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Extracellular matrix-derived extracellular vesicles promote cardiomyocyte growth and electrical activity in engineered cardiac atria

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ABSTRACT

Extracellular matrix (ECM) plays a critical role in the provision of the necessary microenvironment for the proper regeneration of the cardiac tissue. However, specific mechanisms that lead to ECM-mediated cardiac regeneration are not well understood. To elucidate the potential mechanisms, we investigated ultra-structures of the cardiac ECM using electron microscopy. Intriguingly, we observed large quantities of micro-vesicles from decellularized right atria. RNA and protein analyses revealed that these contained exosomal proteins and microRNAs (miRNAs), which we referred to herein as ECM-derived extracellular vesicles (ECM-EVs). One particular miRNA from ECM-EVs, miR-199a-3p, promoted cell growth of isolated neonatal cardiomyocytes and sinus nodal cells by repressing homeodomain-only protein (HOPX) expression and increasing GATA-binding 4 (Gata4) acetylation. To determine the mechanisms, we knocked down Gata4 and showed that miR-199a-3p actions required Gata4 for cell proliferation in isolated neonatal cardiomyocytes and sinus nodal cells. To further explore the role of this miRNA, we isolated neonatal cardiac cells and recellularized into atrial ECM, referred here has engineered atria. Remarkably, miR-199a-3p mediated the enrichment of cardiomyocyte and sinus nodal cell population, and enhanced electrocardiographic signal activity of sinus nodal cells in the engineered atria. Importantly, antisense of miRNA (antagomir) against miR-199a-3p was capable of abolishing these actions of miR-199a-3p in the engineered atria. We further showed in Ang II-infused animal model of sinus nodal dysfunction that miR-199-3p-treated cardiac cells remarkably ameliorated and restored the electrical activity as shown by normalization of the ECG, in contrast to untreated cells, which did not show electrical recovery. In conclusion, these results provide clear evidence of the critical role of ECM, in not only providing a scaffold for cardiac tissue growth, but also in promoting atrial electrical function through ECM-derived miR-199a-3p.

1. Introduction

Heart failure is the leading cause of death worldwide [1]. Although there are many advanced therapies for heart disease, heart transplantation is to date the only cure for heart failure. However, securing transplantable hearts from human donors have significant limitations. Thus, much effort to derive the heart using stem cells are under way [2–4]. Viable cardiac tissues are not only comprised of cells, but also of a complex meshwork known as the extracellular matrix (ECM) [5–7]. In addition to providing structural support for the cells within a tissue, the ECM plays a critical

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role in cellular division, growth, development, and preservation of mechanical properties [8–10]. The ECM is composed of proteoglycans, water, minerals, and fibrous proteins including collagen, elastin, fibronectin, and laminin [11–14]. Proteoglycans consist of a protein core that is surrounded by long chains of starch-like molecules called glycosaminoglycans [11]. Collagen is a stretch-resistant fiber that provides tensile strength to tissues whereas elastin permits tissues to return to their original shape after stretch [12]. Fibronectin supports cell position, division and migration; and laminin forms sheet-like network that provides adhesion between dissimilar tissues [13,14].

Importantly, ECM also contains various factors for tissue growth and homeostasis [15,16]; however, our understanding of this aspect of ECM is less clear. In this study, we found extracellular vesicles (EVs) with a large amount of miRNA from cardiac ECM that we referred to herein as ECM-derived extracellular vesicles (ECM-EVs). A microRNA (miRNA) is a small non-coding RNA molecule containing about 22 nucleotides that mediates RNA silencing and post-transcriptional regulation of gene expression [17,18]. miRNAs can be released into extracellular space and taken up by neighboring cells [19]. From miRNA profiling studies, multiple miRNAs, including miR-1, miR-17, miR-21, miR-92, miR-133, miR-199a-3p, miR-208, miR-499, miR-590, miR-15, or miR-29, have been reported to modulate cardiac regeneration [20]; and similar array of miRNAs are present in ECM-EVs. We therefore hypothesized that ECM-EVs would play an important role in cardiac tissue regeneration. To this end, we analyzed miRNA profiles in ECM-EVs and investigated the effects of these ECM-EVs in the growth and electrical activity of the engineered cardiac tissue.

2. Materials and methods

2.1. Decellularization of mouse hearts

All mice were in C57BL/6 background and purchased from Charles River. All animal experiments were approved and performed by the NIH guidelines (Guide for the care and use of laboratory animals) and the Ewha Womans University Animal Care Committee. 4 week-old male mice were anesthetized with 375 mg/kg 2,2,2-tribromoethanol (T48402, Sigma-Aldrich) by intraperitoneal injection, thoracic cavity opened, and the heart was carefully excised. After cannulation of the aorta, hearts were secured by tying below the innominate artery and perfused retrogradely by the nonrecirculating langendorff technique with 1% SDS (15525017, Invitrogen) in deionized water for 15 min. After decellularization, cardiac extracellular matrix (ECM) was perfused with phosphate-buffered saline (PBS; 10010023, Invitrogen) for 10 min and kept in RNA Later (R0901, Sigma-Aldrich), 3% buffered glutaraldehyde (G5882, Sigma-Aldrich) or 10% formalin (HT5011, Sigma-Aldrich) prior to experiments.

2.2. Transmission electron microscopy

We fixed the myocardium and ECM samples with 3% buffered glutaraldehyde (G5882, Sigma-Aldrich) for 2 h and processed into resin (02334, Polysciences, German). After embedding, the resin block was thin-sectioned by ultramicrotomy. Sections of 50–70 nm thickness were collected on metal mesh and stained with electron dense particles before imaging of ultrastructures, using the transmission electron microscope (H-7650, Hitachi-Science & Technology, Japan).

2.3. Immunofluorescence

Cardiac tissues, decellularized hearts or recellularized engineered atria were placed in 10% formalin for 12 h. After formalin fixation and paraffin embedding, 3-μm sections were cut on silane-coated glass slides. Antibodies were conjugated with alexa fluor 594 (A20185, Life technologies), 350 (A20180, Life technologies), or 488 (A20181, Life technologies) using Alexa Fluor antibody labeling kit. Slides were incubated with antisera directed against Laminin (1:400; ab11575, Abcam), Thy1 (1:400; ab92574, Abcam), TBX3 (1:400; ab89220, Abcam), CD9 (1:400; ab97999, Abcam), or MLCA2 (1:400; MYL-7; ab68086, Abcam) and with detection reagent of an autophagosome (MAK138; Sigma-Aldrich) or terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; Abcam) for 45 min. After being washed with PBS, slides were mounted with 4,6-diamidino-2-phenylindole (DAPI) histology mounting medium (F6057, Sigma-Aldrich) and visualized using Zeiss LSM 510 confocal microscope (Carl Zeiss, German).

2.4. Separation and quantitation of exosomal micro-RNAs

Tissue or cardiac ECM samples were frozen in liquid nitrogen and then pulverized to powder. For ECM analysis, ECM was collected from seven cardiac atria per sample. Each sample was mixed with 100 μl of dilution buffer (EX-COM-SP, MBL, USA) and centrifuged at 10000 g for 10 min at 4 °C to pellet bulky debris. The supernatant was then centrifuged at 100000 g for 30 min at 4 °C. The final supernatant was collected and extracellular vesicles were captured using antibody (ExoCap™ CD9 Capture Kit, MBL, USA). The particle sizes were confirmed between 30 nm and 100 nm using Nanoparticle Tracking Analysis (NTA). Total RNA was extracted via the mirVana miRNA isolation kit (AM1560, Ambion). Quantification and quality of small RNAs were assessed using the Agilent Small RNA Kit (Agilent Technologies, USA). RNA species were separated on a preparative scale by Agilent 2100 Bioanalyzer (Agilent Technologies, USA).

2.5. Analysis of miRNA using real-time PCR

The extracted exosomal miRNA were determined using mmu-miR-1 (477820_mir), mmu-miR-17 (481556_mir), mmu-miR-21 (482709_mir), mmu-miR-92 (000430_mir), mmu-miR-133 (478511_mir), mmu-miR-199a-3p (480983_mir), mmu-miR-208 (477819_mir), mmu-miR-499 (481865_mir), mmu-miR-590 (482796_mir), mmu-miR-29 (478587_mir) or mmu-miR-15 (481554_mir) primer/probe set from Life Technology. Real-time PCR was performed with TaqMan universal PCR Master Mix on an ABI Real time PCR System 7000 (Applied Biosystems, USA). PCR conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. For each experimental sample, the relative abundance value was normalized to the value derived from the spike in control (cel-miR-2-3p, 478291_mir, Life Technology) of the same sample. Relative mRNA levels were quantified using the comparative 2-ΔΔCT method.

2.6. H_2O_2 perfusion into cardiac ECM

To determine releasable-EVs from the heart, cardiac ECM was perfused retrogradely by the langendorff technique with 0.01% H_2O_2 (H3410, Sigma-Aldrich) or PBS buffer. The coronary effluent was collected in timed fractions (2 min) over 45 min and assayed for CD9 protein levels using western blot or miRNA using real time-PCR. Vivaspin 500 (Sartorius, German) was used to increase protein sample concentrations.

Isolation of neonatal cardiomyocytes, sinus nodal cells and fibroblasts from cardiac atria.

Neonatal cardiomyocytes were prepared using methods adapted from studies described previously [21]. Briefly, 2 week-old mice