

## MicroRNAs in Angiogenesis and Vascular Smooth Muscle Cell Function

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The Human Genome Project was barely completed in 2003 before a new quest was launched: this time, in a search for short stretches of RNA called microRNA. The belief that these miniature molecules, which had only been discovered a decade earlier, were major regulators of genetic function gained momentum in the intervening years and sparked a worldwide interest in the physiologic and pathologic roles of microRNAs in the human body.

These single-stranded, noncoding molecules of RNA, spanning to lengths of only 25 nucleotides, appear to modulate a range of cellular events by modifying the primary function of messenger RNA (mRNA): protein synthesis. The first regulatory RNA, lin-4, was discovered by Victor Ambros in 1993. Later, Andy Fire, Craig Mello, and David Baulcombe discovered small interfering RNA (siRNAs) and established the role of small noncoding mRNA in regulating cell function under a variety of conditions. The field has exploded in directions that no one could have predicted. We now know that small RNAs are expressed in most eukaryotic cells and regulate a remarkable number of cellular functions.<sup>1</sup>

The exact mechanism of action of microRNAs is unclear; however, it is believed that these molecules bind to select, noncoding regions of mRNA through traditional Watson and Crick base pairing, repressing or increasing expression of the genetic transcript and its corresponding protein. It is theorized that each molecule of microRNA regulates more than 100 distinct molecules of mRNA, with unknown but potentially far-reaching effects in the human body.<sup>2</sup> Already multiple reports implicate an array of distinct microRNAs in cardiac and skeletal muscle myogenesis, apoptosis, regeneration, hypertrophy, fibrosis, and cardiac function.

Investigators use microRNA expression profiling to identify specific microRNAs in a host of human and animal cells. Quantitative mass spectrometry measures protein levels in cells following the introduction of a specific microRNA molecule, and experiments that amplify or block its expression allow researchers to observe its unique physiologic and pathologic effects. To date, a collection of approximately 650 individual microRNA molecules have been identified, and researchers are slowly piecing together a picture of how these tiny masters operate in healthy and disease states.<sup>2</sup>

What has been learned so far is that microRNA can function to maintain health or disease. A clear illustration is their role in angiogenesis, the process by which new blood

vessels develop from existing vessels in response to external signals detected by vascular endothelial cells. Emerging research has revealed that microRNAs are important modulators of angiogenesis, which can be physiologic (as observed in wound healing) or pathologic (as in tumorigenesis).<sup>3,4</sup>

Although the exact molecular pathways governing vascular formation have yet to be unraveled, recent reports provide insights on the actions of specific microRNAs in regulating the actions of endothelial cells and the surrounding smooth muscle cells that are fundamental to vascular support and function.

### Control of Angiogenesis by MicroRNA-92a

The process of angiogenesis begins within the cells of the vascular endothelium, a dynamic interface exquisitely sensitive and highly responsive to changes in its external environment. Advanced molecular biology in the 1990s pointed to the endothelial cell as a primary mediator of vascular activity. However, accumulating data now indicate that like the wizard behind the curtain, molecules of microRNA lie hidden within the endothelial cells, controlling a constellation of cellular responses, including the promotion or inhibition of angiogenesis.<sup>3,4</sup>

The role of specific microRNA molecules in angiogenesis was investigated by Angelika Bonauer and colleagues in Frankfurt, Germany. The team studied a cluster of specific microRNAs detected by expression profiling of human endothelial cells, most with known function in angiogenesis. However, the actions of one particular molecule, microRNA-92a, remained a mystery.<sup>4</sup>

In an in vitro study designed to determine its function, investigators forced the overexpression of microRNA-92a in human endothelial cells. The team noted that fundamental aspects of angiogenesis were inhibited, including the formation of vascular sprouts and networks. Also observed were reduced endothelial cell migration and impaired attachment to fibronectin, a vascular wall protein produced by proliferating vessels.<sup>4</sup>

To determine the effects of forced overexpression of microRNA-92a in vivo, the investigators implanted human umbilical vein endothelial cells into mice. A reduction in invading endothelial cells and formation of new blood vessels resulted. Forced overexpression of microRNA-92a in zebrafish produced severe defects in vessel formation.<sup>4</sup>

Next, the team set out to discover if inhibition of microRNA-92a would increase blood vessel growth. Inhibition was achieved in vitro through the application of an antisense oligoribonucleotide to hybridize to microRNA-92a, essentially blocking its nucleotides from binding to its target mRNA. The result was an increase in sprout formation. An in vivo model of microRNA-92a inhibition produced by the

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systemic administration of a complementary oligonucleotide into mice exhibited enhanced angiogenesis, as indicated by an increase in invading cells and new vessel formation.<sup>4</sup>

In vivo testing continued with mouse models of ischemia. A model of hind limb ischemia displayed significantly increased levels of microRNA-92a, indicating increased expression in skeletal muscle following ischemic injury. Predictably, inhibition of microRNA-92a in a model of limb ischemia caused the ischemic tissues to revascularize and blood flow to improve. Inhibition of microRNA-92a in a mouse model of acute myocardial infarction improved left ventricular function and reduced infarct size.<sup>4</sup>

The investigators concluded that the microRNA-92a found in vascular endothelial cells normally acts to suppress function of proangiogenic proteins by inhibiting the mRNAs that code for them. Because inhibition of microRNA-92a appears to augment the angiogenic process, it represents an attractive target for therapeutic intervention in vascular disease states characterized by ischemia.<sup>4</sup>

### Regulation of Vascular Smooth Muscle Cell Proliferation and Function

According to two recent reports, microRNAs also regulate the function of vascular smooth muscle cells (VSMCs).<sup>2,5</sup> These cells form layers within the vessel wall and control blood flow by contracting or relaxing in response to external stimuli. Only mature, differentiated VSMCs have contractile function; their premature fibroblast precursors can only proliferate or differentiate. However, recent advances in vascular biology have revealed that VSMCs exhibit a high degree of plasticity, shifting back and forth between the proliferative and the contracting phenotypes.

Under normal physiologic conditions, VSMCs rarely proliferate. However, in the presence of injury, low-density lipoprotein deposits, or other assaults on the vascular endothelium, VSMCs begin to grow and divide. Over time, as endothelium function deteriorates, unchecked proliferation can lead to pathologic changes in the vascular walls.<sup>5</sup>

The switch between the proliferative and the contractile phenotypes is governed by two regulatory proteins. The first is called serum response factor (SRF), which influences VSMC development in response to specific cofactors present at various points in cellular development. The second, myocardin, functions as a powerful coactivator of SRF, and it is the interaction between these two proteins that directs the VSMCs toward proliferation or differentiation.<sup>2</sup>

When levels of myocardin decrease, as occurs in vascular disease, the VSMCs are thrown off course and begin to proliferate, a finding that points to myocardin as the primary regulatory of VSMC development. But, what regulates the regulator? Kimberly R. Cordes, in collaboration with Deepak Srivastava of the Gladstone Institute of Cardiovascular Disease (GICD) in San Francisco, conducted a study that provided some insights. Cordes and associates from GICD and the Aab Cardiovascular Research Institute at the University of Rochester School of Medicine and Dentistry found that in a mouse model, injury to carotid arteries resulted in VSMC proliferation that progressively narrowed the vascular lumen. When compared with healthy carotids in the same

animals, investigators noted a marked decrease in the expression of microRNA-143 and microRNA-145 in the diseased vessels and substantial downregulation of their expression in the thickened vessels.<sup>2</sup>

Previous studies revealed that the SRF-myocardin interaction directly activated expression of both microRNA-143 and microRNA-145 during early and late stages of VSMC development. Cordes and the team now questioned the role of the two microRNAs in myocardin-directed differentiation of VSMCs. In search of an answer, they used an antisense oligonucleotide to inhibit microRNA-145 in fibroblasts and observed that the ability of myocardin to transform these precursors was also blocked. Additional testing confirmed that the presence of microRNA-145 was required for myocardin to convert the fibroblasts into mature differentiated smooth muscle cells.<sup>2</sup>

Inhibition of microRNA-143 barely impacted myocardin's effects. However, both microRNA-143 and microRNA-145 work with a complex of coregulators to guide cellular development. Cordes says, "the two small RNAs work together to regulate a network of genes involved in smooth muscle phenotypic switching by repressing genes involved in SMC proliferation and promoting SMC differentiation." For example, in its active state, microRNA-143 blocked the expression of ELK-1, a factor that stimulates proliferation of fibroblasts with little effect on myocardin. MicroRNA-145 represses Kuppel-like factor (Klf4), which is produced in proliferating cells following injury, and acts to inhibit the expression of myocardin.

Whereas these findings indicate that both microRNA-143 and -145 are part of an elaborate, interactive mesh of molecular regulators that govern smooth muscle plasticity, only microRNA-145 was shown to modulate myocardin, the chief director of VSMC fate. Because this interplay between microRNA-145 and myocardin appears to be vital in maintaining VSMC in their mature form, additional studies are needed to uncover the cues to which microRNA-145 itself responds. "Because of their tissue specificity and multiple targets," Cordes states that further research, "will uncover the mechanisms that lead to many forms of cardiovascular disease." Accordingly, Cordes concludes that microRNA "will be novel therapeutic targets for treating human disease."

The role of microRNA-143 and microRNA-145 in VSMC differentiation was also the focus of a recently published study by researchers led by Thomas Braun and Thomas Boettger at the Max-Planck-Institut für Herz- und Lungenforschung, Germany.<sup>5</sup> Says Boettger, "We did this research to get insight into the physiologic role of the microRNAs-143 and -145 in vivo, as well as to get insights into regulatory interactions that are essential for smooth muscle function."

Using standard hybridization techniques, the researchers demonstrated that expression of both microRNA-143 and microRNA-145 was strongly associated with VSMC number in a broad sampling of mouse tissues at various developmental stages. To determine the function of both microRNAs, which clustered together on chromosome 18, the team deleted the stretch of DNA harboring the genetic sequences that coding for each and observed the effects on VSMC behavior.<sup>5</sup>

Analysis of the large arteries of the mutant mice revealed a substantial decrease in mature cells with contractile function and an increase in the number of noncontracting, proliferating precursors. These findings indicate that the VSMCs transition from a state of contractility to one of proliferation in response to the loss of microRNA-143 and microRNA-145. Investigators also discovered neointimal lesions in the absence of typical atherosclerotic characteristics, suggesting that proliferating cells accumulate in the vascular lumen and contribute to the pathology underlying atherosclerosis.<sup>5</sup> The presence of neointimal lesions helps reinforce the theory that proliferating VSMCs advance the atherosclerotic process.<sup>5</sup>

Based on these observations, the study investigators concluded that both microRNA-143 and microRNA-145 directly participate in the regulation of smooth muscle cell differentiation and act to maintain the cells in the mature, contractile state. “We have shown that the microRNA-143/145 cluster is essential for maintenance of the contractile phenotype of the VSMCs but not for smooth muscle determination and development,” states Boettger.

As with microRNA-92a, deciphering the function of microRNA-143 and microRNA-145 allows scientists to potentially alter the course of a vascular disease by manipulating the expression of these potent molecules. Inhibition of

microRNA-92a-mediated suppression of angiogenesis, for example, may improve tissue recovery following myocardial infarction or other ischemic diseases. Conversely, compounds designed to mimic the actions of microRNA-143 and microRNA-145 may inhibit the overproliferation of smooth muscle cells that occurs in atherosclerosis and vascular injury. Further elucidation of the function of these and other microRNAs in cardiovascular health and disease will likely uncover a vast array of targets for therapeutic intervention.

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