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Long-distance intercellular connectivity between cardiomyocytes and cardiofibroblasts mediated by membrane nanotubes

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Aims Intercellular interactions between cardiomyocytes (CMs) and cardiofibroblasts (FBs) are important in the physiological and pathophysiological heart. Understanding such interactions is important for developing effective heart disease therapies. However, until recently, little has been known about these interactions. We aimed to investigate structural and functional connections between CMs and FBs that are distinct from gap junctions.

Methods and results By membrane dye staining, we observed long, thin membrane nanotubular structures containing actin and microtubules that connected neonatal rat ventricular CMs and FBs. By single-particle tracking, we observed vehicles moving between CMs and FBs within the membrane nanotubes. By dual colour staining, confocal imaging and flow cytometry, we observed mitochondria exchange between CMs and FBs in a coculture system. By combined atomic force microscopy (AFM) and confocal microscopy, we observed calcium signal propagation from AFM-stimulated CM (or FB) to unstimulated FB (or CM) *via* membrane nanotubes. By membrane and cytoskeleton staining, we observed similar nanotubular structures in adult mouse heart tissue, which suggests their physiological relevance.

Conclusions As a novel type of CM to FB communication, membrane nanotubes observed *in vitro* and *in vivo* provide structural and functional connectivity between CMs and FBs over long distances.

Keywords Cardiomyocytes • Cardiofibroblasts • Membrane nanotubes • Mitochondria • Calcium

1. Introduction

Mammalian heart function is regulated by the cooperative and dynamic interactions of two major cell types, cardiomyocytes (CMs) and cardiofibroblasts (FBs).[1] CMs occupy nearly 75% of the normal myocardial tissue volume; however, >60% of the cells are predominantly FBs.[2–4] FBs are interspersed within the layers of CMs and play a key role in regulating normal myocardial function and in adverse myocardial remodelling that occurs with hypertension, myocardial infarction and heart failure.[1,5] Thus, clarifying the structural and functional interactions between CMs and FBs is essential for understanding the pathophysiological heart and the development of safe and effective cell therapies.[6]

Contrary to the classical view that CMs and FBs are isolated from each other, increasing studies have revealed an intimate relationship both structurally and electrically between the two cell types. Functional gap junctions were observed between cultured CMs and FBs, which could underlie the ability of FBs to serve as conductors for electrical excitation and to affect CM electrophysiologic features.[3] In normal cardiac tissue, each CM is closely related to the surrounding FBs with numerous anatomical contacts, such as ion channels and cell junctions.[3,4,7] Despite this research progress, much is unknown about the organization of FBs and their interaction with CMs.

Long, thin connections have been characterized in cultured PC12 cells.[8] These were named tunnelling nanotubes or membrane nanotubes and were found to provide novel biological cell-to-cell

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communication over a long distance.[8] Membrane nanotubes have been identified between several types of cells, both *in vitro* and *in vivo*,[8–14] with diameters ranging from 50 to 200 nm in cultured neural cells to 700 nm in macrophage cell lines.[11] Membrane nanotubes mediate membrane continuity between connected cells and permit long-distance direct intercellular transfer of diverse components and signals, such as membrane components,[15] Ca^{2+},[16] mitochondria,[9,12] bacteria,[17] murine leukaemia virus,[18] HIV-1,[19] prions,[13] and quantum dots.[20] However, transport of some small cytoplasmic molecules such as calcein was impeded, which indicates a mechanism of selective transfer.[10]

In this study, to better understand the interactions between CMs and FBs, we used a coculture system of neonatal rat ventricular CMs and FBs and discovered membrane nanotubes that mediated a long-distance structural and functional connectivity between CMs and FBs. We also observed similar thin nanotubular structures in adult mouse heart tissue, which suggests the physiological relevance of these membrane nanotubes.

2. Methods

2.1 Isolation and culture of neonatal rat ventricular CMs and FBs

The experimental procedures described here were approved by the Committee on Ethics of Animal Experiments (LA2010-034) and were conducted in accordance with the Guidelines for Animal Experiments, Peking University Health Science Center, and the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996).

Neonatal rat ventricular CMs and FBs were isolated as described.[21] In brief, 1-day-old Sprague–Dawley rats (obtained from the Animal Department of Peking University Health Science Center) were anaesthetized with 1.0% isoflurane (Baxter Healthcare Corp., New Providence, NJ, USA) and then were sacrificed. The hearts were immediately embedded in freezing Dulbecco's modified Eagle's medium (DMEM, Gibco). CMs and FBs were dispersed from the neonatal rat hearts by digestion with 0.25% Trypsin (Gibco) at 37°C. Then the isolated CMs and FBs were cultured separately for 48 h in DMEM supplemented with 10% fetal bovine serum (Hyclone) and antibiotics (50 mg/mL streptomycin, 50 U/mL penicillin) at 37°C in a 95% O_2 and 5% CO_2 atmosphere. A high purity of CMs (>91%) and FBs (>95%) was confirmed by positive staining for sarcomeric α-actinin (Sigma) and vimentin (Sigma), respectively.

2.2 Living cell membrane and mitochondria labelling

WGA Alexa Fluor® 488 conjugate (WGA) (Molecular Probes), CellTracker Green (Molecular Probes) and LysoTracker Red (Molecular Probes) were used to stain membrane, cytoplasmic entity and lysosomes of live cells, respectively.

To study the intercellular membrane connectivity, a plate of CMs was incubated with red membrane dye FM (Molecular Probes) and a plate of FBs with green membrane dye WGA for 10 min at 37°C. After cells were washed in phosphate-buffered saline (PBS) three times, they were dissociated in 0.25% Trypsin-EDTA. Then the suspensions were mixed and incubated for 24 h before observation.

To visualize mitochondria exchange between CMs and FBs, a plate of CMs were loaded with mitochondrial-selective fluorescent probe MitoTracker Green (MitoGreen) and a plate of FBs were loaded with MitoTracker Red (MitoRed) (Molecular Probes) for 30 min at 37°C. Then the cells were washed in PBS three times. After CMs and FBs were dissociated in 0.25% Trypsin-EDTA, the suspensions were mixed and incubated for 24 h before observation.

The red fluorescent protein-tagged Smad3 expression plasmid was a gift from Dr Wei Liang (Institute of Biophysics, Chinese Academy of Sciences) and was transfected with Lipofectamine 2000 (Molecular Probes) for 24 h according to the manufacturer's instruction.

2.3 Immunofluorescence

Cells were washed in PBS once, permeabilized in PBS containing 0.5% Triton X-100 for 10 min and fixed with 4% formaldehyde in PBS for 20 min. Then cells were incubated with anti-tubulin TUBA1 polyclonal antibodies (Proteintech Group), anti-EEA1 antibodies (BD Biosciences), anti-non-phosphorylated connexin 43 antibodies (Zymed), anti-α-SMA antibodies (Sigma), anti-vimentin antibodies (Sigma) or anti-α-actinin antibodies (Sigma) overnight at 4°C. After three washes in PBS, cells were incubated with corresponding Alexa Fluor-conjugated secondary antibodies (Molecular Probes) for 1 h at 4°C. Rhodamine phalloidin (Molecular Probes) was used to stain F-actin.

C57BL/6 male mice (12 weeks old) used in this study were provided by Peking University Health Science Center Animal Department. After mice had been anaesthetized with 0.125% tribromoethanol, hearts were harvested, washed and fixed in 4% paraformaldehyde overnight at 4°C, embedded in paraffin, and sectioned at 5 μm. Membrane, vimentin, α-actinin, and cytoskeleton staining was as described above.

The cells and heart tissue slices stained with different fluorescent dyes were viewed under a confocal microscope with a 100×/1.40NA oil objective and excitation wavelengths at 488, 559 or 633 nm (FluoView FV1000 Olympus, Japan).

2.4 Scanning electron microscopy

The cocultured CMs and FBs on coverslips were fixed with 2.5% glutaraldehyde, dehydrated in graded series of ethanol, and then by critical point drying. In order to eliminate the influences of sputter on the tiny structures, the samples without any coating were viewed under low vacuum mode by using environmental scanning electron microscope (FEI Quanta 200).

2.5 Flow cytometry

Mitochondria exchange between CMs and FBs was measured by flow cytometry with both a 488-nm Argon-ion laser and a 635-nm red diode laser (Beckman Coulter). Excitation at 488 nm was used to detect MitoGreen and 633 nm was used to detect MitoRed.

2.6 Calcium signal stimulation and detection

Cells were loaded with Fluo-4 NW (Molecular Probes) at 37°C for 30 min, then at room temperature for 30 min. Cells were physically stimulated with an atomic force microscopy (AFM) tip by use of a Bioscope (Veeco, Santa Barbara, CA) operating in the contact mode in liquid. Silicone nitride cantilever with spring constants of about 0.06 N/m were used. Calcium signals were viewed under an inverted fluorescence microscope (Olympus IX71, Japan) equipped with a CSU10 spinning disk confocal laser-scanner unit (Yokogawa Electronic, Tokyo) and a 40× objective (Olympus). The cells were excited with a 488-nm argon-ion laser (Melles Griot, CA), and the fluorescence signals were directed to an electron multiplying charge-coupled device (EMCCD) (Andor iXon DU-897 BV).

2.7 Vehicle tracking

Moving vehicles were observed with the use of a fluorescence microscope equipped with a 100×/1.45NA Plan Apochromat objective (Olympus, Japan) and a 14-bit back-illuminated EMCCD. The microscope was also equipped with a cell incubation system (INU-ZIL-F1, TOKAI HIT), which ensured live cell imaging at 37°C in 5% CO_2. Movies were acquired

by use of MetaMorph (Molecular Devices). Single vehicles within membrane nanotubes were tracked with the use of MetaMorph or Image J (NIH).

3. Results

3.1 Membrane nanotubes physically connect neonatal rat ventricular CMs and FBs

To study the intercellular interaction of neonatal rat ventricular CMs and FBs, we cocultured the two cell types. Intercellular WGA-positive nanotubular structures were observed between CMs and FBs double stained for the plasma-membrane dye WGA[22,23] and CM-specific sarcomeric α-actinin or FB-specific vimentin (*Figure 1A* and *B*). Also, WGA-positive membrane nanotubes were observed between CMs and α-SMA-positive FBs (*Figure 1C*). Although membrane nanotubes were observed between CMs and FBs, CMs and CMs, FBs and FBs, respectively, here we focus only on the nanotubular structures between CMs and FBs for understanding the interaction between CMs and FBs. Some FBs (passage 1 or 2) were positive for both vimentin and α-SMA expression; those with increased α-SMA did not show prominent stress fibres (*Figure 1C* and Supplementary material online, *Figure S1*). The increased α-SMA expression may only indicate the phenotype conversion from FBs to myofibroblasts.[24] Thus we used FBs to represent both the α-SMA expression and negative FBs in this study.

The mean length of WGA-stained membrane nanotubes between CMs and FBs was 13.9 ± 10.4 μm ($n = 73$ of 39 pairs of cells). Time-lapse movies confirmed the membrane nanotube between a beating CM and a resting FB (Supplementary material online, *Movie 1*). Furthermore, membrane nanotubes between cells were observed by scanning electron microscopy (*Figure 1D*). Next, we examined the location of the gap junction protein connexin 43 (Cx43), one of the main cardiac connexins.[4] Joints of nanotubes and connected cells showed no punctate Cx43 (*Figure 1E*).

Time-lapse microscopy revealed that membrane nanotubes between CMs and FBs could be formed and elongated when the two contact cells moved apart (Supplementary material online, *Figure S2A* and *Movie 2*). Besides, membrane protrusions launched by FBs could arrive to adjacent CMs or FBs and could induce the transient formation of membrane nanotubes between the adjacent cells (Supplementary material online, *Figure S2B* and *Movie 3*).

3.2 Membrane, cytosolic and structure connectivity of neonatal rat ventricular CMs and FBs mediated by membrane nanotubes

To study the intercellular membrane connectivity, we created a coculture system of CMs labelled with red membrane dye FM and FBs labelled with green membrane dye WGA. *Figure 2A* shows a membrane nanotube labelled with both red and green fluorescence along its axis between the CM and FB. The red dye-labelled CM contained green dye and the green dye-labelled FB contained red dye

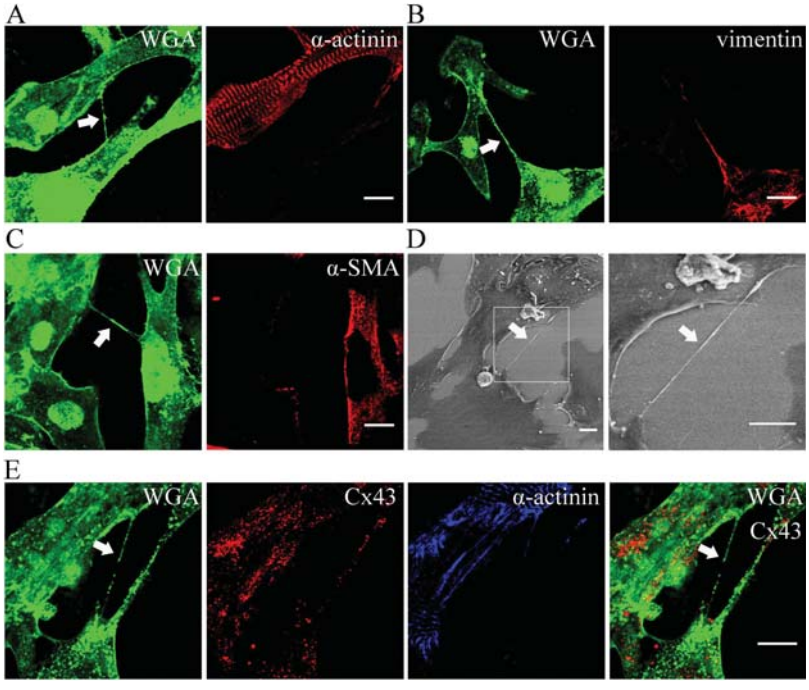

Figure 1 Membrane nanotubes physically connect neonatal rat ventricular cardiomyocytes (CMs) and cardiofibroblasts (FBs). By double staining the cocultured CMs and FBs for green membrane dye WGA Alexa Fluor® 488 conjugate (WGA) and CM-specific sarcomeric α-actinin (*A*), WGA and FB-specific vimentin (*B*), WGA and α-smooth muscle actin (SMA) (*C*), membrane nanotubes (arrows) were observed between CMs and FBs. (*D*) Scanning electron microscopy of membrane nanotubes (arrows) between cells, with the boxed region enlarged and shown at the right. (*E*) The cells were fluorescently labelled for WGA, Cx43 and α-actinin. Scale bar: 10 μm.

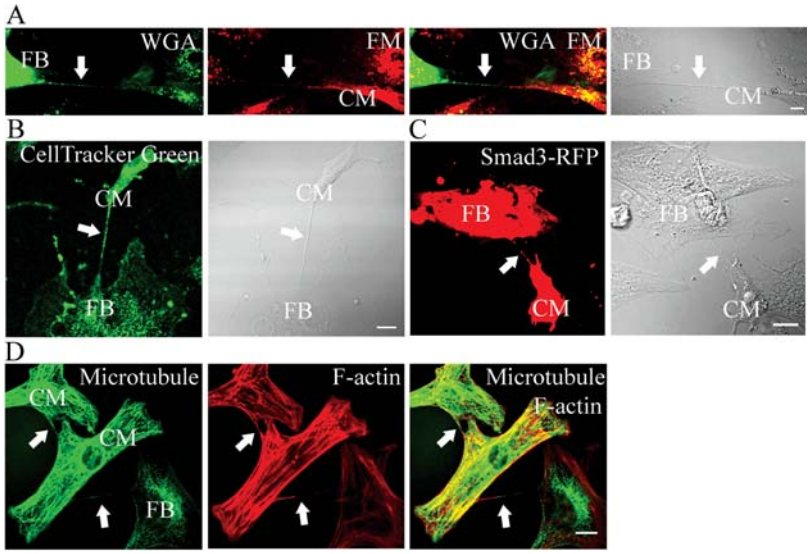

Figure 2 Membrane, cytosolic and structure connectivity between neonatal rat ventricular CMs and FBs *via* membrane nanotubes. (*A*) After a plate of CMs was labelled with red membrane dye FM and a plate of FBs was labelled with green membrane dye WGA, the two cell types were cocultured for 24 h. One membrane nanotube (arrows), with both red and green dye, was established between the CM and FB. The corresponding bright-field image is shown in (d). (*B*) Cocultured CMs and FBs were loaded with cytosolic entity fluorescent probe CellTracker Green. (*C*) Cocultured CMs and FBs were transfected with Smad3-RFP. (*D*) The cells were duel labelled for microtubules and F-actin with α-tubulin antibodies and rhodamine phalloidin. Arrows point to the membrane nanotubes between CMs and FBs in all images. Scale bar: 10 μm.

(*Figure 2A*). Both the nanotube and the connected CM and FB cells were stained with the cytosolic entity fluorescent probe CellTracker Green (*Figure 2B*) to indicate the cytosolic connectivity between CM and FB. After transfection, cytosolic protein molecules with red fluorescence protein-tagged Smad3 were observed within the connecting membrane nanotubes between CMs and FBs, which indicated the cytosolic protein molecules' connectivity between CM and FB (*Figure 2C*). After dual-labelling the cells with α-tubulin antibodies and rhodamine phalloidin, both microtubules and F-actin were observed within the membrane nanotubes between CMs and FBs (*Figure 2D*).

3.3 Intercellular organelle transfer between neonatal rat ventricular CMs and FBs *via* membrane nanotubes

In the cocultured CMs and FBs, time-lapse microscopy revealed small-swelled vehicles that moved within membrane nanotubes between adjacent CMs and FBs (*Figure 3*, $n = 41$ of 18 pairs of cells). *Figure 3A* is a snapshot of a time-lapse movie (Supplementary material online, *Movie 4*) of two vehicles moving from a beating CM to a resting FB through the connected membrane nanotube. The upper vehicle (vehicle 1) moved toward the FB for >5 μm, with a mean velocity of 4.76 ± 3.78 μm/min (*Figure 3A* and *B*). The lower vehicle (vehicle 2) moved along the membrane nanotube toward the FB for 3 μm, with a mean velocity of 3.54 ± 3.31 μm/min for the first 33 min. Then, vehicle 2 moved into the FB with a mean velocity of 2.87 ± 2.30 μm/min and finally was incorporated into the FB. *Figure 3C* (Supplementary material online, *Movie 5*) shows vehicles moving from a FB toward a beating CM through the connected membrane nanotubes.

To determine the identities of the observed moving vehicles, cells were labelled with LysoTracker and anti-EEA1 antibodies, markers for lysosomes and early endosomes, respectively. Both labels were found within the relatively thick membrane nanotubes between CMs and FBs (Supplementary material online, *Figure S3*). To determine whether mitochondria could be transported between CMs and FBs by membrane nanotubes, CMs were labelled with the mitochondrial-selective fluorescent probe MitoGreen and FBs were labelled with MitoRed, then cocultured for 24 h. Some cells were positive for both dyes (*Figure 4A*). The nanotubes between CMs and FBs contained both MitoRed and Green (*Figure 4A*). After FBs were stained with MitoRed and WGA, MitoRed-labelled vehicles were observed within a membrane nanotube between the two connected FBs (*Figure 4B*). When CMs were stained with MitoRed and WGA, MitoRed-labelled vehicles were observed within a membrane nanotube between the two connected CMs (*Figure 4C*). Mitochondria exchange between CMs and FBs was further confirmed by flow cytometry. After CMs and FBs were loaded with MitoGreen or Red, respectively, and then cocultured, a portion of the cells was positive for both dyes (*Figure 4D–d*). As a control, the supernatant of the cocultured cells was added to other cells. As shown in *Figure 4D–e*, cells stained with dyes were rare.

3.4 Intercellular calcium signal propagation between neonatal rat ventricular CMs and FBs *via* membrane nanotubes

To investigate whether calcium signals could propagate *via* membrane nanotubes between CMs and FBs, we loaded the cocultured CMs and FBs with the calcium signal indicator Fluo-4. On mechanical stimulation with an AFM tip, cytosolic calcium release was evoked immediately in CMs and FBs and peaked in seconds. Interestingly, the calcium

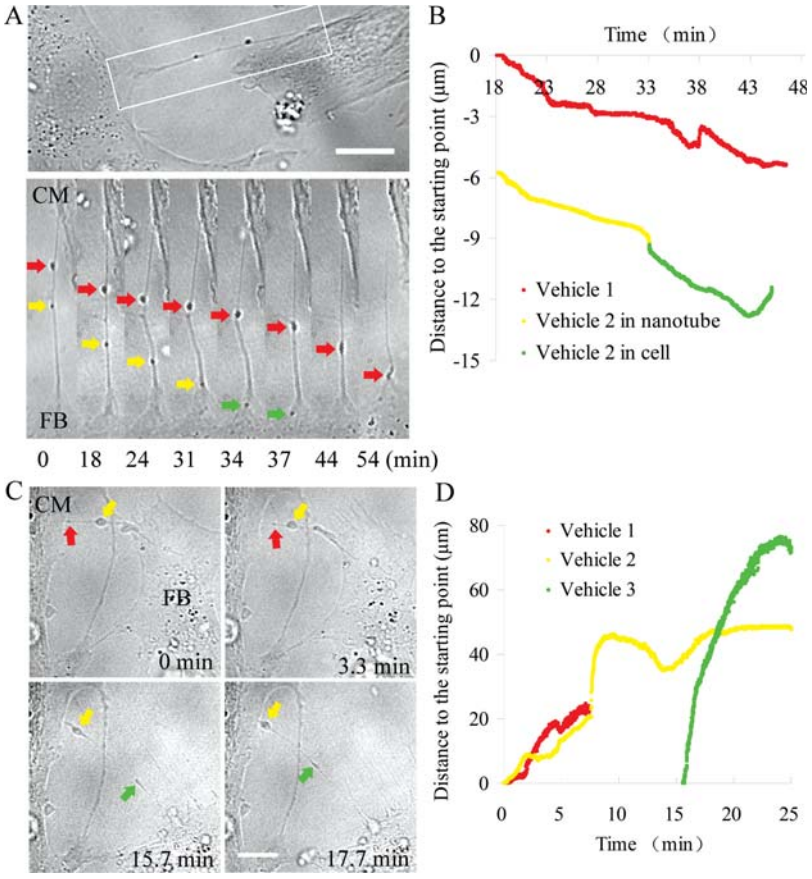

Figure 3 Intercellular vehicle transfer between neonatal rat ventricular CMs and FBs *via* membrane nanotubes. (*A*) A snapshot of a time-lapse movie (Supplementary material online, *Movie 4*) of 2 vehicles moving from a beating CM to a FB through the connected membrane nanotube. The red arrows point to vehicle 1. The yellow arrows point to vehicle 2 moving within the membrane nanotube and finally incorporated into the FB (green arrows). (*B*) Distance to the recoding start point at different times for the two vehicles. (*C*) A snapshot of a time-lapse movie (Supplementary material online, *Movie 5*) of three vehicles moving between a FB and a CM *via* a membrane nanotube. The red arrows point to vehicle 1 moving from the beating CM to the FB within the membrane nanotube. The yellow arrows and green arrows point to vehicle 2 and vehicle 3 moving from the FB to the CM within the membrane nantoube. (*D*) Distance to the recoding start point at different times for the three vehicles. Scale bar: 10 μm.

signal bursting out of the stimulated CM (or FB) was observed to transmit to the nanotube-connected FB (or CM), as shown in *Figure 5A* (Supplementary material online, *Movie 6*) ($n = 25$ pairs of CM and FB). These nanotube-connected cells reached a calcium signal peak seconds after the stimulated cells did (*Figure 5A*). When a network of cells was connected by a network of membrane nanotubes, the cytosolic calcium release in the stimulated cell could propagate to the surrounding cells one by one through these connected membrane nanotubes (Supplementary material online, *Figure S4* and *Movie 7*). However, the adjacent unconnected cells showed no response when the stimulated and membrane nanotube-connected cells exhibited calcium signal bursts (*Figure 5B* and Supplementary material online, *Movie 8*). In several cases, the membrane nanotube-connected cells exhibited an even higher calcium signal amplitude than did the stimulated cells (*Figure 5A* and *B*, Supplementary material online, *Figure S4*). Besides propagating *via* membrane nanotubes, calcium signals could also propagate between these closely contacted cells (Supplementary material online, *Figure S5* and *Movie 9*). Of note, the calcium signal propelled forward in a linear way among these tightly contacted cells.

3.5 Thin structures similar to membrane nanotubes were found within adult mouse heart tissue

To confirm the physiological relevance of the membrane nanotubular structures between cocultured CMs and FBs *in vitro*, mouse heart tissue slices were triple labelled for WGA, CM-specific α-actinin, and FB-specific vimentin. As shown in *Figure 6*, a thin FB process extended between adjacent muscle bundles to connect CMs and FBs. Consistent with cultured cells, these thin structures were positive for both F-actin and microtubules (Supplementary material online, *Figure S6*).

4. Discussion

Clarifying the structural and functional interactions between CMs and FBs is essential for understanding the pathophysiologic heart and for developing safe and effective cell therapies.[6] In the present study, we demonstrated a novel biological interaction, through membrane nanotubes, between cocultured neonatal rat ventricular CMs and

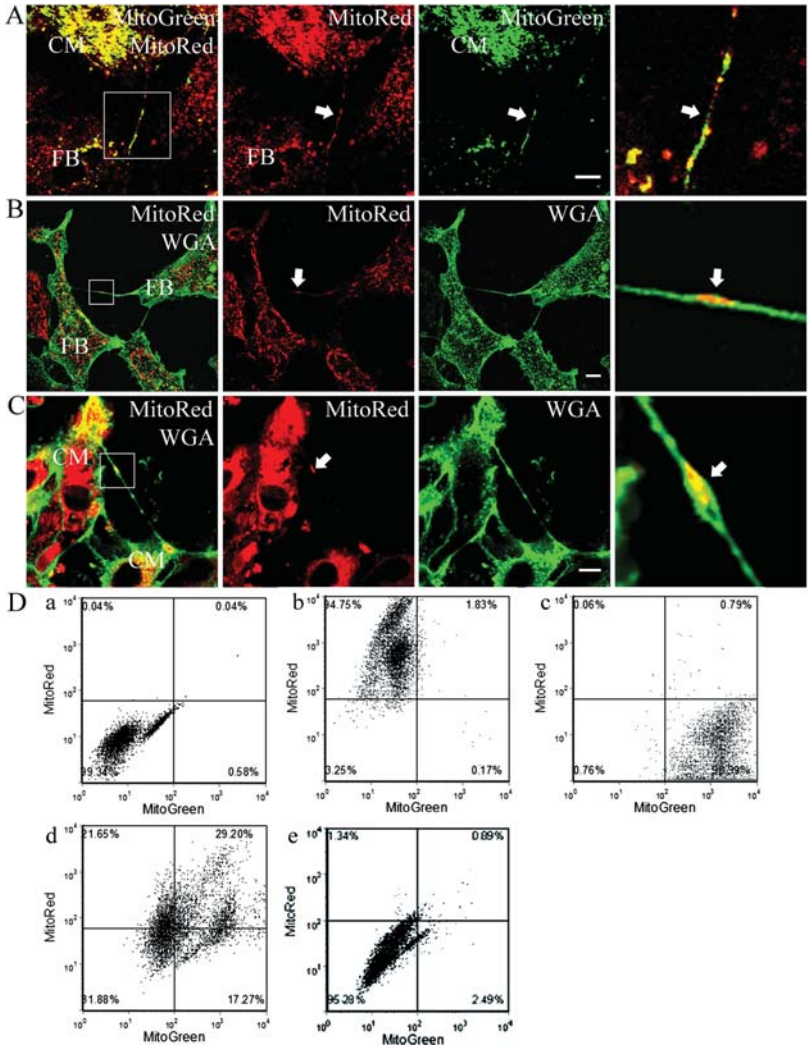

Figure 4 MitoTracker-labelled mitochondria transfer between neonatal rat ventricular CMs and FBs. (*A*) After CMs were labelled with MitoGreen and FBs were labelled with MitoRed, the two cell types were cocultured for 24 h. The nanotube (arrows) between CM and FB contained both MitoRed and Green. Red dye was found in CMs and green dye in FBs. The white box region was enlarged and shown at the right. (*B*) FBs were dual labelled with MitoRed and WGA. One MitoRed-labelled vehicle was observed within the WGA-positive membrane nanotube (arrows). The white box region was enlarged and shown at the right. (*C*) CMs were dual labelled with MitoRed and WGA. One MitoRed-labelled vehicle was observed within the WGA-positive membrane nanotube (arrows). The white box region was enlarged and shown at the right. (*D*) Flow cytometry of the mitochondria exchange between MitoTracker-labelled CMs and FBs. The results of MitoTracker-free cells (a), MitoGreen-labelled cells (b), MitoRed-labelled cells (c), cocultured MitoGreen-labelled CMs and MitoRed-labelled FB (d), cells cultured with the supernatant from the cocultured MitoGreen-labelled CMs, and MitoRed-labelled FBs (e). Scale bar: 10 μm.

FBs. The nanotubular structures contained both F-actin and microtubules, which mediated structural connectivity between CMs and FBs. Cell organelles such as mitochondria can exchange and calcium signals can propagate between CMs and FBs through these membrane nanotubes. These data suggest functional connectivity between CMs and FBs mediated by membrane nanotubes over long distances. In addition, similar membrane thin structures were found in adult mouse heart tissue, which suggests the *in vivo* relevance of membrane nanotubes.*

Intercellular communication is essential for the development and maintenance of multi-cellular organisms.[25] Cells use various mechanisms, such as synapses and gap junctions, to communicate with each other for communication between cells in close proximity.[26] However, membrane nanotubes represent a novel biological principle of cell-to-cell communication over long distances.[8] Membrane nanotubes have been reported in several types of cells, including endothelial progenitor cells with cardiac myocytes and mesenchymal stem cells with cardiac myocytes.[9,12] In the present study, membrane nanotubes could be established between rat ventricular CMs and FBs over long distances, which is consistent with the membrane nanotubes previously reported in other cell types.[8,9,12,13,17] This type of intercellular connection was different from the known gap junctions detected between CMs and FBs in rabbit sinoatrial node.[7] Membrane nanotubes allowed direct communication between the cytoplasm of CMs and FBs even several micrometres away and enabled lengthy trafficking of cargo vesicles, mitochondria and small molecules such as

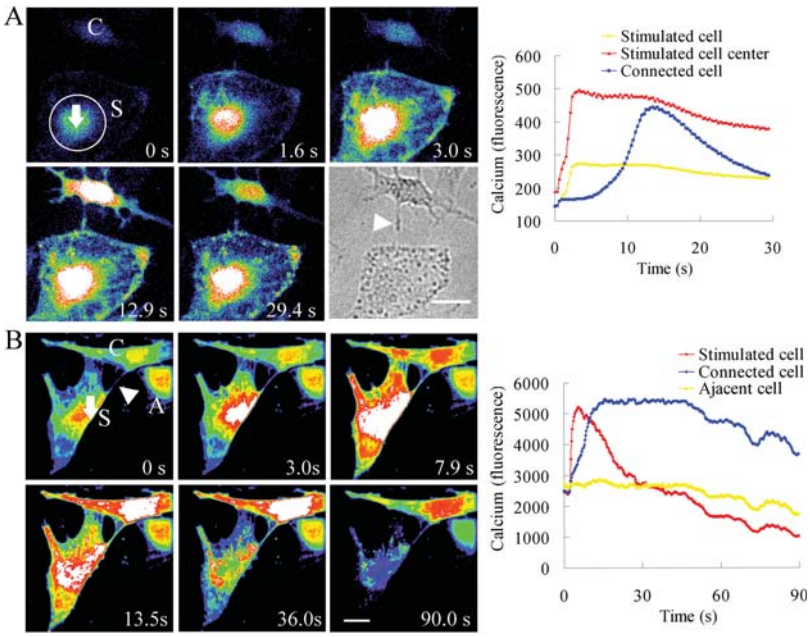

Figure 5 Intercellular calcium signal propagated between neonatal rat ventricular CMs and FBs *via* membrane nanotubes. After the cocultured CMs and FBs were loaded with calcium indicator Fluo-4, a cell was mechanically stimulated with an AFM tip. The calcium signal propagation between cells was recorded by confocal microscopy. (*A*) Calcium signal bursting out in the mechanically stimulated FB was observed to propagate to the membrane nanotube-connected CM *via* the membrane nanotube (Supplementary material online, *Movie 6*). The stimulated FB and the membrane nanotube-connected CM are named S and C, respectively. The relative calcium fluorescence intensity of CM, the centre area and whole area of FB are shown at the right. (*B*) Calcium signal propagation between two membrane nanotube-connected cells but not the adjacent unconnected cell (Supplementary material online, *Movie 8*). The stimulated cell, the membrane nanotube-connected cells and the adjacent unconnected cell are named S, C and A, respectively. The relative calcium fluorescence intensity of the three cells is shown at the right. Arrowheads pointed to membrane nanotubes in all images. Scale bar: 10 μm.

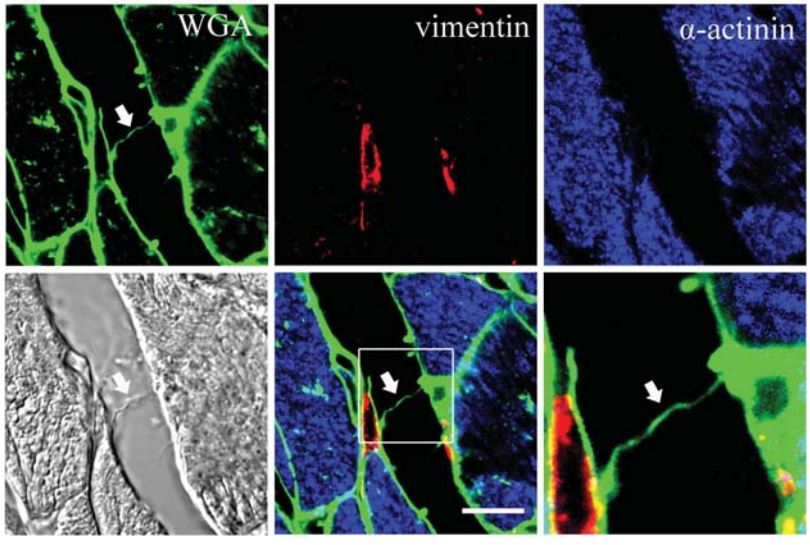

Figure 6 Thin structures similar to membrane nanotubes were found in adult mouse heart tissue. Confocal micrographs of rat cardiac muscle triple labelled for WGA, vimentin and α-actinin. Arrows point to a thin, long FB process similar to membrane nanotubes. Scale bar: 10 μm.

calcium. However, gap junctions were limited to tightly contacted cells and facilitated trafficking of only small molecules up to 1.2 kDa.[27]

Our results confirm that the membrane nanotubes mediated both membrane and cytosolic connectivity between CMs and FBs. Membrane nanotubes were supported inside by the cytoskeleton of both microtubules and F-actin, which is consistent with previous reports in human prostate cancer cells, macrophages, and H9c2 cells.[17,20,25] These cytoskeletal filaments were suggested to be important elements of the intercellular cargo transportation through membrane nanotubes.[8,12,17] Retrograde actin flow, the centripetal flow of newly polymerized actin in the order of 1–5 μm/min,[28] was probably responsible for the cargo vehicle transportation along membrane nanotubes between CMs and FBs. These large intercellular vehicles could be cell organelles such as lysosomes, endosomes, or mitochondria.[8,9,12,13,17] We also found these cell organelles within membrane nanotubes between CMs and FBs.

Both confocal and flow cytometry results confirmed bidirectional exchange of mitochondria between CMs and FBs. Although the exact mechanism of such mitochondria exchange is unclear, the presence of mitochondria within membrane nanotubes suggested that membrane nanotubes might be one of the mechanisms responsible for the exchange. Aerobic respiration of cells with dysfunctional mitochondria could be rescued by the transfer of whole mitochondria or mitochondrial DNA from these undamaged cells.[29] Mitochondria transfer between CMs and endothelial progenitor cells or mesenchymal stem cells was suggested as a new mechanism for stem cell differentiation and for failed myocardium repair.[9,12] Mitochondria fusion is an important mechanism for protecting the mitochondrial genome, which would be depressed in heart failure.[30] Thus, in heart failure or myocardial infarction, membrane nanotubes may provide a long-distance rescue channel between dysfunctional CMs and normal CMs or FBs by facilitating mitochondria exchange, which needs further research.

Using combined AFM and confocal microscopy, we observed that after AFM indentation, the calcium signal bursting out in the stimulated CMs and FBs could promptly propagate to the connected CMs or FBs *via* membrane nanotubes, as was previously reported in immune cells.[16,19] At the same time, the calcium signal could propagate between these closely contacted cells, probably *via* gap junctions.[31] In several cases, the membrane nanotube-connected cells exhibited even higher amplitude of calcium signal than the mechanically stimulated cells, so the membrane nanotube-dependent calcium signal propagation was not merely due to passive transport of calcium ions. IP3 receptors may actively propagate intercellular calcium signals along membrane nanotubes *via* calcium-induced calcium release, acting as amplification sites to overcome the limitations of passive diffusion.[32] Besides, mechanical stimulation-induced depolarization of stimulated cells could probably activate the voltage-gated calcium channels and elicit the transient calcium signals in the connected cells via membrane nanotubes.[33] The calcium signal propagation *via* membrane nanotubes is considered one of the most clear-cut indicators of a physiological role for membrane nanotubes.[34] The coordinated intercellular calcium oscillations offer a rapid mechanism of local intercellular communication and are critical in synchronizing cellular activities.[35] For CMs, intracellular calcium is the central regulator of cardiac contractility, and alterations in CM calcium are important in both the mechanical dysfunction and in arrhythmogenesis associated with congestive heart failure.[36] Thus, membrane nanotubes and gap junctions, which mediate long-distance and short-range calcium signal intercellular connectivity between CMs and FBs, respectively, coordinately regulate cardiac contractility and fight against pathological conditions.

The physiological relevance of membrane nanotubes depends on whether they exist *in vivo*. Long membrane tethers have been observed between immune cells in lymph nodes.[37] By studying live *Drosophila* wing imaginal discs, both apical and lateral cell protrusions were shown to extend in between cells at some distance.[38] Membrane nanotubes observed between dendritic cells within mouse cornea provided the first clear evidence of their existence in mammalian tissues *in vivo*.[11] By immunofluorescence labelling for WGA, F-actin, and microtubules, thin FBs processes similar to membrane nanotubes in cultured CMs and FBs were also observed in adult mouse heart tissues. Unlike the straight features of membrane nanotubes in 2-D-cultured cells, these thin connections within the heart generally adopted tortuous morphologies, which is consistent with previous observations in mouse cornea and 3-D-cultured T cells.[11,19] Previous studies have found that numerous FB processes extended and linked between CMs within the heart, and these processes could mediate the lateral spread of the dye lucifer yellow toward the rabbit sinoatrial node from a scrape-loading site.[4] Scanning electron microscopy and high-voltage transmission electron microscopy confirmed that FBs had long filopodia and, in some cases, appeared to be in contact with other FBs and with CMs.[39] These thin FB processes or long filopodia were similar to the thin structures we report here. Thus, similar to the membrane nanotubes found in cultured CMs and FBs, these thin processes within the heart might also connect FBs and CMs into a structural and functional network. The exact role of these thin connections in the heart is of great interest for future studies.

In summary, this study demonstrates a long-distance connectivity between neonatal rat ventricular CMs and FBs *via* membrane nanotubes, which brings a new outlook on the relationship between CMs and FBs. Cells were weaved into a structurally connective yet functionally supportive network. In addition, the membrane nanotubular connections between CMs and α-SMA-expressed FBs suggested that membrane nanotubes may participate in cardiac fibrosis, which needs further research.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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