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Original article

Role of miR-145 in cardiac myofibroblast differentiation

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ABSTRACT

Following a myocardial infarction (MI), fibroblasts differentiate to myofibroblasts, which possess some of the characteristics of smooth muscle cells (SMCs) and contribute to wound healing. Previous studies suggested that the miR-143/-145 cluster plays a critical role in SMC differentiation. Therefore, we determined whether miR-145 promoted differentiation of cardiac fibroblasts to myofibroblasts. Following coronary occlusion in mice, myocardial miR-145 expression was downregulated at 3 days but was restored at 7 days. In vitro studies showed that hypoxia also downregulated miR-145 in cardiac fibroblasts. The number of α-smooth muscle actin (α-SMA) positive cells in fibroblast cultures was employed to determine their transdifferentiation to cardiac myofibroblasts and was increased by 73.5% after transient transfection with miR-145. Ultrastructural analysis of α-SMA stress fibers revealed that ~95% of the α-SMA^{+} cells treated with miR-145 organized their actin-filament bundles with a specific orientation compared to only 15% in the scrambled control group. This orientation of the SMA bundles and their integration with the filamentous actin fibers of the cytoskeleton permit infarct wound contraction. Structural and functional studies showed that miR-145 induced a myofibroblast phenotype, and miR-145 also potentiated the production of mature collagen by myofibroblasts. Repression of KLF5, a target of miR-145, was validated by a chimeric luciferase construct tagged with the full-length 3′-UTR of KLF5. A dramatic decrease in KLF5 and a corresponding increase in myocardin expression were observed after transfecting cultured fibroblasts with miR-145. Similar results were found in vivo: the transient decrease in miR-145 expression 3 days post-MI was associated with an increase in KLF5 and a decrease in myocardin. In addition, in vivo delivery of a miR-145 antagomir 1 day prior to and 2 and 6 days after MI decreased myofibroblast formation and increased scar size. The antagomir also reversed the suppressed expression of KLF5 protein in the scar region at day 7 after MI. In summary, we describe a novel association between miR-145 and fibroblast differentiation toward myofibroblasts. These observations provide a new approach to promote endogenous scar healing and contracture by stimulating the transdifferentiation of cardiac fibroblasts to myofibroblasts.

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1. Introduction

In the unstressed myocardium, cardiac-resident fibroblasts have no contractile microfilaments or stress fibers, exhibit few actin-associated cell–cell or cell–matrix contacts, and produce minimal amounts of extracellular matrix [1]. In the aftermath of a myocardial infarction (MI), fibroblasts are activated, proliferate, and then differentiate to myofibroblasts, which have both fibroblast and smooth muscle cell (SMC) characteristics and contribute to wound healing and scar contracture. Fully differentiated myofibroblasts express α-smooth muscle actin (α-SMA) and other SMC markers [2,3], and they are able to synthesize and secrete fibrillar collagen types I and III [4]. They also possess contractile properties that may contribute to a smaller and stronger scar, preventing infarct expansion and ventricular dilatation. As the scar matures, myofibroblasts undergo apoptosis and are removed from the healed infarct region, which is then composed predominantly of cross-linked collagen and other matrix proteins, with a low cellular content. Virag and Murry found that proliferation of fibroblasts and endothelial cells in the mouse heart peaks within 4 days of the infarction and decreases to less than 0.5% at 2 weeks [5]. These dynamic changes in cardiac myofibroblast proliferation, migration, and differentiation during MI remodeling are similar to vascular smooth muscle cell (VSMC) invasion into a vascular neointimal lesion. miR-143 and -145

Abbreviations: MI, myocardial infarction; miRNA, microRNA; VSMC, vascular smooth muscle cell; α-SMA, alpha smooth muscle actin; SMC, smooth muscle cell; SMHC, smooth muscle myosin heavy chain; TGF-β, transforming growth factor beta.

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have been demonstrated to control VSMC activity [6,7], and therefore we determined whether miR-145 influences the response of cardiac fibroblasts to MI.

A large number of miRNAs are associated with the cardiac response to injury [8]. However, very few studies have investigated the influence of miRNAs on cardiac remodeling after coronary artery ligation [9]. Infarct contraction during healing has been proposed to result from phenotypic conversion of cardiac fibroblasts to myofibroblasts [10]. The signaling mechanisms controlling this process have not been fully elucidated, but miRNAs are excellent candidates to provide epigenetic orchestration of these events. A similar response has been reported in VSMCs, and miRNAs have been demonstrated to modulate the phenotypic response of VSMCs to injury [6]. The role of miR-143 and -145 in the regulation of SMC differentiation in vitro, as well as the effects of targeted deletion of the miR-143/-145 gene cluster on SMC phenotype have been determined in a murine model [6]. Furthermore, Cheng et al. reported that miR-145, as a novel VSMC phenotypic modulator, was able to control vascular neointimal lesion formation [7]. These studies indicate that miR-143 and -145 modulate the balance of VSMC synthesis/proliferation and contraction/differentiation [6,7,11,12].

We postulated that a similar response might control the phenotypic differentiation of cardiac fibroblasts to myofibroblasts. We attempted to determine if miR-145 converted cardiac fibroblasts from a proliferative to a differentiated, functional phenotype. Identifying one of the mechanisms responsible for this conversion could provide a new therapeutic approach to induce infarct scar contraction and prevent heart failure by impeding progressive scar thinning and expansion.

2. Materials and methods

Please refer to the online Supplemental Methods for details.

2.1. Animal procedures

The Animal Care Committee of the University Health Network approved all experimental procedures, which were carried out according to the Guide for the Care and Use of Laboratory Animals (NIH, revised 1996). Female C57BL/6 mice (Charles River) 8–12 weeks of age were used for both in vitro and in vivo studies.

Mice were intubated and ventilated with 2% isoflurane. Through a left thoracotomy, the pericardium was entered and the left anterior descending coronary artery was ligated. Cardiac function was evaluated by echocardiography. Left ventricular (LV) end-diastolic and end-systolic diameters and areas were measured, and fractional shortening and fractional area contraction were calculated as follows: $(\%FS) = [(LVEDd - LVEDs) - LVEDd] \times 100$ and $(\%FAC) = [(LVEAd - LVEAs) - LVEAd] \times 100$. Twenty eight days after MI, the hearts were arrested and fixed at physiologic pressures. Hearts were then cut into 1 mm sections and photographed for morphometry.

2.2. miR-145 antagomir for in vivo studies

The miR-145 antagomir used for the in vivo studies was a chemically modified oligonucleotide with a sequence complementary to mature mmu-miR-145. All nucleosides were 2′-OMe modified, had two bases at the 5′ terminal and four bases at the 3′ terminal, and contained a phosphorothioate internucleoside bond (Integrated DNA Technologies). The molecules contained a 3′ cholesterol attached via a hydroxyprolinol linker. Sequences are 5′-mA*mG*mGmGmAmUmUm CmCmUmGmGmGmAmAmAmAmCmUmG*mG*mA*mC*-3′ (miR-145 antagomir), 5′-mA*mG*mGmGmAmUmUmCmCmUmGmGmGmAmAm CmAmUmAmG*mA*mG*mC*-3′ (mutant miR-145 antagomir). The “m” represents the 2′-O-methyl-modified oligonucleotides, the “*” represents a phosphorothioate linkage, and underlined letters are a mutated seed sequence. Mice received either the miR-145 antagomir or the mutant (mismatch control) at a dose of 80 mg/kg body weight through tail vein injection 1 day before and 2 and 6 days after MI. Tissues were collected at 3 and 7 days after MI and compared to sham control mice.

2.3. Histological analysis

Heart samples were perfusion-fixed in 10% formalin and sectioned. Sections were stained for α-SMA (Sigma) and counter-stained with DAPI. Whole heart sections were scanned using an Olympus VS120 fluorescence slide scanner and analyzed using CellSense software. Scar area was manually defined, and α-SMA$^+$-coated blood vessels were manually subtracted. The α-SMA$^+$ signal was reported as the percentage area of the total defined scar area.

2.4. Cell culture

To isolate cardiac fibroblasts, mouse hearts were digested with 0.1% collagenase type II (Worthington) for 30 min at 37 °C. The isolated cells were resuspended in Iscove's Modified Dulbecco's Medium (IMDM; Gibco), with 10% fetal bovine serum (FBS), 100 U/mL penicillin G, and 100 μg/mL streptomycin. After 3–5 days of culture, the non-adherent cells were washed off. Adherent cardiac fibroblasts were expanded to three passages before transfection with the miR-145 mimic, antimir, or scrambled miRNA.

Mouse cardiomyocytes were isolated and cultured as previously described [13], and transfection with the miR-145 mimic was carried out within 1 week of cell isolation.

Mouse aortic SMCs were prepared by the method of Smith and Brock [14] with minor modifications.

2.5. miR-145 mimic and antimir transfection

Chemically modified sense RNA (miR-145 mimic) or antisense RNA (miR-145 antimir) was synthesized by Qiagen. The sequence was 5-GUCCAGUUUUCCCAGGAAUCCCU-3 (Syn-mmu-miR-145). Transfection with the miR-145 mimic or antimir was performed using HiPerFect Transfection Reagent (Qiagen). Briefly, 5 nM of miR-145 mimic or antimir was mixed with 20 μL HiPerFect in 100 μL serum-free culture medium for 10 min at room temperature to form transfection complexes. The cells were incubated with the transfection complexes for 48 h.

2.6. Cotransfection of KLF5 plasmid and miR-145 antimir

Mouse KLF5 plasmid (Origene) was amplified using the maxiprep kit (Qiagen) following the manufacturer's instructions. Mouse cardiac fibroblasts were seeded onto either 60 mm dishes at a density of 5×10^5 cells/dish or 35 mm dishes at a density of 2.5×10^5/dish and cultured overnight in DMEM (10% fetal calf serum) without antibiotics. Cells were cotransfected with 1 μg of KLF5 plasmid or the control vector in the presence or absence of the miR-145 mimic using Lipofectamine 2000 (Invitrogen).

2.7. KLF5 luciferase reporter assay

We constructed a chimeric luciferase reporter system tagged with the full-length 3′-UTR region of human KLF5 harboring the seed-match sequence with (3′-mUTR) or without two nucleotide mutations (3′-UTR). HEK 293 cells were transfected with 200 ng of pMIR-REPORT and 8 ng of pRL-SV40 renilla luciferase control vector in the presence or absence of the mimic using Lipofectamine 2000 (Invitrogen). Luciferase activity was assayed after 48 h and expressed as a percentage of the luciferase activity of the 3′-mUTR transfected group.

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