Myofibroblast-derived Exosomes Contribute to Development of a Susceptible Substrate for Atrial Fibrillation

Shichao Li, Yuanfeng Gao, Ye Liu, Jing Li, Xiyan Yang, Roumu Hu, Jia Liu, Yuan Zhang, Kun Zuo, Kuibao Li, Xiandong Yin, Mulei Chen, Jiuchang Zhong\*, Xinchun Yang\*

Heart Center & Beijing Key Laboratory of Hypertension, Beijing Chaoyang Hospital, Capital Medical University, 8th, Gongti South Road, Chaoyang District, Beijing, 100020, PR China.

Shichao Li and Yuanfeng Gao are co-first authors.

\* Corresponding author.

**Supplementary data**

**Supplemental Methods**

1. Cell culture

Neonatal rat atrial fibroblasts and cardiomyocytes were isolated and cultured with the procedures similar to described previously [1, 2]. Briefly, 1- to 3-day-old neonatal Sprague-Dawley rats were decapitated and their hearts were aseptically removed, put into cold (4℃) calcium-free PBS. The atria were dissected, finely minced, and digested with 0.07% (w/v) trypsin (Sigma; tryptic activity: 1:250) and 0.04% (w/v) collagenase type Ⅱ (Worthington) under constant stirring at 37℃. The solution of the atrial tissue was then transferred to a centrifuge tube containing isopyknic DMEM/F12 plus 10% FBS. A new enzyme solution was added to the tissue, and several digestion procedures were repeated before the collected primary cells were passed through a cell strainer (75 μm nylon mesh) and centrifuged at 700 g for 10 min. The supernatant was discarded, and the cells were resuspended in DMEM/F12 containing 10% FBS and cultured for 90 min at 37℃. Subsequently, the supernatant (containing the atrial cardiomyocytes) was collected and plated in DMEM/F12 containing 10% FBS, 1% penicillin/streptomycin, and 0.1 mM 5-bromodeoxyuridine (Sigma). The attached atrial fibroblasts were cultured in the same medium but without 5-bromodeoxyuridine at 37℃ in 5% CO2.

2. Immunofluorescence staining

The primary antibodies used for immunofluorescence staining were as follows: anti-vimentin (Cat#: V6389, 1:80, Sigma-Aldrich), anti-cardiac troponin I (cTNI) (Cat#: ab47003, 1:200, Abcam), anti-CD31 (Cat#: ab119339, 1:50, Abcam), anti-α-smooth muscle actin (α-SMA) (Cat#: ab5694, 1:200, Abcam), and anti-Cav1.2 (Cat#: ab84814, 1:400, Abcam).

3. Exosome isolation

The fetal bovine serum was depleted of contaminating bovine exosomes by ultracentrifugation at 100,000 g for 14 h at 4℃. Conditioned medium was collected for 24 h, and exosomes were isolated by several centrifugation and filtration steps. Briefly, the conditioned medium was centrifuged at 300 g for 10 min, 2,000 g for 10 min, and 10,000 g for 30 min, followed by filtration through a 0.22 μm filter. The supernatant was then ultracentrifuged at 110,000 g for 70 min, followed by an additional washing step of the exosome pellet with PBS at 110,000 g for 70 min (Ultracentrifuge: Beckman, L-80XP; Rotor: swinging-bucket rotor, SW32Ti). All centrifugations were performed at 4℃.

4. Western blot analysis

The primary antibodies used for Western blot were as follows: anti-α-SMA (Cat#: ab5694, 1:400, Abcam), anti-fibronectin (Cat#: sc-6952, 1:100, Santa Cruz), anti-connective tissue growth factor (Cat#: 23936, 1:1000, Proteintech), anti-collagen type Ia1 (Cat#: 14695, 1:1000, Proteintech), anti-collagen type IIIa1 (Cat#: 22734, 1:1000, Proteintech), osteopontin (Cat#: ab8448, 1:1000, Abcam), anti-CD63 (Cat#: ab108950, 1:500, Abcam), anti-TSG101 (Cat#: ab125011, 1:1000, Abcam), anti-calnexin (Cat#: ab133615, 1:1000, Abcam), anti-HSC70 (Cat#: ab51052, 1:1000, Abcam), anti-Cav1.2 (Cat#: ab84814, 1:1000, Abcam), and anti-GAPDH (Cat#: ab181603, 1:10000, Abcam).

5. qRT-PCR analysis

The sequences of the primers were as follows: rno-miR-21-5p: F 5’-GCGGTAGCTTATCAGACTG-3’, R 5’-TGCGTGTCGTGGAGTC-3’; rno-miR-21-3p: F 5’-GGAACAACAGCAGTCGATGG-3’, R 5’-CAGTGCGTGTCGTGGAGTC-3’; U6: F 5’-GCTTCGGCAGCACATATACTAAAAT-3’, R 5’-CGCTTCACGAATTTGCGTGTCAT-3’; rno-miR-93-5p: F 5’-GGCAAAGTGCTGTTCGTG-3’, R 5’-CAGTGCGTGTCGTGGAGT-3’. Amplification was carried out as follows: initial denaturation at 95℃ for 10 min, followed by 40 cycles of 95℃ for 10 s, and 60℃ for 60 s. Melting curve analysis was carried out by increasing the temperature from 60℃ to 99℃ at a rate of 0.05℃/s.

**Supplemental References**

1 Lu Y, Zhang Y, Wang N, Pan Z, Gao X, Zhang F, Zhang Y, Shan H, Luo X, Bai Y, Sun L, Song W, Xu C, Wang Z, Yang B: MicroRNA-328 contributes to adverse electrical remodeling in atrial fibrillation. Circulation 2010;122:2378-2387.

2 Bang C, Batkai S, Dangwal S, Gupta SK, Foinquinos A, Holzmann A, Just A, Remke J, Zimmer K, Zeug A, Ponimaskin E, Schmiedl A, Yin X, Mayr M, Halder R, Fischer A, Engelhardt S, Wei Y, Schober A, Fiedler J, Thum T: Cardiac fibroblast-derived microRNA passenger strand-enriched exosomes mediate cardiomyocyte hypertrophy. J Clin Invest 2014;124:2136-2146.

**Figure legends to supplemental figures**

Supplemental Fig.1. Purity of neonatal rat fibroblast and cardiomyocyte. The high purity of fibroblast and cardiomyocyte cell cultures derived from neonatal rat atriums was confirmed by immunofluorescence staining. (a) Atrial fibroblasts were stained with vimentin (fibroblast marker, red), CD31 (endothelial cell marker), cardiac Troponin I (cTNI, cardiomyocyte marker), and DAPI (nucleus, blue). n=3. Scale bars: 100 μm. (b) Atrial cardiomyocytes were stained with cardiac Troponin I (cTNI, cardiomyocyte marker, green) and DAPI (nucleus, blue). n=4. Scale bars: 50 μm.