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ORIGINAL RESEARCH ARTICLE

Cardiomyocyte-derived exosomal microRNA-92a mediates post-ischemic myofibroblast activation both *in vitro* and *ex vivo*

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Abstract

Aims We hypothesize that specific microRNAs (miRNAs) within cardiomyocyte-derived exosomes play a pivotal role in the phenoconversion of cardiac myofibroblasts following myocardial infarction (MI).

Methods and results We used an established murine model of MI, obtained *in vivo* via ligation of the left anterior descending coronary artery. We isolated adult cardiomyocytes and fibroblasts, and we assessed the functional role of cardiomyocyte-derived exosomes and their molecular cargo in the activation of cardiac fibroblasts. We identified and biologically validated miR-92a as a transcriptional regulator of mothers against DPP homologues 7 (SMAD7), a known inhibitor of α-smooth muscle actin (α-SMA), established marker of myofibroblast activation. We found that miR-92a was significantly ($P < 0.05$) upregulated in cardiomyocyte-derived exosomes and in fibroblasts isolated after MI compared with SHAM conditions ($n \geq 6$/group). We tested the activation of myofibroblasts by measuring the expression levels of αSMA, periostin, and collagen. Primary isolated cardiac fibroblasts were activated both when incubated with cardiomyocyte-derived exosomes isolated from ischemic cardiomyocytes and when cultured in conditioned medium of post-MI cardiomyocytes, whereas no significant difference was observed following incubation with exosomes or medium from sham cardiomyocytes. These effects were attenuated when an inhibitor of exosome secretion, GW4869 (10 μM for 12 h) was included in the experimental setting. Through means of specific miR-92a mimic and miR-92a inhibitor, we also verified the mechanistic contribution of miR-92a to the activation of cardiac fibroblasts.

Conclusions Our results indicate for the first time that miR-92a is transferred to fibroblasts in form of exosomal cargo and is critical for cardiac myofibroblast activation.

Keywords Epigenetics; Exosomes; MicroRNA; Myofibroblast

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Background

The mechanistic role of microRNAs (miRNAs, miRs) in cardiovascular pathophysiology is well established.[1–3] Substantial evidence has shown that miRNAs can be included in extracellular microvesicles/exosomes, thereby protecting them from ribonuclease-dependent degradation, allowing cell–cell communication.[4–6]

Cardiac fibroblasts and their activated counterpart (myofibroblasts) have emerged as essential cellular components of normal and postischemic cardiac function.[7–9]

Aims

We hypothesize that cardiomyocyte-derived exosomal miRNAs are involved in the phenoconversion of quiescent fibroblasts to activated myofibroblasts in myocardial infarction (MI).

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Methods

An extended version is available in the Supporting Information.

In vivo studies

All *in vivo* procedures were approved by the Einstein Institutional Animal Care and Use Committee. MI was obtained (see data in Please upload the attached document as Data S1.Supporting Information, *Table S1*) via ligation of the coronary artery, as previously described by our group.[10]

Isolation of cardiomyocytes, fibroblasts, and exosomes

Cardiac fibroblasts and cardiomyocytes were isolated as previously described.[7,10] Exosomes were obtained from primary isolated cardiomyocytes via serial centrifugation: The medium was collected and centrifuged first at 300 *g* for 3′ and then at 2000 *g* for 10′; supernatants were centrifuged at 10 000 *g* for 30′. Exosomes were then isolated from the supernatant by ultracentrifugation at 100 000 *g* for 70′. The pellet was re-centrifuged at 100 000 *g* for 2 h. Exosomes were characterized via immunoblot assessing the presence of established markers as well as the absence of contamination.[4] Immunoblotting was performed as we previously described and validated;[10,11] antibodies are listed in Supporting Information, *Table S2*.

Biological validation of miRNA targets and quantitative real-time PCR

To assess the effects of miR-92a on SMAD7 gene transcription, we used a luciferase reporter containing the 3′ untranslated region (UTR) segment, WT and mutated, of the predicted miRNA interaction sites. Levels of miR-92a were measured using individual TaqMan microRNA assays. Data were normalized using the synthetic spike-in C. elegans oligonucleotide cel-miR-39. Cellular expression of αSMA, collagen I, collagen III, and periostin was determined as previously described;[12] for all non-miRNA probes, the relative amount of specific mRNA was normalized to GAPDH.[11] Primer sequences are in Supporting Information, *Table S3*.

Statistical analysis

Data are expressed as means ± SEM. Significance was tested using the nonparametric Mann–Whitney *U* test or two-way ANOVA followed by Tukey–Kramer multiple comparison test, as appropriate. Significant differences were established at a $P < 0.05$.

Results

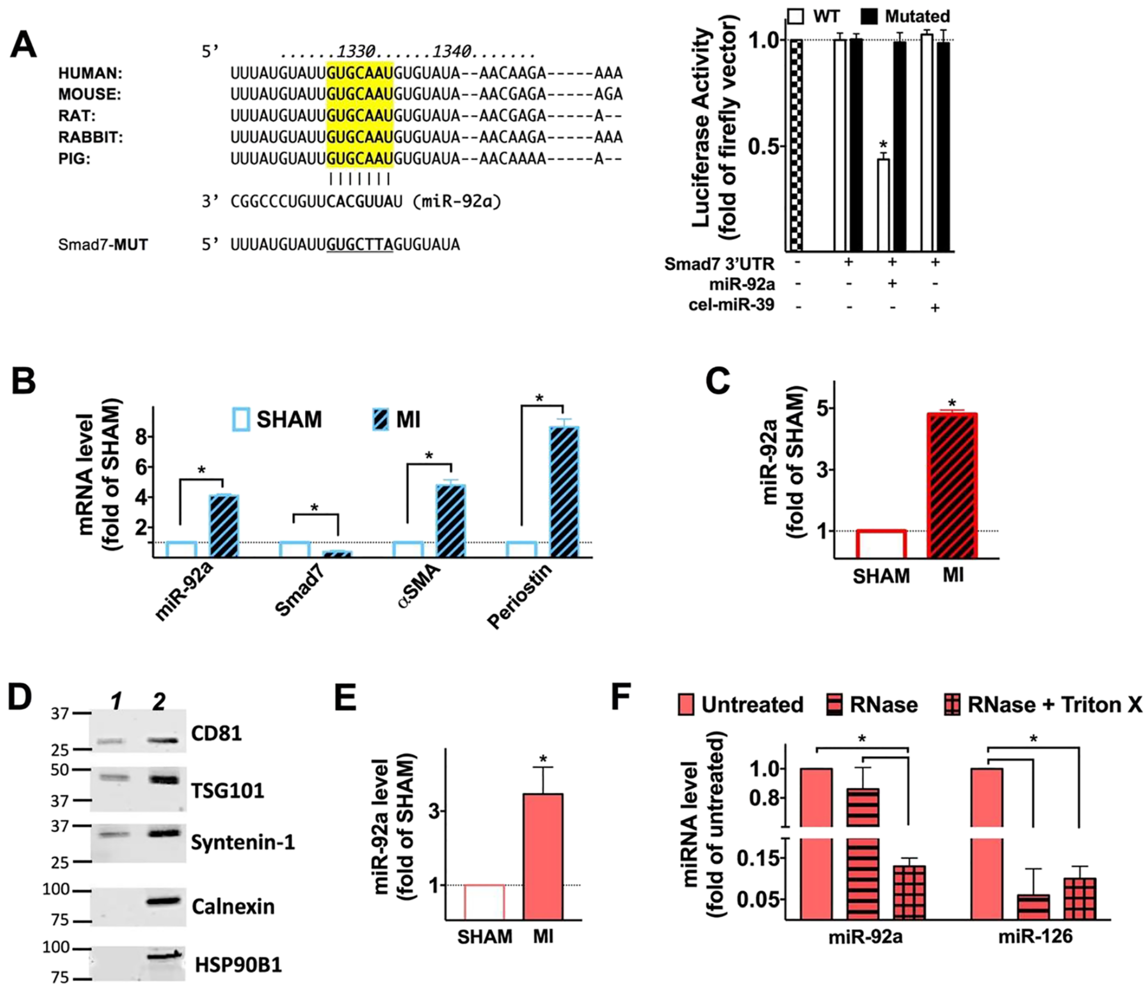
Ischemic injury upregulates miR-92a in cardiac myofibroblasts

After MI, cardiac fibroblasts become activated, and an established marker of this activation is the increased expression of α-smooth muscle actin (αSMA). Through means of a bioinformatic approach, we identified miR-92a as a potential target of a crucial inhibitor of αSMA expression, namely, mothers against DPP homologues 7 (SMAD7), and we validated the interaction between this miR and the 3′ UTR of SMAD7 via luciferase assay (*Figure 1A*). Our findings are consistent with previous studies performed in H9c2 cells.[13] Using an established murine model of MI, we then determined via quantitative real time PCR (RT–qPCR) that miR-92a was significantly upregulated in cardiomyocytes, fibroblasts, and in cardiomyocyte-derived exosomes following MI (*Figure 1B, C, D*). We also proved that miR-92a was contained in cardiomyocyte-derived exosomes (*Figure 1D–F*); to verify that mir-92a was actually confined inside exosomes, we treated the samples with RNase, showing that miRNA levels were not affected by RNase treatment, unless when in presence of the detergent Triton X-100 (*Figure 1F*). The increased expression of αSMA in cardiac fibroblasts further confirmed the actual myofibroblast phenoconversion (*Figure 1B*).

Exosomes isolated from ischemic cardiomyocytes activate fibroblasts

After having obtained primary cardiomyocytes from MI and SHAM mice, we isolated exosomes from these cells, and we incubated them with fibroblasts primarily isolated from SHAM mice for 72 h: We observed a significant increase in miR-92a level in fibroblasts treated with exosomes from MI cardiomyocytes but not in fibroblasts incubated with exosomes from SHAM cells (*Figure 2A*). Similarly, trascription of genes activated in myofibroblasts, including αSMA, collagen, and periostin, was significantly upregulated in fibroblasts incubated with exosomes from MI cardiomyocytes compared with fibroblasts incubated with exosomes from SHAM cardiomyocytes (*Figure 2A*); we also verified by immunoblot that αSMA was significantly upregulated at the protein level (Supporting Information, *Figure S1*). These experiments confirm that the exosomal miRNA cargo was actually transferred from cardiomyocytes to fibroblasts.

Figure 1 Functional role of miR-92a in fibroblast activation. (A) miR-92a targets SMAD7; complementary nucleotides between the target region of Smad7 3′ untranslated region (UTR) (in yellow) and miR-92a are conserved across different species; Luciferase activity (right panel) was measured 48 h after transfection, and values are shown as fold change of the luciferase activity detected using the firefly vector without Smad7 3′ UTR (checkered bar); a mutated Smad7 3′ UTR (Smad7 MUT) and a cel-miR-39 mimic have been used as further controls. (B–E) Upregulation of miR-92a following cardiac ischemic injury. We found a significant upregulation of miR-92a in cardiac fibroblasts (B) and cardiomyocytes (C) and in exosomes obtained from cardiomyocytes (D,E) isolated 7 days after MI ($n \geq 6$ mice/group); Smad7 mRNA was markedly downregulated while αSMA was upregulated in fibroblasts post-MI (B). Representative immunoblots (D) showing the presence of marker proteins typically enriched in exosomes, namely, CD81, Tumor Susceptibility Gene 101 (TSG101), and syntenin-1, and the absence of contamination from other cellular components using Heat Shock Protein 90 Beta Family Member 1 (HSP90B1, a.k.a. GRP94) and calnexin; lane 1: exosomes; lane 2: whole cells. Exosome preparations were re-suspended in 300-ml PBS and spiked with 20 ρmol of a synthetic oligonucleotide corresponding to the mature sequence of miR-126 (exogenous miRNA used as control); samples were then treated or not with Triton X-100 (1%) and incubated with or without RNase A (0.5 U) and T1 (15 U) for 30′ at 37°C before RNA extraction (F). Mean ± SEM of at least three independent experiments; $^{*}P < 0.05$.



Conditioned medium of post-MI cardiomyocytes activates fibroblasts

Culturing fibroblasts from SHAM mice in conditioned medium of cardiomyocytes isolated from post-MI mice significantly increased levels of miR-92a, αSMA, and periostin (*Figure 2B*), compared with fibroblasts cultured in medium from SHAM cardiomyocytes; of note, these effects were attenuated by GW4869 (10 μM for 12 h), an inhibitor of exosome secretion.[14] Furthermore, we used specific miR mimics (*Figure 2C*) and inhibitors (*Figure 2D*) to verify the mechanistic role of miR-92a in the activation of cardiac fibroblasts.

Conclusions

In the present study, we demonstrate that miR-92a is upregulated in cardiac myocytes after MI and is subsequently transferred to cardiac fibroblasts within exosomes. In the

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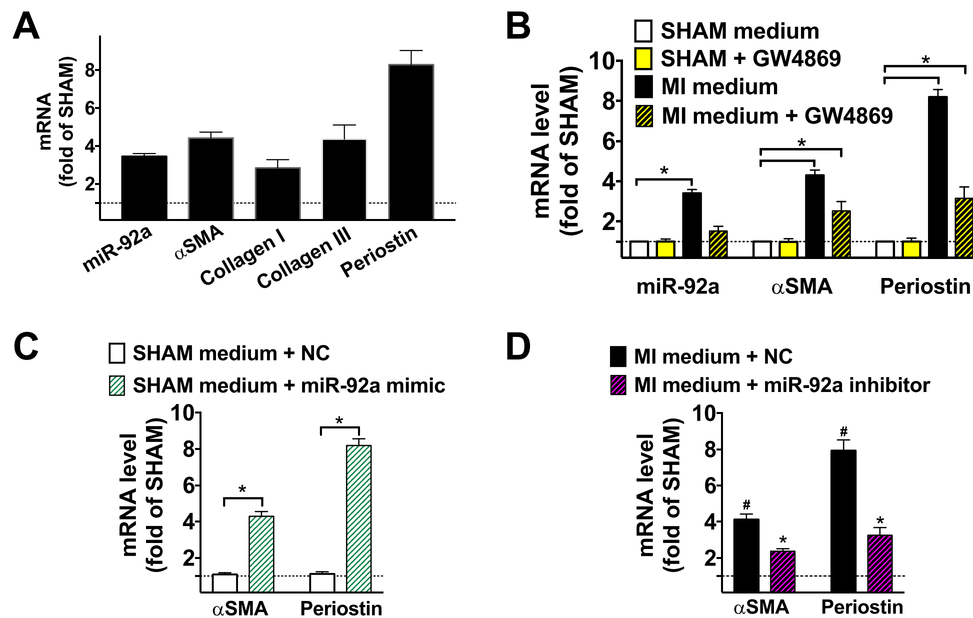
Figure 2 Effects of cardiomyocyte-derived exosomes on fibroblast activation. (A) Fibroblasts were incubated with exosomes obtained from cardiomyocytes isolated from SHAM and myocardial infarction (MI) mice ($n \geq 6$ mice/group) 7 days post-surgery; such incubation induced an upregulation of miR-92a, αSMA, collagens I and III, and periostin. Mean ± SEM of at least three independent experiments (all significantly different compared with SHAM, $P < 0.05$). (B) Effects of conditioned cardiomyocyte medium on the activation of fibroblasts; conditioned medium from cardiomyocytes isolated ($n \geq 6$ mice/group) 7 days post-MI induced an upregulation of miR-92a, αSMA, and periostin. However, the addition of an inhibitor of exosome release (GW4869 10 μM for 12 h) significantly attenuated these responses. Mean ± SEM of at least three independent experiments; $^*P < 0.05$ vs. SHAM. (C,D) Effects of miR mimics and inhibitors on fibroblast activation. Conditioned medium from cardiomyocytes isolated ($n \geq 6$ mice/group) 7 days post-MI or SHAM surgery was added to fibroblasts transfected with miR-92a mimics (C) and inhibitors (D), or negative control miR (NC). Mean ± SEM of at least three independent experiments; $^*P < 0.05$; in Panel D, $^*P < 0.05$ vs. MI medium + NC, $^\#P < 0.05$ vs. SHAM.



fibroblast, miR-92a relieves the SMAD7-mediated inhibition of αSMA transcription, triggering the phenoconversion to myofibroblast. The transcriptional regulation of αSMA by SMAD7 has been previously described by other investigators in diverse cell types.[15,16] Our findings are consistent with previous studies exploring fibroblast-myocyte communication;[17] however, most of these investigations focused on the influence of fibroblasts on cardiomyocyte function,[18,19] whereas we are investigating the opposite relationship. Intriguingly, miR-92a has been implied in the pathophysiology of heart failure.[20]

Our experiments clearly indicate that miR-92a is essential in the activation of cardiac myofibroblasts. Indeed, obtaining exosomes *ex vivo* from primary isolated cardiomyocytes, is proving the exact source of such vesicles. Of note, when inhibiting the release of exosomes using GW4869, the transcriptional regulation of αSMA was not completely blunted, suggesting that other mechanisms are involved in myofibroblast activation. Further studies are necessary to appraise the translational potential of our findings, which pave also the way to future investigation on the potential role of exosomal miRNAs in long-distance communications.

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Conflict of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Supporting information.

Figure S1. Evaluation of αSMA protein expression in fibroblasts.

Table S1. Characteristics of SHAM and myocardial infarction (MI) mice.

Table S2. Antibodies used in the study.

Table S3. Sequences of oligonucleotide primers (*mus musculus*) and product sizes.

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