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

Xujun Wang, Marco Bruno Morelli, Alessandro Matarese, Celestino Sardu and Gaetano Santulli

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 ≥ 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

Received: 5 June 2019; Revised: 25 November 2019; Accepted: 9 December 2019
*Correspondence to: Gaetano Santulli, Department of Medicine, Division of Cardiology, Albert Einstein College of Medicine, Montefiore University Hospital, New York, NY 10461, USA. Email: gsantulli001@gmail.com
†These authors contributed equally to this study.

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).
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’ and 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 miRNA 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 levels in fibroblasts treated with exosomes from MI cardiomyocytes but not in fibroblasts incubated with exosomes from SHAM cells (Figure 2A). Similarly, transcription 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 S2). These experiments confirm that the exosomal miRNA cargo was actually transferred from cardiomyocytes to fibroblasts.
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 peristin (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
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.\textsuperscript{15,16} Our findings are consistent with previous studies exploring fibroblast-myocyte communication;\textsuperscript{17} however, most of these investigations focused on the influence of fibroblasts on cardiomyocyte function,\textsuperscript{18,19} whereas we are investigating the opposite relationship. Intriguingly, miR-92a has been implied in the pathophysiology of heart failure.\textsuperscript{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.

**Acknowledgements**

We thank N.G. Frangogiannis, R.N. Kitsis, and J. Shu, Albert Einstein College of Medicine, for helpful discussion and technical support.

**Conflict of interest**

None declared.

**Funding**

The Santulli’s Lab is supported in part by the NIH (R01-HL146691, R01-DK033823, R00-DK107895, P30-DK020541, R01-DK123259 to G.S.).

**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.
Cardiomyocyte-derived exosomal microRNA-92a mediates post-ischemic myofibroblast activation both in vitro and ex vivo

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.

References

1. Wronska A, Kurkowska-Jastrzebska I, Santulli G. Application of microRNAs in diagnosis and treatment of cardiovascular disease. Acta Physiol (Oxf) 2015; 213: 60–83.

2. Bink DJ, Lozano-Vidalan N, Boon RA. Long Non-Coding RNA in vascular disease and aging. Non-Coding RNA 2019; 5: 26.

3. Santulli G. MicroRNA: from molecular biology to clinical practice. New York: Springer Nature; 2016.

4. Stahl PD, Raposo G. Extracellular vesicles: exosomes and microvesicles, integrators of homeostasis. Physiology (Bethesda) 2019; 34: 169–177.

5. Montecalvo A, Larregina AT, Shufesky WJ, Stolz DB, Sullivan ML, Karlsson JM, Baty CJ, Gibson GA, Erdos G, Wang Z, Milosevic J, Tkacheva OA, Divito SJ, Jordan R, Lyons-Weiler J, Watkins SC, Morelli AE. Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. Blood 2012; 119: 756–766.

6. Santulli G. Exosomal microRNA: the revolutionary endogenous Innerspace nanotechnology. Sci Transl Med 2018; 10: eaav9141.

7. Morelli MB, Shu J, Sardu C, Matarese A, Santulli G. Cardiosomal microRNAs are essential in post-infarction myofibroblast phenoconversion. Int J Mol Sci 2019; 21: 201.

8. Villalobos E, Criollo A, Schiattarella GG, Altimirano F, French KM, May HI, Jiang N, Nguyen NUN, Romero D, Roa JC, Garcia L, Díaz-Araya G, Morselli E, Ferdous A, Conway SJ, Sadek HA, Gillette TG, Lavandero S, Hill JA. Fibroblast primary cilia are required for cardiac fibrosis. Circulation 2019; 139: 2342–2357.

9. Díaz-Araya G, Vivar R, Humeres C, Boza P, Bolivar S, Muñoz C. Cardiac fibroblasts as sentinel cells in cardiac tissue: Receptors, signaling pathways and cellular functions. Pharmacol Res 2015; 101: 30–40.

10. Santulli G, Xie W, Reiken SR, Marks AR. Mitochondrial calcium overload is a key determinant in heart failure. Proc Natl Acad Sci U S A 2015; 112: 11389–11394.

11. Santulli G, Wronska A, Uryu K, Diacovo TP, Bolivar S, Muñoz C. Cardiosomal microRNAs are essential in post-infarction myocardial diseases: exosomes and microvesicles, integrators of homeostasis. Physiological to clinical practice

12. Lombardi A, Gambardella J, Du XL, Felder J, Fox H, Doebele C, Ohtani K, Z, Milosevic J, Tkacheva OA, Divito SJ, Jordan R, Lyons-Weiler J, Watkins SC, Morelli AE. Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. Blood 2012; 119: 756–766.

13. Zhang B, Zhou M, Li C, Zhou J, Li H, Morelli MB, Shu J, Sardu C, Matarese A, Santulli G. Cardiosomal microRNAs are essential in post-infarction myofibroblast phenoconversion. Int J Mol Sci 2019; 21: 201.

14. Villalobos E, Criollo A, Schiattarella GG, Altimirano F, French KM, May HI, Jiang N, Nguyen NUN, Romero D, Roa JC, Garcia L, Díaz-Araya G, Morselli E, Ferdous A, Conway SJ, Sadek HA, Gillette TG, Lavandero S, Hill JA. Fibroblast primary cilia are required for cardiac fibrosis. Circulation 2019; 139: 2342–2357.

15. Santulli G, Xie W, Reiken SR, Marks AR. Mitochondrial calcium overload is a key determinant in heart failure. Proc Natl Acad Sci U S A 2015; 112: 11389–11394.

16. Wei J, Ghosh AK, Sargent JL, Komura K, Wu M, Huang QQ, Jain M, Whitfield ML, Feghali-Bostwick C, Varga J. PPARgamma downregulation by TGFβs in fibroblast and impaired expression and function in systemic sclerosis: a novel mechanism for progressive fibrogenesis. PLoS ONE 2010; 5: e13778.

17. Kakkar R, Lee RT. Intramyocardial fibroblast myocyte communication. Circ Res 2010; 106: 47–57.

18. Cartledge JE, Kane C, Dias P, Tesfom M, Clarke L, McKee B, Al Ayoubi S, Chester A, Yacoub MH, Camelliti P, Terracciano CM. Functional crosstalk between cardiac fibroblasts and adult cardiomyocytes by soluble mediators. Cardiovasc Res 2015; 105: 260–270.

19. Salvarani N, Maguy A, De Simone SA, Miragoli M, Jousset F, Rohr S. TGF-beta1 (Transforming Growth Factor-beta1) Plays a pivotal role in cardiac myofibroblast arrhythmogenicity. Circ Arrhythm Electrophysiol 2017; 10: e004567.

20. Bonauer A, Carmona G, Iwasaki M, Mione M, Koyanagi M, Fischer A, Burchfield J, Fox H, Doebele C, Ohtani K, Chavakis E, Potente M, Tjwa M, Urbich C, Zeiber AM, Dimmeler S. MicroRNA-92a controls angiogenesis and functional recovery of ischemic tissues in mice. Science 2009; 324: 1710–1713.