<td>SMGs</td>
<td>Sprouty-related protein</td>
</tr>
<tr>
<td>SREBP</td>
<td>sterol regulatory element-binding protein</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
</tbody>
</table>

---

**Figure 1. MiRNAs involved in the cholesterol homeostasis.**

Liver miRNAs with regulatory function in cholesterol metabolism, fatty acid and triglyceride biosynthesis, and homeostasis are shown in red. In brief, miR-33 regulates cholesterol homeostasis and inhibits fatty acid oxidation. Similarly, miR-122 and miR-370 reduce fatty acid oxidation but promote fatty acid and cholesterol synthesis. Only their direct targets are depicted and the processes they control are shown in gray. Validated direct and indirect targets are listed in Table 1.
Carboxylase-α (ACACA or ACC1) are central players in fatty acid synthesis.

**MiR-122**

Besides miR-33, miR-122 is involved in the regulation of lipid metabolism. By far the most abundant miRNA in the liver, miR-122 maintains the hepatic cell phenotype and plays a prominent role in cholesterol and fatty acid metabolism. Different technologies were successfully applied to block miR-122 activity in vivo, including the cholesterol-conjugated-2′-OMe antisense oligonucleotides, the unconjugated but chemically modified 2′-OMe antisense oligonucleotides and locked nucleic acid antisense oligonucleotide (LNA) anti-miRs. Phase II clinical trials are currently under way to explore the effectiveness of an inhibitor of miR-122 for hepatitis C. In mice, miR-122 inhibition resulted in sustained reduction of total plasma cholesterol levels, increased hepatic fatty acid oxidation, and a decrease in hepatic fatty acid and cholesterol synthesis rates. Microarray analysis revealed reduced hepatic expression of a range of genes involved in the regulation of lipid biosynthesis (Figure 1) such as acetyl-CoA carboxylase β (ACC2), stearoyl-CoA desaturase (SCD1), and ATP citrate lyase (ACLY). Interestingly, most of the identified genes seem to be indirect targets of miR-122 because they lack seed sequences for miR-122, providing an explanation for their observed downregulation in response to miRNA inhibition whereas the opposite would be expected for direct miRNA targets. Notably, miR-122 suppresses AMP activated protein kinase (AMPK), a master regulator of metabolism that promotes ATP-generating pathways, for example, fatty acid oxidation, but inhibits energy storage, that is, fatty acid synthesis. Although the mechanism is still unknown, an increase in AMPK activation could induce the switch in energy usage by inhibiting ACC2. In mice and in nonhuman primates—African green monkeys—-injection of LNA anti-miR-122 caused a dose dependent and sustained reduction in total plasma cholesterol by 30% without apparent toxicity. Decreases in both HDL and apolipoprotein A1, as well as in LDL and apolipoprotein B were observed.

**MiR-370 Regulates MiR-122**

Intriguingly, there is interplay between miR-122 and miR-370. Transfection of a hepatocellular carcinoma cell line (HepG2 cells) with miR-370 induced the expression of miR-122 while the effect of miR-370 on the expression of the lipogenic genes was negated by antisense miR-122. Moreover, miR-370 acting via miR-122 upregulates initially the expression of SREBP-1c, diacylglycerol O-acyltransferase 2 (DGAT2), and subsequently the expression of other genes that affect lipid metabolism thus promoting fatty acid and triglyceride biosynthesis but antagonizing fatty acid catabolism. The net effect is hepatic triglyceride accumulation. Also, a direct involvement of miR-370 was proposed in the regulation of CPT1A (Figure 1), an important enzyme that mediates the transport of long-chain fatty acids across the mitochondrial membrane and regulates β-oxidation. Bioinformatic analysis revealed that miR-370 targets the 3′ untranslated region (3′UTR) of CPT1A and represses its expression. Hence, stimuli that upregulate miR-370 and/ or miR-122 may lead to accumulation of hepatic triglycerides by promoting lipogenesis and inhibiting fatty acid oxidation.

**MiR-378/378**

Besides the hepatic miRNAs mentioned above, miR-378/378 increases on adipocyte differentiation. Located in the first intron of peroxisome proliferator-activated receptor gamma coactivator-1 β (PGC1b), these miRNAs are coordinately expressed with their host gene during adipogenesis. Forced overexpression of miR-378/378 during adipogenesis induces triacylglycerol accumulation due to increased de novo lipogenesis. Assessment by qPCR identified significant expression changes in genes...
related to adipocyte differentiation and lipid synthesis, for example, Kruppel-like factor 5 (KLF5), fatty acid binding protein 4 (FABP4), stearoyl-CoA desaturase (SCD-1), and resistin.\(^1\) Of note, these are not direct targets of miR-378/378*.

Instead, miRNA378/378* increases the transcriptional activity of CCAAT-enhancer-binding proteins (or C/EBPs) \(\alpha\) and \(\beta\) on adipocyte gene promoters.

**MiR-335, miR-125a**

Elevated levels of miR-335 were detected in the liver and white adipose tissue of obese mice.\(^19\) MiR-125a is overexpressed in the liver and the adipose tissue of spontaneously diabetic Goto-Kakizaki rats and normoglycemic Brown-Norway rats.\(^20\) However, no validated targets have been described.

**MiRNAs in Glucose Homeostasis**

**Pancreas-Specific Dicer Knockout**

Dicer is an endoribonuclease that processes double-stranded RNA, including pre-miRNAs. Pancreas-specific Dicer knockout mice survive until birth but fail to grow and die by postnatal day 3. Severe defects in all pancreatic lineages and impaired Notch signaling led to increased cell death during pancreatic development.\(^21\) Dicer-1 deficient \(\beta\)-cells showed reduced insulin expression while \(\beta\)-cell differentiation was not affected. Even in \(\beta\)-cells of adult mice, inactivation of miRNAs led to a striking diabetic phenotype.\(^22\)

**MiRNAs Regulate Insulin Expression**

Dicer deletion in \(\beta\)-cells upregulates a set of transcriptional repressors such as the transcription factor Sox6 and the class E basic helix-loop-helix protein 22 (Bhlhe22) resulting in inhibition of insulin expression. These transcriptional repressors, as suggested by computational analysis, are putative targets of a panel of miRNAs including miR-24, miR-26, miR-182 and miR-148. Knockdown of any of these miRNAs in cultured \(\beta\)-cells or in isolated primary islets suppressed insulin promoter activity and insulin mRNA levels (Figure 2).\(^22\)

**MiR-375 Suppresses Insulin Secretion**

MiR-375 is highly expressed in pancreatic islets and directly regulates insulin exocytosis, independently of changes in glucose metabolism or alterations in the transmembrane Ca\(^{2+}\) fluxes and intracellular Ca\(^{2+}\) signaling.\(^23\) In silico analysis correctly predicted myotrophin, phosphoinositide-dependent protein kinase-1 (PDK1), and vesicle transport through interaction with t-SNAREs homolog 1A (VT1A) as direct targets of miR-375 (Figure 2).\(^23,24\) Overexpression of miR-375 suppressed glucose-induced insulin secretion, and conversely, inhibition of endogenous miR-375 function enhanced insulin secretion suggesting that miR-375 is a negative regulator of \(\beta\)-cell exocytosis. MiR-375 also maintains a normal pancreatic \(\alpha\)- and \(\beta\)-cell mass. MiR-375 null mice are normoinsulinemic but hyperglycemic and glucose intolerant. A reduction of \(\beta\)-cell mass antagonizes their enhanced insulin secretion per \(\beta\)-cell. The primary consequence is hyperactivation of the glucagon axis, as evidenced by an increase in \(\alpha\)-cells, higher glucagon release from isolated islets, and elevated fasted and fed plasma glucagon concentrations. Downstream effects of glucagon are expression of genes regulating gluconeogenesis and hepatic glucose production.

Although the increase in \(\alpha\)-cell numbers could be the result of a compensatory response to altered \(\beta\)-cell mass or to chronic hyperglycemia, miR-375 emerges as an essential regulator of glucose homeostasis, \(\alpha\)- and \(\beta\)-cell turnover and adaptive \(\beta\)-cell expansion in response to elevated insulin demand with insulin resistance. Further support to this notion was provided by the genetic deletion of miR-375 in obese mice (ob/ob) that led to profound loss of \(\beta\)-cells and a severe diabetic state.\(^25\) However, miR-375 knockout mice only partially mimic the phenotype observed after Dicer deletion during pancreas development,\(^21\) suggesting that other miRNAs contribute to the developmental defect in \(\beta\)-cell growth and differentiation.

**MiR-9, MiR-96, and MiR-124a Are Fine-Tuners of Insulin Release**

MiR-9 is mandatory for maintaining appropriate granuphilin levels and optimal secretory capacity in \(\beta\)-cells. Onceluc-2 (OC2), Figure 2), the granuphilin (synaptotagmin-like 4) repressor, is a direct target of miR-9.\(^26\) Computational analysis and subsequent experimental validation revealed that miR-9 affects the insulin secretion by down-regulating OC2 and therefore releasing its inhibitory effect on granuphilin, a protein that significantly enhances basal, but strongly inhibits K\(^{+}\)-induced insulin secretion. Granuphilin deletion in mice results in abnormally high insulin levels on stimulation.\(^27\) In contrast, overexpression of miR-9 in insulin-secreting cells suppresses glucose-induced exocytosis via upregulation of granuphilin. Likewise, miR-96 increases granuphilin, but independently of OC2. Additionally, miR-96 decreases Noc2, a Rab effector and positive regulator of insulin secretion.\(^28\) Rabphilins (Rab proteins) represent a family of small GTP-binding proteins that facilitate exocytosis. Noc2 interacts with 2 members of the Rab family with a key role in insulin secretion, Rab3 and Rab27, and inhibits G protein Gi/o signaling.\(^29\) Impaired Ca\(^{2+}\)-triggered insulin secretion in Noc2 knockout mice is reversed by treatment with pertussis toxin, an inhibitor of Gi/o signaling. Noc2 is also targeted by miR-124a.\(^30\) In addition, miR-124a increases the expression of other components of the secretory machinery such as synaptosomal-associated protein 25 (SNAP25), Rab3A and synapsin-1A. Overexpression of miR-124 promotes insulin secretion under basal conditions but attenuates insulin secretion in response to glucose stimulation.

**MiR-34a and MiR-146 Induce \(\beta\)-Cell Apoptosis**

MiR-34a and miR-146a are elevated in pancreatic islets from diabetic obese mice and significantly affect the survival of \(\beta\)-cells and insulin exocytosis. In vitro treatment of an insulin-secreting mouse cell line (MIN6B1 cells) and pancreatic islets with palmitate induced miR-34a and miR-146 expression in a dose-dependent manner. Activation of p53 upregulated MiR-34a. The latter was proposed to mediate \(\beta\)-cell apoptosis and to impair nutrient-induced insulin secretion.\(^31\) Inhibition of miR-34a and miR-146 could partially rescue the apoptotic response but failed to restore normal insulin secretion. Reporter gene assays demonstrated that miR-34a directly targets the 3’UTR of vesicle-associated membrane protein 2 (VAMP2) and B-cell lymphoma 2 (Bel-2).

**MiR-103 and MiR-107 Are Negative Regulators of Insulin Sensitivity**

Unlike the pancreatic miRNAs mentioned above, miR-103 and miR-107 are highly expressed in the liver of ob/ob and diet-
induced obese mice. Both miRNAs differ by only one nucleotide in their mature sequence. Silencing of miR-103/107 led to improved glucose homeostasis and insulin sensitivity, whereas overexpression of miR-103/107 in either liver or adipose tissue induced insulin resistance and glucose intolerance. Caveolin-1, a key component of caveolae and a critical regulator of insulin receptor signaling was among the predicted targets of miR-103/107 and mediated the effect of miR-103/107 in insulin signaling.32 Similarly, in liver biopsies from patients with diabetes and insulin resistance, an elevation of miR-103 and miR-107 was observed.

The Lin28/Let-7 Axis Regulates Glucose Metabolism
An unexpected role in glucose metabolism has recently emerged for the tumor suppressor family of let-7. In a transgenic mouse models, overexpression of Lin28a/b—a protein that binds to the let-7 pre-miRNA and blocks the production of the mature let-7 miRNA—promoted an insulin-sensitized state that reduced diabetes development after a high-fat diet. In contrast, loss of Lin28a or overexpression of let-7 in skeletal muscle led to insulin resistance and impaired glucose tolerance. Let-7 mediated repression of multiple components of the insulin-PI3K-mTOR pathway including insulin-like growth factor 1 receptor (IGF1R), insulin receptor (INSR), and insulin receptor substrate 2 (IRS2).37 In genome-wide association studies, let-7 targets were enriched for genes containing single-nucleotide polymorphisms associated with type 2 diabetes and control of fasting glucose.

MiRNAs Influence the Phenotype of Vascular Smooth Muscle Cells

SMC-Specific Dicer Knockout
Dicer is essential for normal development, differentiation and contractile activity of vascular SMCs. Deleting a floxed Dicer allele using Cre recombinase under control of the SM22 promoter resulted in extensive hemorrhages and embryonic lethality at E16.5.34 In the aorta, embryos showed outward hypertrophic remodeling and dysorganization of the elastic lamellae. Loss of actin filaments, contractile dysfunction of umbilical arteries and reduced expression of SMC specific genes was observed. Overexpression of miR-145 partially rescued this phenotype.34

MiR-143/145 Cluster
This bicistronic unit that encodes miR-143 and miR-145 is highly expressed in SMCs and critical for maintaining their contractile phenotype. MiR-143 and miR-145 are downregulated in synthetic SMCs,35–38 while transforming growth factor β1, a strong activator of SMC differentiation, stimulates miR-143/145 expression in a dose- and time-dependent manner.40 The transcription of both miRNAs is under the control of 2 independent signaling pathways: Serum response factor/myocardin/Nkx2.5,37,38 as well as Jag-1/Notch signaling.46 Overexpression of miR-145 led to reduced neointima formation in balloon-injured arteries.35 Further work demonstrated that miR-145 promotes a contractile phenotype with upregulation of SMC markers by suppressing Kruppel-like factor 4 and 5 (KLF4 and KLF5), ETS-like transcription factor 1 (Elk-1) and Ca²⁺/calmodulin-dependent protein kinases II6 (CamKIIδ), and increasing myocardin. Myocardin, a potent SMC-specific nuclear coactivator,38 orchestrates contractile SMC-specific gene transcription (Figure 3).

MiRNAs in the Vasculature
Besides their role in metabolism, miRNAs play a prominent role in vascular smooth muscle cells (SMC, Figure 3) and endothelial cells (EC, Figure 4).
Neither miR-143 nor miR-145 are essential for SMC differentiation in vivo: Mice lacking both miR-143 and miR-145 are viable and miR-143 deficient mice have functional SMCs. MiR-143−/− mice show no obvious abnormalities during embryonic development. MiR-145−/− mice, however, have a lower vascular tone and reduced blood pressure. Like miR-145−/− SMCs,36,37,41 SMCs from miR-143/miR-145 double knockout mice have diminished contractile activity. They also display a synthetic phenotype and form spontaneous neointimal lesions in femoral arteries of 18-month-old double knockout mice.37 After ligation of the carotid artery, neointima formation was reduced in miR-143−/− mice and almost abolished in miR-145−/− and miR-143/miR-145 double knockout mice. This protection against vascular injury apparently results from reduced SMC migration due to dysregulation of actin dynamics and the presence of feedback loops to modulate cytoskeletal assembly.37 Key regulators of podosome formation, such as platelet-derived growth factor (PDGF) receptor α, protein kinase C ε and fascin, were identified as direct targets of miR-143 and miR-145.42 A proteomic analysis of aortas from global miR-143/miR-145−/− mice also revealed angiotensin-converting enzyme (ACE-1) as another target of the miR-143/miR-145 cluster. Pharmacological inhibition by ACE inhibitors or angiotensin I receptor blockers partially reversed vascular dysfunction and normalized gene expression in miR-143/miR-145−/− mice.41

**MiR-21, MiR-133, MiR-221, and MiR-222**

Four other miRNAs have been implicated in the phenotypic modulation of SMCs: unlike miR-143/miR-145, miR-21, miR-221, and miR-222 were upregulated in neointimal lesions. MiR-21 promotes SMC proliferation while inhibiting apoptosis by down-regulation of the phosphatase and tensin homolog (PTEN) and up-regulation of Bcl-2 (Figure 3). These miR-21 effects were confirmed in balloon-injured rat carotid arteries.43,44 MiR-221 and miR-222 contribute to SMC dedifferentiation. Knockdown of miR-221 and miR-222 in rat carotid arteries suppressed SMC proliferation and neointimal lesion formation after angioplasty. MiR-221 and miR-222 appear to be essential for PDGF-mediated cell proliferation, by repressing c-Kit, p57Kip2 and the cyclin-dependent kinase inhibitor p27Kip1 (Figure 3). Inhibition of c-Kit reduced the expression of myocardin.45,46 MiR-133 blocked the phenotypic switch of SMCs by targeting the transcription factor Sp-1 as predicted by bioinformatics47 and derepression of myocardin from KLF4. Its expression was regulated by extracellular signal–regulated kinase 1/2 (ERK1/2) and inversely correlated to SMC proliferation.

**MiRNAs Control the Angiogenic Potential of Endothelial Cells**

**EC-Specific Dicer Knockout**

EC-specific inactivation of Dicer revealed a regulatory role for endothelial miRNAs in postnatal angiogenesis. Knockdown of Dicer in cultured ECs altered the expression of key regulators such as Tie-2, vascular endothelial growth factor (VEGF) receptor 2, Tie-1, endothelial nitric oxide synthase (eNOS) and interleukin-8, as well as reducing their proliferative and angiogenic potential. Surprisingly, it activated eNOS and enhanced the release of nitric oxide.48 Two transgenic mouse models were generated harboring an endothelial-specific deletion of Dicer: 1) A conditional floxed Dicer allele and expression of Cre-recombinase under the regulation of Tie2 promoter/ enhancer (Tie2-Cre; Dicer flox/flox mice). 2) A tamoxifen-inducible system to express
Cre-recombinase under the regulation of vascular endothelial cadherin promoter (VECad-Cre-ERT<sup>2</sup>; Dicer<sup>lox/lox</sup> mice). The mutant mice were indistinguishable from their littermate controls. Intradermal injection of an adenovirus expressing murine VEGF164 resulted in an impaired angiogenic response. Likewise, the angiogenic response to limb ischemia and wound healing was reduced in these mice.<sup>49</sup>

**MiR-17<sup>−/−</sup> Cluster and Angiogenesis**

The angiogenic response after vascular injury is modulated by the miR-17<sup>−/−</sup> cluster. Originally identified in tumors and highly expressed in ECs this cluster is a typical example of a polycistronic miRNA. The pri-miRNA gives rise to six mature miRNAs: miR-17, miR-18a, miR-19a, miR-20, miR-19b-1, and miR-92–1.<sup>50</sup> Through a complex series of duplication and loss of individual members, miRNA genes just like their protein-encoding counterparts can be found in multiple genomic loci. Three paralog miR-17<sup>−/−</sup> miRNA clusters have been reported in humans that appear to be closely linked to the early evolution of the vertebrates.<sup>51</sup> Mice deficient for miR-106b<sup>25</sup> or miR-106a<sup>49</sup> survive till adulthood display defective cardiac neovascularization after myocardial infarction.<sup>56,57</sup> Thus, miR-126 regulates vascular integrity and angiogenesis. Gene expression profiles by microarray analysis identified putative targets of miR-126 and the proangiogenic effect of miR-126 was, at least in part, attributed to the repression of the Sprouty-related protein 1 (SPRED-1) and phosphoinositol-3 kinase regulatory subunit 2 (PI3KR2/PI85-B) (Figure 4), 2 negative regulators of Ras/MAPK and PI3K signaling pathways.<sup>58,59</sup> Binding of VEGF and fibroblast growth factor (FGF) to their receptors on ECs leads to activation of MAPK and PI3K signaling pathways, and initiates a proangiogenic gene transcription program. Expression of miR-126 also appears to safeguard vascular integrity by inhibiting the expression of inflammatory mediators such as vascular cell adhesion molecule 1 (VCAM-1), hence limiting leukocyte adhesion and inflammation.<sup>60</sup> Additionally, miR-126 in ECs regulates vascular remodeling by modulating the expression of stromal cell-derived factor-1 (SDF-1).<sup>61</sup> In vivo, elevated levels of SDF-1 were proposed to mediate the recruitment and mobilization of Sca-1+/Lin- progenitor cells to the ischemic region.

In the zebrafish genome, 2 homologs of miR-126 were identified, miR-126a and miR-126b, that differ by only 1 nucleotide in their mature sequence.<sup>62</sup> Silencing miR-126 by antisense morpholino resulted in a loss of vascular integrity and hemorrhages during embryogenesis. More than 20% of the embryos developed cranial and pericardial hemorrhages, indicating the presence of blood cells but a loss of vascular integrity. Similar to mice, miR-126 altered PI3K and ERK signaling by targeting PIK3R2 and SPRED1, respectively. Hence, loss of miR-126 function attenuated MAPK signaling and impaired VEGF and FGF signaling.<sup>56,57</sup> In zebrafish, P21-activated kinase 1 (PAK1) was also reported as a direct target of miR-126a/b. PAK1 expression increased in endothelial cells when miR-126a/b were knocked down. Importantly, overexpression of the active form of human PAK1 caused cranial hemorrhages while knockdown of PAK1 effectively rescued hemorrhages induced by inhibition of miR-126a/b.<sup>63</sup>

Intriguingly, a loss of circulating miR-126 and other miRNAs was observed in subjects with type 2 diabetes. In vitro, high glucose concentrations did not affect endothelial expression of miR-126, but a significant reduction was observed in the miR-126 content of endothelial apoptotic bodies.<sup>64</sup> Further studies are required to investigate the cellular origin of circulating miR-126. The potential of miRNAs as cardiovascular biomarkers is beyond the scope of the present review and has been reviewed elsewhere.<sup>64</sup>

**Flow-Dependent Regulation of MiR-126, MiR-92a, MiR-21, and MiR-10a**

MiR-126 and miR-92a are directly regulated by hemodynamic forces. Atheroprotective flow patterns lead to an increase in the mecano-sensitive zinc finger transcription factor KLF2, a crucial integrator for maintaining endothelial func-
KLF2 induces miR-126 but represses miR-92a. MiR-126 was reported to regulate flow-stimulated angiogenesis and the blood flow-dependent formation of the aortic arch blood vessels in zebrafish. In response to hemodynamic forces, KLF2a induces miR-126 that subsequently activates VEGF signaling. Hence, miR-126 facilitates the integration of a physiological stimulus such as flow with growth factor signaling in ECs to guide angiogenesis.

On the other hand, oscillatory stress occurring in atherosusceptible areas enhances the binding of c-Jun to the promoter of miR-21 and induces the transcription of miR-21. Sustained upregulation of miR-21 contributes to the proinflammatory profile of ECs in the vascular territories prone to atherosclerosis through downregulation of peroxisome proliferators activated receptor-α (PPARα), a direct target of miR-21 (Figure 4) as predicted by bioinformatics analysis. Its inhibition results in derepression of activator protein 1 (AP-1) activation and upregulation of proinflammatory mediators like vascular adhesion molecule 1 and monocyte chemotactic protein-1. MiR-21 is also a negative modulator of angiogenesis. Overexpression of miR-21 reduced endothelial cell proliferation, migration and tube formation capacity mediated through inhibition of RhoB.

Moreover, expression of miR-10a was lower in atherosusceptible regions. Homeobox A1 (HOXA1), a direct target of miR-10a, mediates the expression of monocyte chemoattractant protein-1, interleukin-6, interleukin-8, VCAM-1 and E-selectin susceptible regions. Homeobox A1 (HOXA1), a direct target of miR-10a, mediates the expression of monocyte chemoattractant protein-1, interleukin-6, interleukin-8, VCAM-1 and E-selectin.

**Hypoxia Induces MiR-210 in ECs**

Hypoxia is a key trigger of angiogenesis. Hypoxia inducible factor 1 α upregulates transcription of miR-210 contributing to the proangiogenic phenotype of hypoxic endothelial cells. Overexpression of miR-210 in normoxic conditions stimulated endothelial tube formation in Matrigel assays. Likewise, inhibition of miR-210 blocked tube formation in hypoxic ECs. Thus far, validation of computational target prediction confirmed ephrin-A3 (EFNA3) as a direct target of miR-210. MiR-210 was also proposed to be a mediator of increased stem cell survival after ischemic preconditioning, through suppression of caspase 8 associated protein 2.

**MiR-221/MiR-222 Cluster in Angiogenesis and Inflammation**

Highly expressed in ECs, this miRNA cluster modulates the angiogenic activity of stem cell factor through direct targeting of its receptor c-Kit. As a consequence, the angiogenic properties of ECs are altered and their ability to form new vascular capillaries is reduced. Upregulation of miR-221 was reported in response to hyperglycemia. Elevated miR-221 correlated with reduced expression of c-kit and impaired endothelial cell migration. This miRNA family is downregulated in response to inflammatory stimuli. Further studies revealed that signal transducer and activator of transcription 5A (STAT5a), a predicted target of miR-222, is involved in vascular remodeling triggered by inflammation.

**MiR-23~27~24 Cluster in Angiogenesis**

The miR-23~27~24 cluster is abundant in ECs. In a mouse model of laser-induced choroidal neovascularization, miR-23 and miR-27 were essential for pathological angiogenesis, by promoting angiogenic signaling through targeting Sprouty2 and the transmembrane semaphorin protein Sema6A (Figure 4), as correctly predicted by bioinformatics. Inhibition of miR-23 and miR-27 represses angiogenesis in vitro and postnatal retinal vascular development in vivo. Increased expression of this cluster is detected in response to proinflammatory stimuli, suggesting a link between inflammation and angiogenesis in choroidal neovascularization.

MiR-24 is upregulated in ECs after myocardial infarction. Detailed studies highlighted its role in the ischemic response of ECs. MiR-24 was shown to induce EC apoptosis, to abolish endothelial capillary network formation on Matrigel and to inhibit cell sprouting from endothelial spheroids. Initially proposed by in silico analysis, the transcription factor GATA2 and PKA kinase were validated as novel targets of miR-24. Inhibition of miR-24 in mice reduced the infarct size and enhanced vascularization resulting in better cardiac function and survival. In contrast, miR-24 was reported to act as inhibitor of cardiomyocyte apoptosis. Reduced miR-24 expression was detected in the ischemic border zone of the murine left ventricle after MI. MiR-24 inhibited apoptosis in cardiomyocytes in vitro and in vivo using a mouse model of MI. This effect was, at least in part, mediated by direct repression of BH3-only domain-containing protein (Bim). Thus, the 2 studies disagree regarding a protective versus detrimental effect of miR-24 after MI and implicate different target cells – proapoptotic effects in ECs versus antiapoptotic effects in cardiomyocytes.

**The Understudied MiRNA-Ome of Platelets**

Platelets play a key role in cardiovascular pathologies. Besides their main physiological function to form hemostatic thrombi and prevent blood loss, platelets also contribute to the maintenance of vascular integrity, angiogenesis and vascular repair on injury. Harboring a small, but diverse, transcriptome, platelets have recently been shown to contain abundant quantities of small miRNAs. The relative abundance of miRNAs in platelets translates into a markedly increased miRNA/mRNA ratio versus nucleated cells. Although platelets lack a nucleus and genomic DNA, platelets contain Dicer and Ago2 complexes and may control expression of specific transcripts. Thus far, studies have confirmed that mRNA expression in platelets is under the control of platelet miRNAs. A close association between the miRNA-mRNA coexpression profiles and platelet activity was observed and a set of miRNAs were predictive for enhanced platelet aggregation. Moreover, a panel of three miRNAs were selected to demonstrate direct downregulation of their predictive targets: miR-200b inhibited protein kinase cAMP-dependent regulatory type IIβ (PRKAR2B), miR-495 downregulated kelch-like 5 (KLHL5), whereas miR-107 decreased the circadian locomoter output cycles protein kaput (CLOCK).
Likewise, in studies where global knockout mice or systemic inhibitors were used (Figure 5), platelet function ought to be taken into consideration. Although speculative, altered platelet function could offer an alternative explanation for some of the reported cardiovascular pathologies, for example, systemic edema, multifocal hemorrhages, and ruptured blood vessels after genetic deletion of miR-126 in animal models or reduced infarct size and enhanced vascularization after systemic inhibition of miR-24 in a mouse model of myocardial injury.

**MiRNA Target Identification**

The known miRNA targets are listed in Tables 1 and 2. More comprehensive screening techniques will be essential to delineate the biological effects of miRNAs. Phenotypes after systemic inhibition or genetic deletion of miRNAs are unlikely to be the result of individual targets, even if the miRNA is expressed in a highly tissue-specific manner.

**Bioinformatic Tools for Prediction of Direct MiRNA Targets**

Mature miRNAs typically modulate gene expression through binding to the 3’UTRs of their transcripts, although targeting of the 5’UTRs or coding sequences has also been reported.\(^8^\)\(^-^\)\(^\text{87}\) Individual miRNAs contain binding sites for multiple miRNAs, allowing redundancy and creating miRNA-miRNA cotargeting networks.\(^8^\) Nucleotides 2–8 of the miRNA, termed the “seed” sequence, are essential for canonical base pairing and target recognition. Each miRNA has numerous targets. Several prediction algorithms (miRanda, microCosm, TargetScan, PicTar, Diana-MicroT) have been developed and are based on the following criteria:

<table>
<thead>
<tr>
<th>Table 1. MiRNAs Involved in Glucose and Cholesterol Homeostasis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MiRNAs</strong></td>
</tr>
<tr>
<td>miR-33</td>
</tr>
<tr>
<td>miR-122</td>
</tr>
<tr>
<td>miR-370</td>
</tr>
<tr>
<td>miR-378/378*</td>
</tr>
<tr>
<td>miR-375</td>
</tr>
<tr>
<td>miR-9</td>
</tr>
<tr>
<td>miR-96</td>
</tr>
<tr>
<td>miR-124a</td>
</tr>
<tr>
<td>miR-34a</td>
</tr>
<tr>
<td>miR-103, miR-107</td>
</tr>
<tr>
<td>Let-7</td>
</tr>
<tr>
<td>miR-24, miR-26, miR-182, miR-148</td>
</tr>
</tbody>
</table>
Table 2. MiRNAs Involved in Maintaining Vascular Integrity

<table>
<thead>
<tr>
<th>MiRNAs</th>
<th>Validated Targets</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-10a</td>
<td>HOXA1, MAP3K7, bTRC</td>
<td>Fang et al.68 2010</td>
</tr>
<tr>
<td>miR-17–92</td>
<td>TSP-1, CTGF, Sir1, ITGA5, Map2k4</td>
<td>Bonauer et al.53 2009; Dewes et al.118 2006</td>
</tr>
<tr>
<td>miR-21</td>
<td>ERK, PTEN, RhoB, PPARα, Bcl2, PDCD4</td>
<td>Davis et al.44 2008; Ji et al.43 2007; Sabatel et al.66 2011; Zhou et al.77 2011</td>
</tr>
<tr>
<td>miR-23–27–24</td>
<td>Sprouty2, Sema6A, GATA2, PAK4</td>
<td>Fiedler et al.79 2011; Jennewein et al.76 2011; Zhou et al.77 2011</td>
</tr>
<tr>
<td>miR-126</td>
<td>SPRED1, PI3KR2, VCAM1, SDF-1, PAK1</td>
<td>Fish et al.57 2008; Harris et al.60 2008; Wang et al.56 2008; van Solingen,67 2011; Zhou et al.77 2011</td>
</tr>
<tr>
<td>miR-133</td>
<td>Sp1</td>
<td>Torella et al.47 2011</td>
</tr>
<tr>
<td>miR-143,</td>
<td>KLF4, KLF5, Myocardin, CaMKIIβ, Elk1</td>
<td>Cordes et al.38 2009; Xin et al.32 2009; Cheng et al.17 2009</td>
</tr>
<tr>
<td>miR-145</td>
<td>PDGF-Rα, PKCε</td>
<td>Quintavalle et al.46 2010</td>
</tr>
<tr>
<td>miR-210</td>
<td>Ephrin A3</td>
<td>Fasanaro et al.71 2008</td>
</tr>
<tr>
<td>miR-221,</td>
<td>cKit, p27Kip1, p57Kip2, STAT5A</td>
<td>Davis et al.46 2009; Liu et al.45 2009; Poliseno et al.14 2006; Dentelli et al.76 2010</td>
</tr>
<tr>
<td>miR-222</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(1) The partial complementarity of the miRNA and the mRNA sequences
(2) The thermodynamics of the duplex
(3) The conservation of the binding sites across species

Cell type context and other parameters that determine miRNA-mRNA interactions, ie, the actual cellular concentrations of miRNAs and mRNAs or sequences in the vicinity of the target site that enable the binding of modulators of miRNA activity, are currently not taken into consideration. Due to our poor understanding of miRNA-target interaction beyond seed pairing, the prediction algorithms have low sensitivity and specificity with a false-positive rate of at least 40%. Moreover, experimental validation demonstrated that numerous miRNA targets do not comply with the conventional target prediction rules and thus would not be identified by bioinformatic prediction tools. Importantly, only a handful of targets may have physiological relevance. Many of the computationally predicted miRNA targets were proposed to act as competitive inhibitors of miRNA function by preventing miRNA binding to their authentic targets by sequestration. Further support to this notion was provided by the discovery of a human pseudogene that can regulate mRNA sequences by competing for miRNA binding therefore acting as a natural “miRNA sponge.”

Experimental Strategies to Identify Direct MiRNA Targets
The predominant mechanism of action for reduced protein synthesis by miRNAs is the destabilization of target mRNAs. However, miRNA and mRNA levels do not always correlate. In heart failure, for example, miRNAs were more sensitive than mRNAs to the acute functional status of end-stage heart failure. Although, the mRNA signature was always sensitive to the mechanical unloading status of end-stage hearts. Therefore, detecting changes at the mRNA level to predict miRNA targets is not always reliable. Different methods of high-throughput identification for miRNA targets have been proposed:

Labeled MiRNA Pull-Down (LAMP) Assay
Affinity based target identification was initially described using miRNAs synthesized with a 3’ biotin group. After cell lysis the miRNA-protein-mRNA complexes are purified on streptavidin-agarose beads and the associated mRNAs are isolated and identified by microarray. This technique was successfully used to identify targets of miR-10a. Binding to the 5’UTR of ribosomal protein mRNAs of miR-10a was detected and its function as a positive global regulator of protein synthesis was established. An improved version of the affinity based identification assay was recently described. The LAMP assay is an in vitro approach that utilizes labeling of the pre-miRNA with digoxigenin (DIG). The DIG labeled precursor is mixed with cell extracts and immunoprecipitated by anti-DIG antibody. The obtained DIG-labeled miRNA and bound mRNA complexes are isolated and the complementary DNAs are subcloned and sequenced or amplified by reverse transcription polymerase chain reaction to search for putative target genes of the miRNA. Although this system has the advantage that it does not specifically target the 3’UTR sequences, DIG labeling may interfere with the cleavage by Dicer or strand processing resulting in nonspecific binding.

High-Throughput Sequencing by Cross-Linking and Immunoprecipitation (Ago HITS-CLIP)
Nucleic acids are cross-linked by ultraviolet radiation and immunoprecipitated with an antibody against the Argonaute (Ago) component of the RNA-induced silencing (RISC) complex. RISC-protected RNA fragments are then analyzed by high-throughput RNA sequencing. Ago HITS-CLIP provides a general platform for exploring the specificity and range of miRNA action in vivo, and identifies precise sequences for targeting relevant miRNA-mRNA interactions. HITS-CLIP data can offer unique information for the identification of exact cross-link sites and determine protein-RNA interactions at single-nucleotide resolution. As the reverse transcriptase skips the cross-linked amino acid-RNA adduct, crosslinking results in a nucleotide deletion. Genome-wide analysis of these cross-linking–induced mutation sites provides a general and more precise means of mapping protein-RNA interactions in living tissues.
Cell-Based Cross-Linking and Immunoprecipitation (CLIP)
A modification of the CLIP method was developed to determine at high resolution and transcriptome-wide the binding sites of cellular miRNA-containing ribonucleoprotein complexes. In this PAR-CLIP approach (photoactivatable-ribonucleoside–enhanced cross-linking and immunoprecipitation), 4-thiouridine is incorporated into transcripts of cultured cells and the cross-linked sites are identified by thymidine to cytidine transitions in the complementary DNAs prepared from immunopurified ribonucleoprotein complexes.

Immunoprecipitation of Tagged Components of the RISC Complex (RISC-IP)
Pull-down of myc-tagged Ago is another strategy to identify putative targets of miRNAs. Experimental validation of the targets identified by this method is high, suggesting that it is an effective technique to determine potential miRNA binding sites. However, overexpression of Ago was reported to increase miRNA production, which may compromise endogenous miRNA-mRNA interactions. A refined version of this assay, RISC-IP-RNA-sequencing, assesses targets by overexpressing the miRNA of interest.

Proteomics and Metabolomics to Assess Direct and Indirect MiRNA Effects
MiRNAs act as rheostats that fine tune protein output. Despite having a modest effect on individual targets, miRNAs can exert potent biological effects. A single miRNA is able to regulate the expression of multiple targets often within the same biological pathway. Recent evidence suggests that miRNAs function by generating thresholds in target gene expression. Alternatively, miRNAs may act as both positive and negative regulators of cellular processes to ensure the precision and robustness of biological systems against perturbations. This complex regulatory effect is often indirect in nature and thus cannot be predicted by conventional bioinformatic tools. It will be paramount to delineate the function of miRNAs not just by compiling lists of direct targets, but by providing a comprehensive assessment of protein and metabolite changes, most of which may be secondary in nature.

Proteomics is the method of choice since some miRNA targets are not regulated by miRNA degradation but inhibition of translation. Different proteomics approaches can be employed as reviewed previously. To minimize false-positives, it is advisable to search for commonalities of protein changes after transfection of the precursor as well as inhibition of the endogenous miRNAs. Using pulsed stable isotope labeling by amino acids in cell culture (pSILAC), newly synthesized proteins can be pulse-labeled with different isotopes of amino acids and readily discerned from preexisting proteins. In Hela cells, hundreds of genes were directly repressed by individual miRNAs but inhibition was usually less than 30% and rarely reached more than a fourfold reduction in protein levels. Noteworthy, some targets were repressed without detectable changes in mRNA levels, but targets with translational repression by more than one third also displayed corresponding mRNA destabilization. Exploring different aspects of cellular processes, for example, microarrays to interrogate the transcriptional signal, proteomics related to translational and post-translational mechanisms and metabolomics as a comprehensive screening method for changes in small molecules will be critical in understanding the various biological effects of miRNAs. In the cardiovascular setting, proteomic analysis was recently employed to identify targets of miR-21 in human angiogenic progenitor cells (APCs). Difference in-gel electrophoresis followed by mass spectrometric analysis of regulated proteins identified superoxide dismutase 2 as a target. MiR-21 further repressed Sprouty2, leading to ERK MAP kinase–dependent reactive oxygen species formation and APC migratory defects. Additional experiments revealed that the elevated levels of miR-21 in APCs from patients with coronary artery disease and high asymmetrical dimethyline (ADMA) correlate with low superoxide dismutase 2 expression and impaired migratory capacity that can be rescued by miR-21 antagonism. Generally, studies on APCs, previously referred to as endothelial progenitor cells, have to be interpreted with caution given that these early outgrowth cultures tend to be contaminated with platelets.

Concluding Remarks
MiRNAs are attracting considerable interest and emerged as important regulators and potential biomarkers in the cardiovascular system. However, miRNAs have numerous targets often within the same biological pathway. Moreover, multiple miRNAs can target the same gene and interact with each other forming miRNA-miRNA cotargeting networks. To add to this complexity, cotargeting networks consist of miRNAs that are responsible for controlling a given set of targets in different contexts rather than miRNAs that are coexpressed so as to regulate their common targets at the same time and place. Yet, miRNA effects tend to be explained based on our traditional view of linear signaling pathways. This oversimplification creates the illusion of simplicity to obtain "mechanistic insights," but ignores the reality of biological complexity. Importantly, cell type–specific effects of miRNAs have been proposed, for example, miR-21 in cardiac fibroblasts targets Sprouty1 and PTEN while regulating cardiomyocyte apoptosis by controlling expression of programmed cell death 4 (PDCD4). Similarly, the miR-17-92 cluster is antiangiogenic via downregulation of integrin subunit α5 and Sirtuin 1, but proangiogenic in tumor cells by targeting thrombospondin 1 and connective tissue growth factor (CTGF). The redundancy in miRNA function and the presence of compensatory mechanisms has been highlighted by discrepancies between pharmacological intervention studies and transgenic animal models. Inconsistencies were also observed between the use of LNA anti-miRs versus antago-miRs of miR-21, a miRNA that is upregulated in fibroblasts of failing and hypertrophic hearts. Knockdown of miR-21 with cholesterol-modified miR-21 antago-miRs inhibited cardiac remodeling and fibrosis. In contrast, the inhibition of miR-21 by LNA anti-miRs did not ablate the fibrotic and hypertrophic cardiac response to stress. Several issues have to be taken into consideration, in particular oligonucleotide length. On systemic delivery, unconju-
gated tiny LNAs show uptake and successful miRNA silencing in a variety of tissues. However, they appear to be cleared more rapidly from the heart compared with other organs.

In summary, given the ever-expanding number of noncoding RNAs, understanding their function represents a formidable task. Technologies, such as metabolomics and proteomics, allow a more comprehensive assessment of miRNA effects and provide exciting opportunities for new pathogenic insights into cardiovascular diseases. Novel therapeutic strategies will face the major challenge of developing standardized methods for miRNA inhibition that combine high transfection efficiency with targeted delivery.

Sources of Funding
M.M. is a Senior Fellow of the British Heart Foundation. This work was supported by Diabetes Research UK (BDA 100004115), the Juvenile Diabetes Research Foundation (17-2011-658) and the Department of Health via a National Institute for Health Research (NIHR) Biomedical Research Centre award to Guy’s & St Thomas’ NHS Foundation Trust in partnership with King’s College London and King’s College Hospital NHS Foundation Trust.

Disclosures
The authors filed patent applications owned by King’s College London that detail claims related to circulating miRNAs as cardiovascular biomarkers.

References


Zampetaki and Mayr

521

MiRNAs in Vascular and Metabolic Diseases


MicroRNAs in Vascular and Metabolic Disease
Anna Zampetaki and Manuel Mayr

doi: 10.1161/CIRCRESAHA.111.247445

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/110/3/508

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/