MicroRNA-145 Restores Contractile Vascular Smooth Muscle Phenotype and Coronary Collateral Growth in the Metabolic Syndrome

Rebecca Hutcheson, Russell Terry, Jennifer Chaplin, Erika Smith, Alla Musiyenko, James C. Russell, Thomas Lincoln, Petra Rocic

Objective—Transient, repetitive occlusion stimulates coronary collateral growth (CCG) in normal animals. Vascular smooth muscle cells (VSMCs) switch to synthetic phenotype early in CCG, then return to contractile phenotype. CCG is impaired in the metabolic syndrome. We determined whether impaired CCG was attributable to aberrant VSMC phenotypic modulation by miR-145–mediated mechanisms, and whether restoration of physiological miR-145 levels in metabolic syndrome (JCR rat) improved CCG.

Approach and Results—CCG was stimulated by transient, repetitive left anterior descending artery occlusion and evaluated after 9 days by coronary blood flow measurements (microspheres). miR-145 was delivered to JCR VSMCs via adenoviral vector (miR-145-Adv). In JCR rats, miR-145 was decreased late in CCG (=2-fold day 6; =4-fold day 9 versus SD), which correlated with decreased expression of smooth muscle-specific contractile proteins (=5-fold day 6; =10-fold day 9 versus SD), indicative of VSMCs’ failure to return to the contractile phenotype late in CCG. miR-145 expression in JCR rats (miR-145-Adv) on days 6 to 9 of CCG completely restored VSMCs contractile phenotype and CCG (collateral/normal zone flow ratio was 0.93±0.09 JCR+miR-145-Adv versus 0.12±0.02 JCR versus 0.87±0.02 SD).

Conclusions—Restoration of VSMC contractile phenotype through miR-145 delivery is a highly promising intervention for restoration of CCG in the metabolic syndrome. (Arterioscler Thromb Vasc Biol. 2013;33:727-736.)

Key Words: collateral circulation ■ metabolic syndrome ■ microRNA ■ vascular smooth muscle phenotype

Transient repetitive coronary artery occlusion, characteristic of stable angina, and resultant myocardial ischemia (RI) stimulate coronary collateral growth (CCG) in healthy humans and normal animals. Clinically, patients with stable angina have decreased incidence of fatal myocardial infarction, which is associated with better developed collateral networks. However, CCG is severely impaired in metabolic syndrome patients and in our metabolic syndrome rat model (JCR:LA-cp or JCR).

In the healthy canine model, CCG has been documented to progress through distinct stages. The early stage begins with endothelial activation and accumulation of bone marrow-derived progenitor and inflammatory cells. This is followed by degradation of the basement membrane, vascular smooth muscle cells (VSMCs) phenotype switch from the adult, quiescent, contractile phenotype to the synthetic, proliferative and migratory phenotype, and proliferation and migration of endothelial cells and VSMCs into the lumen of the preexisting native collateral vessel. The later phase is characterized by outward endothelial cell and VSMC migration, luminal expansion, and the VSMCs’ return to the contractile phenotype. Thus, the transient switch of the VSMCs to the synthetic phenotype early in the process and their return to the contractile phenotype late in the process seem to be an important component of normal CCG.

However, VSMC phenotype has never been investigated during collateral development in the metabolic syndrome. The coronary vasculature of metabolic syndrome patients and JCR rats is characterized by neointimal lesions even very early in progression of coronary disease. In JCR rats, these lesions consist primarily of proliferative, synthetic VSMCs, and some macrophages. Consequently, we hypothesized that aberrant VSMC phenotype regulation, specifically, the inability of the VSMCs to assume the contractile phenotype played a causative role in impaired CCG in the metabolic syndrome.

VSMC phenotype is predominantly regulated by the competitive binding of serum response factor (SRF), coactivator, myocardin, and repressors, Krueppel-like factor 4 (KLF4) and phosphorylated Elk-1 (p-Elk-1), to the CARG box in the promoter of the smooth muscle (SM)-specific genes. If myocardin is bound to SRF, SRF binds to the CARG box resulting in the transcription of the SM-specific genes, including the SM-specific
contractile proteins, SM-myosin heavy chain (SM-MHC), SM-α-actin, calponin and caldesmin, and the contractile VSMC phenotype. If, however, KLF4 expression is increased, it displaces SRF, even if bound by myocardin, from the CARG box, and the SM-specific genes will not be transcribed, resulting in the synthetic VSMC phenotype. p-Elk-1 displaces myocardin from SRF; thus, an increase in its abundance also results in the synthetic VSMC phenotype.12

Over the last several years, it has been shown that the major negative regulators of Elk-1 and KLF4 abundance are microRNAs (miRs)-143 and -145, respectively.13 miR-145 directly targets KLF4 and downregulates its expression, thus enabling SRF binding to the CARG box, whereas miR-143 downregulates Elk-1.13 miR-145 also indirectly upregulates myocardin.13 miR-143 and -145 are highly enriched in VSMCs with negligible expression in other cell types.14 Not surprisingly, they have emerged as the major regulators of VSMC phenotype.13 miR-145 was sufficient to stimulate multipotent neural crest cells differentiation in VSMCs.15 Correlating with neointimal lesions marked by synthetic VSMCs, miR-145 was significantly reduced in patients with coronary artery disease.16 Similarly, our preliminary data showed downregulation of miR-145 in VSMCs in JCR rats. However, nothing is known about the possible involvement of miRs in the regulation of collateral development. Therefore, in the present study, we sought to determine whether restoring physiological miR-145 levels would restore the contractile VSMC phenotype and whether this would be sufficient to restore CCG in the metabolic syndrome.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Previous data from our laboratory demonstrate that RI induces maximal CCG in the normal (Sprague-Dawley [SD]) rats but does not stimulate CCG in metabolic syndrome (JCR) rats.3 Nine days of RI stimulate maximal CCG in the SD rats. Prolonging the duration of RI to 14, 21, or 28 days does not induce CCG in the JCR rats (Figure 1 in the online-only Data Supplement).

VSMCs Adopt the Contractile Phenotype in the Later Stages of CCG in Normal but Not in the Metabolic Syndrome Animals
VSMC contractile phenotype was evaluated by measurements of SM-specific contractile protein expression using SM-specific antibodies (Figure 1A). SM-MHC, expressed exclusively by contractile smooth muscle, decreased in the CZ on day 3 of RI in both rat phenotypes (∼75% versus day 0 RI), indicating a switch to a less contractile phenotype. However, in the later stages of CCG, although SM-MHC expression gradually and dramatically increased (5.5±0.5-fold on day 6 RI; 10.1±0.4-fold on day 9 RI) in the CZ of normal (SD) rats, no increase in SM-MHC expression was observed in the CZ of metabolic syndrome (JCR) rats (3.3±0.2-fold on day 6 RI; 3.3±0.2-fold on day 9 RI) (Figure 1B). Thus, the temporal expression of both myocardin and KLF4 correlated with the decrease in SM-MHC and SM-α-actin expression, basal KL4F4 expression was ∼2-fold higher in JCR versus SD rats and increased on day 3 of RI in both rat phenotypes (1.9±0.1-fold in SD; 2.0±0.5-fold in JCR), and then increased on days 6 and 9 of RI (1±0.2-fold) in SD, but not in JCR (2.8±0.15-fold on day 6 RI; 3.3±0.2-fold on day 9 RI) (Figure 1C). The expression of both myocardin and KLF4 correlated with the return of the SD but not JCR VSMCs to the contractile phenotype in the later stages of coronary collateral remodeling. No change in protein expression was observed during the course of CCG in the NZ (Figure II in the online-only Data Supplement).

In the aggregate, these data indicate a switch to less contractile, more synthetic VSMC phenotype in the early phase of CCG in both phenotypes. However, although in the normal phenotype the VSMCs return to the normal, adult contractile phenotype in the later stages of CCG, this does not occur in the metabolic syndrome phenotype. This aberrant VSMC phenotype switching correlates with the failure of native collaterals to enlarge in the metabolic syndrome phenotype as evident by the difference in collateral diameter between SD and JCR rats shown in Figure 1A and Figure VIII in the online-only Data Supplement, which is representative of the average collateral diameter in the 2 rat phenotypes (quantified in Figure 4B). Immunohistochemistry analysis in Figure VI in the online-only Data Supplement illustrates the connection between abundant SM-specific contractile protein expression and collateral expansion (growth) in normal (SD) animals versus low level of SM-specific contractile protein and high level of synthetic marker expression and failure to undergo collateral expansion (growth) in the metabolic syndrome (JCR) animals.

MicroRNA-145 Is Decreased in the Later Stages of CCG in the Metabolic Syndrome Animals
Although only miR-145 has been shown to directly downregulate KLF4 expression and upregulate myocardin available for JCR animals on day 9 of RI (Figure 1B). This increase was, however, not statistically significant and was confined to the lumen of immature collaterals that failed to undergo expansion (Figure 1A) and is likely attributable to the fact that although SM-α-actin is expressed predominantly by contractile smooth muscle, it can be expressed by other cell types relevant to the process of collateral remodeling, including synthetic VSMCs, macrophages, and myeloid progenitor cells.17 No change in SM-MHC or SM-α-actin expression was observed during the course of CCG in the NZ (Figure II in the online-only Data Supplement). Expression of nonmuscle myosin (NMHC-B), a marker of synthetic VSMC phenotype, inversely correlated with SM-MHC expression in both rat phenotypes (Figure 1B).

Because myocardin and KLF4 are major regulators (myocardin-positive; KLF4-negative) of SM-specific contractile protein expression and targets of miR-145, we next evaluated their expression along the time course of CCG in the 2 rat models. Basal myocardin expression was ∼1.5-fold higher in SD versus JCR rats correlating with SM-specific contractile protein expression. Myocardin decreased in both SD and JCR rats on day 3 of RI (∼30% versus day 0 RI), then increased on days 6 (2.2±0.4 fold) and 9 (2.0±0.2 fold) of RI in SD, but not in JCR rats (Figure 1C). Correlating with increased basal SM-MHC and SM-α-actin expression, basal KL4F4 expression was ∼2-fold higher in JCR versus SD rats and increased on day 3 of RI in both rat phenotypes (1.9±0.1 fold in SD; 2.0±0.5 fold in JCR), and then increased on days 6 and 9 of RI (1±0.2 fold) in SD, but not in JCR (2.8±0.15 fold on day 6 RI; 3.3±0.2 fold on day 9 RI) (Figure 1C). Thus, the temporal expression of both myocardin and KLF4 correlated with the return of the SD but not JCR VSMCs to the contractile phenotype in the later stages of coronary collateral remodeling. No change in protein expression was observed during the course of CCG in the NZ (Figure II in the online-only Data Supplement).
Hutcheson et al

miR-145 Restores Collateral Growth

Figure 1. A, SD or JCR rats underwent 9 days of resultant myocardial ischemia (RI). Representative images of consecutive cardiac cross-sections stained with anti-smooth muscle myosin heavy chain (anti-SM-MHC; top left), anti-SM-α-actin (top right), anti-nonmuscle myosin heavy chain (anti-NMHC-β; top right), SM-α-actin (red), and anti-CD31 (green; bottom right). *for wider image field see Figure VIII in the online-only Data Supplement) are shown. The difference in collateral diameter between SD and JCR rats is representative of the average collateral diameter in the 2 rat phenotypes.

B, SD or JCR rats underwent 0, 3, 6, or 9 days of RI. Tissue samples were collected from the NZ or the CZ. SM-MHC (top left), SM-α-actin (top right), and NMHC-β (bottom) expression was determined by Western Blot. Top: Cumulative data, n=7. Bottom: Representative Western blots in the CZ using anti-SM-MHC and anti-SM-α-actin and in the CZ and NZ using anti-NMHC-β antibodies. β-tubulin is the loading control.

C, Same as B except that antimyocardin (left) and anti-Krueppel-like factor 4 (anti-KLF4; right) antibodies were used, n=7. β-tubulin is the loading control.
miR-143 and miR-145 expression during CCG in normal versus metabolic syndrome rats. There was no difference in basal miR-143 expression between SD and JCR rats, and RI did not change miR-143 expression in either rat phenotype. In contrast, baseline miR-145 levels were ≈50% lower in JCR versus SD rats. miR-145 decreased to a similar level (≈50% versus day 0 RI) in both rat phenotypes on day 3 RI. However, in the SD rats, miR-145 levels then increased back toward baseline in the later stages of CCG, reaching baseline on day 6 and increasing nearly 2-fold on day 9 of RI. In contrast, in the JCR rats, miR-145 levels remained decreased (≈50% versus day 0 RI on days 6 and 9 RI) (Figure 2A).

Our in situ hybridization studies show VSMC-specific miR-145 expression as expected. In addition, we consistently demonstrate both stronger intensity of staining and larger numbers of miR-145–positive cells in SD rats on day 9 of RI (Figures 2B and 3A). These results suggest that the observed increase in miR-145 levels on day 9 of RI, versus baseline, in the SD rats is a consequence of a greater total number of contractile VSMCs consistent with collateral vessel enlargement and concomitant medial thickening. In contrast, decreased miR-145 levels in JCR rats on day 9 of RI, versus baseline, are a consequence of both decreased miR-145 expression within individual cells in JCR rats, suggestive of an RI-induced individual VSMC switch toward a less contractile phenotype, which could be a causative factor in lack of collateral expansion, and lack of medial thickening as a consequence of lack of collateral expansion.

**MicroRNA-145 Delivery Restores VSMC Contractile Phenotype in the Later Stages of CCG in the Metabolic Syndrome**

MicroRNA-145 was delivered via an Adv vector (miR-145-Adv). VSMC-specific expression was ensured by placing the miR-145 sequence under the direction of an SM-specific SM22a promoter and confirmed by in situ hybridization (Figure 3A). The amount of miR-145-Adv delivered was adjusted until there was no significant difference between miR-145 levels expressed in treated JCR rats and untreated SD rats (0.34±0.03 JCR RI+miR-145-Adv versus 0.31±0.03 SD RI pg miR-145/200 ng total RNA) (Figure 3A). miR-145-Adv specificity was confirmed by the fact that it did not alter expression of other cardiac- or vascular-enriched miRs, including miR-21 and miR-143 (Figure II in the online-only Data Supplement).

Because myocardin and KLF4 are targets of miR-145, we assessed the effect of miR-145-Adv delivery on their expression. Restoration of miR-145 levels in JCR rats to those found in the SD rats on day 9 of RI by miR-145-Adv delivery restored both myocardin (≈2-fold increase versus NZ for JCR RI+miR-145-Adv and SD RI versus ≈1-fold for JCR RI) and KLF4 (≈1-fold increase versus NZ for JCR RI+miR-145-Adv and SD RI versus ≈3-fold for JCR RI) expression in the CZ of JCR rats to those found in the SD rats (Figure 3B). Although KLF5 is a known regulator of smooth muscle phenotype and a known target of miR-145, miR-145-Adv did not affect KLF5 expression. KLF5 expression was also not changed in response to RI in either SD or JCR rats (Figure VII in the online-only Data Supplement).

Importantly, SM-MHC and SM-α-actin expression on day 9 of RI in the CZ of miR-145-Adv–treated JCR rats equaled that in the untreated SD rats (≈10-fold increase versus NZ for JCR RI+miR-145-Adv and SD RI versus ≈1-fold for JCR RI; Figure 3C), indicating that the expression of physiological miR-145 levels was sufficient to restore the VSMC contractile phenotype in the later stages of CCG in the metabolic syndrome.

miR-145-Adv also significantly increased SM-MHC and SM-α-actin expression in the NZ of JCR rats (≈4- and ≈3-fold, respectively; Figure 3C). Similarly, miR-145-Adv increased myocardin and decreased KLF4 expression ≈1.5-fold in the NZ of JCR rats (Figure 3B). The effects in the NZ were likely lesser in magnitude than in the CZ because transduction efficiency in the NZ was lesser than in the CZ (≈90% of VSMCs...
in the CZ versus 40% of VSMCs in the NZ; Figure III in theonline-only Data Supplement) because of our method of Advdelivery, which apparently favors retention of the constructin the CZ over the NZ. Enhanced green fluorescent protein-Adv delivered to a separate group of SD and JCR rats, atthesame concentration and on the same day of RI because themiR-145-Adv had no effect on SM-specific contractile protein,KLF4, or myocardin expression in the CZ or the NZ,indicating that the observed effects were miR-145 specific(Figure 3B and 3C).

Angiotensin-converting enzyme (ACE) is another relevantverified target of miR-145. 18 To determine whether ACEdownregulation was responsible for the beneficial effectsof miR-145 delivery on CCG in the JCR rats, we examinedthe effects of miR-145-Adv on ACE expression. miR-145-Adv did not effect ACE expression in either SD or JCR rats.
Moreover, ACE expression was not different between the 2 rat phenotypes and did not change in response to RI (Figure IV in the online-only Data Supplement). The beneficial effect of miR-145 on CCG was also not attributable to the amelioration of the metabolic syndrome because miR-145-Adv had no effect on weight, plasma insulin, glucose, triglycerides, high-density lipoprotein, low-density lipoprotein, or blood pressure in JCR rats.

**MicroRNA-145 Delivery Completely Restores CCG in the Metabolic Syndrome**

Finally, we investigated the effects of miR-145-Adv on CCG in the metabolic syndrome. Myocardial blood flow in the CZ and the NZ was measured in the same rats in which the physiological amount of miR-145 (equal to that seen in SD rats on day 9 of RI) delivered via the miR-145-Adv was shown to restore the VSMC contractile phenotype in Figure...
3. On day 9 of RI, mean CZ flow was 2.1±0.05 mL/min per g in miR-145-Adv–treated JCR rats versus 0.15±0.03 mL/min per g in untreated JCR rats and 1.89±0.08 mL/min per g in untreated SD rats. Collateral/normal zone flow ratio was 0.93±0.09 (JCR RI+miR-145-Adv), 0.12±0.02 (JCR RI), and 0.87±0.02 (SD RI; Figure 4C).

This complete restoration of normal blood flow was coupled to increased arteriolar density, which equaled that observed in the normal (SD) rat in response to RI (2.8±0.5 versus 2.6±0.6). Capillary density showed a tendency to increase significantly in some JCR animals (2 of 7) treated with the miR-145-Adv, but there was no overall significant increase in capillary density (Figure 4A). RI alone likewise had no effect on capillary density in JCR rats but caused a significant increase in capillary density in SD rats (=1.8-fold versus baseline). Because wall thickness was comparable between groups on day 9 RI, measurements of wall thickness to lumen diameter ratios in Figure 4B further confirm the significant increase in arteries with expanded lumen in response to miR-145 delivery in JCR rats (wall thickness/lumen diameter was =4 for JCR RI versus =0.25 for JCR RI+miR-145-Adv and SD RI). Because of the anatomy of the coronary vasculature in rats, it is not possible to isolate collateral arteries; therefore, we used the fact that only actively remodeling vessels in the heart would contain proliferating cells to distinguish collaterals from the preexisting, nonremodeling vasculature. Thus, only coronary arteries, which also stained positive for the proliferating nuclear antigen (Figure 4B), were used for these measurements.

Taken together, these results indicate that restoration of physiological miR-145 levels in the later stages of CCG in the metabolic syndrome was sufficient to completely restore blood flow and that CCG, and not angiogenesis, is the major mechanism by which this occurred. In contrast, delivery of miR-145-Adv to normal (SD) animals to overexpress miR-145 significantly decreased CCG (Figure 5A). Delivery of anti–miR-145 to downregulate native miR-145 levels likewise blocked CCG in normal animals (Figure 5B). Overexpression of miR-145 and complete inhibition of native miR-145 expression by antimiR-145 was confirmed by real-time polymerase chain reaction (Figure 5A and 5B). Enhanced green fluorescent protein-Adv delivered to a separate group of SD and JCR rats, at the same concentration and on the same day of RI as the miR-145-Adv, had no effect on CCG (Figures 4A, 4B, 4C, and 5A). Similarly, scrambled antimiR, which did not lower native miR-145 levels, had no effect on CCG (Figure 5B). These results indicate that the observed effects on CCG were miR-145 specific.

**Discussion**

The most important novel findings of our study are as follows: (1) miR-145 expression is decreased at baseline and in the later stages of CCG in the coronary VSMCs of metabolic syndrome animals which correlates with failure of the VSMCs to assume the contractile phenotype and impaired CCG; and (2) restoration of physiological miR-145 expression in the metabolic syndrome enables the VSMCs to assume the contractile phenotype in the later stages of collateral remodeling and completely restores CCG. Our data further demonstrate that the normal physiological level of miR-145 is essential for collateral remodeling. Downregulation of miR-145 in normal animals inhibited CCG. However, although physiological miR-145 amounts completely restored CCG in the metabolic syndrome animals, overexpression of miR-145 severely compromised CCG in normal animals. Greater than physiological levels of miR-145 also induced a hypercontractile and hypertrophied phenotype in VSMCs in pulmonary artery hypertension. Reduction of miR-145 levels by antimiR-145 restored normal VSMC phenotype and reduced pulmonary artery pressure and pulmonary artery hypertension symptoms. Taken together, these observations emphasize the importance of physiological levels of miR-145 for normal vascular structure and function.

Another interesting aspect of our study is that our data convincingly demonstrate that RI alters miR-145, but not miR-143 expression, in both the normal and the metabolic rat models of CCG. miR-143/-145 are transcribed as a bicistronic unit with common regulatory elements and are typically upregulated or downregulated together. However, miR-143 and miR-145 target different downstream effectors. Although miR-145 targets myocardin, KLF4, and KLF5, miR-143 targets Elk-1. Importantly, upregulation of miR-145 was sufficient to induce expression of SM-specific marker genes, and its inhibition was sufficient to downregulate expression of these markers. miR-145 alone has been shown to be sufficient to stimulate multipotent neural crest cells differentiation into VSMCs. These data indicate that miR-145 may be the primary regulator of VSMC phenotype in adult VSMCs. Our results demonstrate that the miR-143/-145 cluster is differentially regulated in CCG and that downregulation of miR-145, but not miR-143, underlies impaired CCG in the metabolic syndrome.

miR-145 is also the most abundant miR in normal, healthy arterial walls, and VSMCs and has been shown to be the major determinant of VSMC phenotype in vivo. Restoration of miR-145 expression limited neointima formation in response to vascular injury by promoting KLF4 downregulation and VSMC contractile protein expression. This is particularly relevant with respect to our results, which show an increase in miR-145 in normal (SD) rats, but not in the metabolic syndrome (JCR) rats, in the later stages of CCG when the neointima resolves and luminal expansion occurs in the normal, but not the metabolic syndrome, phenotype. miR-145 has also been shown to be able to suppress cell proliferation in many human cancers. However, its antiproliferative effects in VSMCs seem to be mediated by restoration of the contractile VSMC phenotype. KLF4 also targets many genes that regulate VSMC proliferation and migration. Therefore, because KLF4 binding to the CArG box and subsequent inhibition of SM-specific gene transcription is regulated by its relative abundance, high expression of the SM-specific genes is most often associated with low VSMC proliferation and migration rates, whereas low expression of SM-specific genes is most often associated with high proliferation and migration rates. miR-145 did not effect KLF5 expression in our study, nor did KLF5 change in response to RI, suggesting that in contrast to KLF4, KLF5 does not play a role in VSMC phenotype regulation during CCG. Other studies have reported differential regulation of KLF4 and KLF5.
For example, KLF4 expression was increased in response to oxidative stress in neonatal myocytes, whereas KLF5 was unchanged.24

A single study explored VSMC phenotype switching during CCG in normal dogs and correlated the synthetic, proliferative phenotype with the early phase and the contractile phenotype with the late phase of CCG.25 However, a definitive cause–consequence relationship between VSMC phenotypic switching and collateral development has not been established. In contrast to upregulating medial thickness via VSMC hypertrophy, miR-145 has been shown to limit intimal thickening by decreasing neointimal formation in response to angioplasty-type vascular injury through promoting VSMC phenotype switching from synthetic to contractile.18 In our study, delivery of miR-145 at the transition from inward to outward remodeling likewise improves CCG in the metabolic syndrome by facilitating synthetic to contractile VSMC phenotype switching and resolution of the neointima, suggesting universal consequences of physiological levels of miR-145 expression in the vasculature.

Very recently, ACE was verified as a target of miR-145.18 The significance of this regulation in vivo was unknown. Our previous studies have shown that angiotensin II type 1 receptor inhibition and inhibition of angiotensin II type 1 receptor–dependent reactive oxygen species production was beneficial for CCG in the metabolic syndrome.9 However, our current results demonstrate that the beneficial effects of miR-145 are independent of ACE downregulation, and therefore likely, of angiotensin II–mediated signaling.

Our study does not definitively prove that the capacity of VSMCs to assume the contractile phenotype in the later stages of collateral remodeling fully accounts for the ability of physiological miR-145 levels to restore CCG in the metabolic syndrome. However, the ability to switch from the synthetic to the contractile VSMC phenotype dictates important functions of the vascular wall in addition to the structural integrity of the tunica media itself. First, as already mentioned, contractile VSMCs also assume low-proliferation rates, which may aid in the clearance of the neointima and luminal expansion. Second, contractile versus synthetic VSMCs produce different extracellular matrix components and proteases, including matrix metalloproteinases, some of which play critical roles in the bioavailability and balance of proangiogenic growth factors and antiangiogenic peptides (growth inhibitors), as well as endothelial cell adhesion and survival. Thus, it is feasible that converting the VSMCs to the physiological, contractile phenotype overcomes a plethora of impediments to successful collateral development, which are present in the metabolic syndrome, including documented endothelial dysfunction, endothelial progenitor cell (EPC) dysfunction, and the altered growth factor/growth inhibitor balance. On the contrary, endothelial dysfunction, a hallmark of metabolic syndrome in humans and animals models including the JCR rat,26 could be the cause of decreased miR-145 levels and consequent propensity toward the synthetic VSMC phenotype. A recent cell culture study has shown that miRs-143 and -145 were in fact expressed as the ratio of collateral/normal zone (CZ/NZ) flows on day 9 of RI. B. Same as A except that rats were treated with anti–miR-145 or scrambled antimiR on day 6 of RI where indicated.
Another limitation of our study is that we did not evaluate the effect of miR-145 delivery on CCG under conditions of maximal vasodilation. We have previously shown that adenosine had no effect on coronary blood flow in the JCR:LA-cp rat but increases it in the donor rat. Thus, we cannot exclude the possibility that altered vasoactive properties of the coronary vasculature could not affect collateral-dependent blood flow in this study.

Nothing is known about the possible involvement of miRs in the regulation of collateral development. This is the first study to demonstrate a definitive and important role not only for miR-145 specifically but also for miRs in general in the regulation of collateral development. Furthermore, the potential for using miRs for the induction of CCG in conditions where it is impaired remains completely unexplored. This study is the first to demonstrate the feasibility of such an approach. Long-term patency of bypass grafts remains a vexing problem in metabolic syndrome and diabetic patients, with frequent need for revascularization. Thus, the intracardiac route of delivery is specifically applicable during open-heart surgery where stimulation of CCG immediately after coronary bypass grafting (CABG) has been shown to significantly increase the long-term patency of the grafts. Because of previous adverse effects using AdV-mediated delivery in humans, AdV-free delivery would be optimal for effective miR-145–based therapy in humans. Such delivery systems are already available for in vivo miR delivery and could be applicable to miR-145 specifically, although with caution, because miR-145 has been shown to be able to convert multiple progenitor cells into VSMCs.

**Sources of Funding**

This study was supported by grants from the American Heart Association 11PRE7060011 to R. Hutcheson and National Institutes of Health R01HL093052 to P. Rocic.

**Disclosures**

None.

**References**


---

**Hutcheson et al miR-145 Restores Collateral Growth**

---
in conjunction with enhanced Akt activation restores coronary collateral
29. Reed R, Potter B, Smith E, Jadhav R, Villalta P, Jo H, Rocic P. Redox-
sensitive Akt and Src regulate coronary collateral growth in metabolic

30. Sung SH, Wu TC, Huang CH, Lin SJ, Chen JW. Prognostic impact of
body mass index in patients undergoing coronary artery bypass surgery.
31. Hu R, Ma CS, Nie SP, et al. Effect of metabolic syndrome on prognosis
and clinical characteristics of revascularization in patients with coronary

Significance
This is the first study to establish an essential role for miR-145, specifically, and microRNAs, in general, in collateral development, as well
as to definitively investigate the importance of vascular smooth muscle phenotype during collateral development in metabolic syndrome.
Furthermore, this study is the first to demonstrate the feasibility of using microRNAs for induction of collateral growth in conditions where it
is impaired, including metabolic syndrome. These results are highly significant because miR-145 expressed at physiological levels (versus
overexpressed) was sufficient to completely restore collateral growth in response to normal stimulus. This is important because miR-145
overexpression is associated with hypercontractile vascular smooth muscle phenotype in pulmonary hypertension. Finally, people with
metabolic syndrome are more likely to require early coronary revascularization complicated by multiple occlusions, high procedural risk,
and early bypass graft closure. Induction of collateral growth by miR-145 delivery in this patient population may be especially beneficial as
adjunct therapy.
MicroRNA-145 Restores Contractile Vascular Smooth Muscle Phenotype and Coronary Collateral Growth in the Metabolic Syndrome
Rebecca Hutcheson, Russell Terry, Jennifer Chaplin, Erika Smith, Alla Musiyenko, James C. Russell, Thomas Lincoln and Petra Rocic

Arterioscler Thromb Vasc Biol. 2013;33:727-736; originally published online February 7, 2013; doi: 10.1161/ATVBAHA.112.301116

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/33/4/727

Data Supplement (unedited) at:
http://atvb.ahajournals.org//subscriptions/

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology 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 Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
**SUPPLEMENTAL MATERIAL**

**DETAILED METHODS**

*Rat model of coronary collateral growth (CCG)/repetitive ischemia (RI).* Male, 10-12 week old Sprague-Dawley (SD; Charles Rivers, Wilmington, MA) (300–350g) or JCR:LA-cp rats (JCR; James C. Russell, University of Alberta, Edmonton, Canada) (650–700g) were used for chronic (0-9 days) implantation of a pneumatic occluder over the left anterior descending coronary artery (LAD). A suture was passed under the proximal portion of the LAD and the occluder was sown onto the surface of the heart. The occluder catheter was externalized between the scapulae. When the occluder is inflated, the suture is pulled towards the surface of the heart and the LAD is occluded. The LAD perfusion territory is termed the collateral-dependent zone (CZ) because perfusion in this area, while the LAD is occluded, depends on the development of coronary collaterals. The animals underwent the RI protocol consisting of eight 40sec occlusions, once every 20min (2h, 20min total) followed by a rest period of 5h, 40min. This 8-hour cycle was repeated 3 times/day for 0-9 days. Surgical procedures were performed in accordance with the Animal Welfare Act and are approved by the IACUC of the University of South Alabama.

The JCR rat is a cross between the lean LA/N Zucker and the spontaneously hypertensive obese (SHROB) rat developed in the laboratory of Dr. Carl Hansen at the National Institutes of Health and sent to Dr. James C. Russell. By 8 weeks of age, the JCR rats develop obesity with fatty liver, insulin resistance with glucose intolerance, complex dyslipidemia (low HDL, high LDL and vLDL), and vasculopathy characterized by decreased endothelium-dependent and -independent vasorelaxation and intimal lesions morphologically identical to early atherosclerotic lesions in humans. By 12 weeks, the rats exhibit widespread atherosclerosis, left ventricular hypertrophy and myocardial and cerebral (micro)infarctions. At 16+ weeks, the rats are prone to stroke and myocardial infarction, and at 18+ weeks, they develop heart failure. Like the development of the metabolic syndrome and cardiovascular disease in humans, the apparent complexity of the cardio-metabolic phenotype exhibited by the JCR rats is suspected to be multifactorial and polygenetic in etiology.1,2

**Adenoviral constructs: miR-145-Adv and EGFP-Adv.** The miR-145-Adv construct was made and purified by ViraQuest Inc., North Liberty, IA. miR-145 precursor sequence CACCTGTGCC TCACGGTCGA GTTTTCACAG GAATCCCTTA GATGCTAAGA TGGGGTTCC TGGAATACT GTTCGGAGT TCATGGTT was inserted into an Adv vector behind the SM22a promoter (gift from Dr. Thomas Lincoln, University of South Alabama, Mobile, AL) to insure smooth muscle (SM)-specific delivery. The EGFP-Adv was on an identical viral backbone with EGFP expression under the direction of the SM22a promoter. The Adv constructs were injected at 1.5x10**12** plaque-forming units (PFU) in isotonic saline (100µL) by direct injection into the LV cavity followed by a 40 sec LAD occlusion as described previously3 on day 4 of RI to express maximal levels of mature miR-145 beginning on day 6 of RI and through the duration of the RI protocol.

**antimiR-145.** LNA-modified antimiR-145 (Exiqon, Woburn, MA) was delivered at 2 mg/kg in 100 µL of sterile saline via intracardiac injection directly into the LV cavity according to modification of previously used protocols for tail vein injection4 on day 6 of RI. Scrambled LNA-antimiR sequence was used as control.

**Western blot.** Unperfused hearts were excised, CZ separated from the non-ischemic, normal zone (NZ), and snap-frozen in liquid nitrogen before homogenization in the modified RIPA lysis buffer containing 0.1% SDS and 1% Triton. Equal amounts of protein (10µg) were separated by SDS-PAGE and transferred to Hybond-ECL nitrocellulose membranes. Anti-SM-MHC (1:1,000, Abcam, Cambridge, MA), anti-SM-α-actin (1:5,000, Sigma, St. Louis, MO), anti-NMH-B (1:2,000, Abcam), anti-Klf4 (1:1,000, (Cell Signaling) Millipore, San Diego, CA), anti-Klf5 (1:500, Abcam), anti-myocardin (1:1,000, Abcam), anti-ACE (1:1,000, Abcam) and anti-β-tubulin (1:5,000, Santa Cruz, CA) were used for Western blotting. Bands were visualized by enhanced chemiluminescence (ECL, Amersham, Piscataway, NJ) and
quantified using Un-Scan-It Image software (Silk Scientific Corporation, Orem, UT). Experiments were n=7 animals per time point (day of RI) and were analyzed by two-way ANOVA followed by Bonferroni correction. p<0.05 determined statistical significance.

**Immunohistochemistry (IHC).** Unperfused, formalin-fixed, paraffin-imbedded cardiac tissue was cut into 10µm sections. Primary anti-SM-MHC (1:200, Abcam, Cambridge, MA), anti-SM-α-actin (1:500, Sigma, St. Louis, MO), anti-NMHC-B (1:200, Abcam), anti-proliferating nuclear antigen (PCNA) (1:200, Millipore, San Diego, CA) and anti-CD31 (1:200, Millipore) and secondary Alexa488- and Alexa568-conjugated (Invitrogen, Carlsbad, CA) antibodies were used. Red (Alexa568) fluorescence was visualized and representative images collected using a Nikon fluorescent microscope equipped with Nikon Elements software. Images are representative of n=7 animals per time point (day of RI) from 10 consecutive cardiac cross-sections per animal and 5 separate fields per slide.

**Histology.** Arteriolar and capillary density, lumen diameter, wall thickness and wall thickness to lumen diameter ratio were measured in cardiac cross-sections on day 9 of RI. Formalin-fixed, paraffin-imbedded cardiac tissue was cut into 5µm sections. For measurements of arteriolar and capillary densities, a 1mm² grid was superimposed over SM-MHC-stained (for arterioles/arteries) and hematoxylin/eosin-stained (for capillaries and arterioles/arteries) cardiac cross sections and vessels <20µM in diameter and >20µM in diameter inside the grid were counted as capillaries and arterioles/arteries, respectively. Fully formed arterioles/arteries were additionally identified by the presence SM-MHC-positive VSMCs. Larger arteries were distinguished from veins by assessment of vessel wall thickness, where wall thickness/lumen diameter ratio >0.25 signified an artery and <0.25 a vein. Average collateral damaeter was determined by averaging all SM-MHC- and PCNA-positive vessels in SD rats and all NMHC-B-, SM-α-actin- and PCNA-positive vessels in JCR rats on day 9 of RI. NMHC-B and SM-α-actin were used as VSMC markers due to lack of contractile, SM-MHC-positive VSMC in the JCR rats. Data were collected from n=7 animals per group from 5 consecutive cross-sections per animal and 5 separate lmm² grids per slide.

Measurements of wall thickness and lumen diameter were obtained from the same hematoxylin/eosin-stained slides and confirmed on PCNA and SM-α-actin co-stained adjacent cross-sections. However, to select for collateral arteries vs. pre-existing vessels, only arteries which also stained positive for PCNA were included in data analysis. Data were collected from n=7 animals per group from 5 consecutive cross-sections per animal and 5 separate fields per slide. Results were analyzed by 2-way ANOVA followed by Bonferroni correction. p<0.05 determined statistical significance.

**microRNA (miR) quantitation.** Total RNA was isolated from the NZ and the CZ with QIAzol™ followed by small RNA isolation with miRNeasy Mini Kits™ (QIAGEN, Valencia, CA). Total and small RNA concentration and quality were determined by absorbance at 260/280 nm. The ratio of 18S and 28S ribosomal RNA and the degree of DNA contamination were assessed by agarose gel electrophoresis with Sybr Green II staining. cDNA synthesis and quantitative RT-PCR were performed with TaqMan miR assays using 250 ng RNA. Absolute quantities of miR-143 and -145 in CZ and NZ cardiac tissue were obtained by quantitative RT-PCR using standards constructed from a dilution series of miR-143 and -145 standards (Integrated DNA Technologies, Coralville, IA). Experiments were n=7 animals per time point (day of RI) and were analyzed by two-way ANOVA followed by Bonferroni correction. p<0.05 determined statistical significance.

**In situ hybridization (ISH).** ISH was performed using the Exiqon miRCURY LNA microRNA ISH Optimization Kit 7 FFPE (Exiqon, Woburn, MA) using double DIG*-labeled miRCURY LNA miR-145 detection probes at a concentration of 10 nM. Scrambled microRNA probe was used for a negative control and 5’-DIG-labeled U6 snRNA was used for a positive control. Hybridization was performed on
formalin-fixed, paraffin-embedded tissue, cut into 5µM cross-sections, mounted on slides and handled according to the manufacturer’s protocol. Images are representative of n=7 animals per time point (day of RI) from 10 consecutive cardiac cross-sections per animal and 5 separate fields per slide.

**Myocardial and collateral-dependent blood flow measurements.** Color microspheres (5x10^5, 15µM diameter) labeled with samarium (day 0 RI (initial surgery) or gold (day 10 RI) were injected into the LV during LAD occlusion. Arterial reference blood samples (carotid) and heart tissue from the NZ and the CZ were collected, weighed and sent to BioPal (Worcester, MA) for analysis. Blood flows to the NZ and the CZ (ml/min/g) were calculated from the formula: Blood Flow=[(radioactive counts in myocardial tissue)X(blood reference withdrawal rate)/(radioactive count in blood reference)]/(weight of myocardial tissue). Blood flows were measured in the following groups of animals: SD RI, JCR RI, JCR RI+miR-145-Adv. Results were expressed as the CZ/NZ flow ratio on day 9 of RI. All experiments were n=7 animals per group. Results were analyzed by 2-way ANOVA followed by Bonferroni correction. p<0.05 determined statistical significance.

**REFERENCES**


Figure I. SD and JCR rats underwent 9, 14, 21 or 28 days of RI as indicated. Coronary blood flow was measured in the CZ and the NZ using microspheres during LAD occlusion and is expressed as the ratio between CZ and NZ flows. *p<0.05 vs. day 0 RI, #p<0.05 vs. SD, n=5.
**Figure II.** A. SD or JCR rats underwent 0, 3, 6 or 9 days of RI. Tissue samples were collected from the NZ or the CZ. SM-MHC (left) and SM-α-actin (right) expression was determined by Western Blot. Top: Cumulative data, n=7. Bottom: Representative Western blots in the NZ using anti-SM-MHC and anti-SM-α-actin antibodies. β-tubulin is the loading control. C. Same as B except anti-myocardin (left) and anti-Klf4 (right) antibodies were used, n=7. β-tubulin is the loading control.
**Figure III.** JCR rats were treated with miR-145-Adv on day 4 of RI where indicated, and SD or JCR rats underwent 9 days of RI. Tissue samples were collected from the NZ and CZ. miR-21 (**A**), miR-143 (**B**) levels were determined by RT-PCR. Levels in the CZ are shown, n=7.
Figure IV. A. SD or JCR rats underwent 0, 3, 6 or 9 days of RI. Tissue samples were collected from the NZ or the CZ. ACE expression was determined by Western Blot. Top: Cumulative data, n=7. Bottom: Representative Western blots in the CZ and NZ using anti-ACE antibodies. β-tubulin is the loading control for CZ samples. B. JCR rats were treated with miR-145-Adv or EGFP-Adv on day 4 of RI where indicated, and SD or JCR rats underwent 9 days of RI. Tissue samples were collected from the NZ and CZ. ACE expression was determined by Western Blot. Top: Cumulative data, n=7. Bottom: Representative Western blots in the CZ and NZ using anti-ACE antibodies. β-tubulin is the loading control.
Figure V. JCR rats were treated with SM22a-EGFP-Adv on day 4 of RI and underwent 9 days of RI. Representative images of EGFP fluorescence in cardiac cross-sections in the NZ and CZ are shown.
**Figure VI.** SD and JCR rats underwent 9 days of RI. Representative images of consecutive myocardial cross sections stained with SM-MHC to identify contractile VSMCs, NMHC-B to identify synthetic VSMCs, SM-α-actin to identify both contractile and synthetic VSMCs, and PCNA to identify proliferating cells as indicated are shown. The difference in collateral diameter between SD and JCR rats is representative of the average collateral diameter in the two rat phenotypes. *Note the difference in 30µM size bars.
Figure VII. A. SD or JCR rats underwent 0, 3, 6 or 9 days of RI. Tissue samples were collected from the NZ or the CZ. Klf5 expression was determined by Western Blot. Top: Cumulative data, n=7. Bottom: Representative Western blots in the CZ and NZ using anti-ACE antibodies. β-tubulin is the loading control for CZ samples. B. JCR rats were treated with miR-145-Adv or EGFP-Adv on day 4 of RI where indicated, and SD or JCR rats underwent 9 days of RI. Tissue samples were collected from the NZ and CZ. ACE expression was determined by Western Blot. Top: Cumulative data, n=7. Bottom: Representative Western blots in the CZ and NZ using anti-Klf5 antibodies. β-tubulin is the loading control.
Figure VIII. SD and JCR rats underwent 9 days of RI. Representative images of consecutive myocardial cross sections stained with SM-α-actin (Alexa-568-conjugated secondary antibody, red) to identify VSMCs and CD31 (Alexa-488-conjugated secondary antibody, green) to identify ECs are shown. The difference in vessel diameter between SD and JCR rats is representative of the average vessel diameter in the two rat phenotypes. *Note the difference in 50µM size bars.