Kissing and nanotunneling mediate intermitochondrial communication in the heart

Xiaohu Huang,1 Lei Sun, Shuangxi Ji, Ting Zhao, Wanrui Zhang, Jiebia Xu, Yue Zhang, Yanru Wang, Xianhua Wang,2 Clara Franzini-Armstrong1,2, Ming Zheng,3* and Heping Cheng1

*State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Molecular Medicine, Peking-Tsinghua Center for Life Sciences, and College of Engineering, Peking University, Beijing 100871, China; 2Center for Biological Imaging, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China; 3Department of Cell and Developmental Biology, University of Pennsylvania Medical School, Philadelphia, PA 19104-6058; and 4Department of Physiology, Health Science Center, Peking University, Beijing 100083, China

Contributed by Clara Franzini-Armstrong, January 15, 2013 (sent for review November 12, 2012)

Mitochondria in many types of cells are dynamically interconnected through constant fusion and fission, allowing for exchange of mitochondrial contents and repair of damaged mitochondria. However, constrained by the myofibrillar lattice, the ∼6,000 mitochondria in the adult mammalian cardiomyocyte display little motility, and it is unclear how, if at all, they communicate with each other. By means of target-expressing photoactivatable green fluorescent protein (PAGFP) in the mitochondrial matrix or on the outer mitochondrial membrane, we demonstrated that the local PAGFP signal propagated over the entire population of mitochondria in cardiomyocytes on a time scale of ~10 h. Two elemental steps of intermitochondrial communications were manifested as either a sudden PAGFP transfer between a pair of adjacent mitochondria (i.e., “kissing”) or a dynamic nanotubular tunnel (i.e., “nanotunneling”) between nonadjacent mitochondria. The average content transfer index (fractional exchange) was around 0.5; the rate of kissing was 1% s−1 per mitochondrial pair, and that of nanotunneling was about 14 times smaller. Electron microscopy revealed extensive intimate contacts between adjacent mitochondria and elongated nanotubular protrusions, providing a structural basis for the kissing and nanotunneling, respectively. We propose that, through kissing and nanotunneling, the otherwise static mitochondria in a cardiomyocyte form one dynamically continuous network to share content and transfer signals.

In most cells, mitochondria are highly dynamic organelles that constantly undergo shape changes through fusion and fission. This results in a form of communication that allows for the exchange and distribution of soluble and membranous components, including metabolic intermediates, signaling messengers, proteins, lipids, and mitochondrial DNAs, and provides a mechanism for repairing damaged mitochondria and maintaining a healthy mitochondrial population (1–3). Disorders of mitochondrial fusion and fission have been associated with developmental defects, neurodegenerative diseases, and cardiovascular diseases (4–7).

In adult mammalian cardiomyocytes, mitochondria occupy ∼40% of the cell volume and are rigidly organized between bundles of myofilaments (interfibrillar mitochondria), under the sarcomera (subsacleralmem mitochondria), and around the nucleus (perinuclear mitochondria). This arrangement and the apparent lack of motility, a prerequisite for mitochondrial fusion in other types of cells, raise the question of whether mitochondria in adult cardiomyocytes communicate with each other dynamically. Mitochondrial dynamics in cardiac cell lines or neonatal cardiomyocytes (8) do not provide a direct answer to this question because mitochondria in these cells are not constrained strictly. Limited studies with adult cardiomyocytes have been inconclusive and even controversial. Whereas low-amplitude and high-frequency mitochondrial fluctuations have been visualized and quantified in living adult cardiomyocytes, no mitochondrial fusion and fission was detected (9). In isolated adult rat heart challenged by short periods of hypoxia (10) and in cardiomyopathy patients (11), elongated mitochondria ranging from three to seven sarcomeres in length have been visualized, whereas in cardiomyocytes from heart failure patients and rat models, increased amounts of small and fragmented mitochondria were detected (12), indicating the occurrence of mitochondrial fusion and fission or other as-yet-unknown type of dynamic regulation. Pharmacological treatment with a mitochondrial fission protein the dynamin-related protein Drp1 inhibitor increases the proportion of elongated mitochondria in rat cardiomyocytes and protects the heart against ischemia–reperfusion injury (13), further suggesting a crucial role of mitochondrial dynamics in cardiac function.

Using mitochondria-targeted expression of photoactivatable green fluorescent protein (PAGFP) in conjunction with confocal microscopy, we investigated mitochondrial dynamics in living adult rat cardiomyocytes over extended periods of time. We found that mitochondria communicate with each other, with the elemental steps manifesting as discrete, sudden content transfer events between adjacent (“kissing”) or long-range mitochondrial pairs (“nanotunneling”). As a result, the entire population of mitochondria forms one dynamically continuous network, such that the membranous or matrix contents of individual mitochondria mix and exchange over the whole cardiomyocyte on a timescale of ~10 h.

Results

Mitochondria Are Dynamically Interconnected in Adult Cardiomyocytes. In adult rat cardiomyocytes stained with the mitochondrial membrane potential indicator tetrathymethyl rhodamine methyl ester (TMRE), optically resolved interfibrillar mitochondria were mostly rod-shaped and each was confined to a single sarcomere of ∼2 μm in length (Fig. S1A) (6). However, unlike mitochondria in many other cell lines, time-lapse imaging revealed little motility over as long as 2 h (Fig. S1A and B), indicating that cardiac intermitochondrial communication, if any, must be achieved by means other than the “collision, fusion, and fission” mechanism common to other cell types.

To investigate the intermitochondrial communication in cardiomyocytes, we expressed a photoactivatable green fluorescent protein in the mitochondrial matrix (mtPAGFP) and detected a 40-fold increase in local mtPAGFP fluorescence upon photoactivation (illuminated by 405-nm laser for 60 ms) (Fig. 1A, Upper). Photoactivated mtPAGFP appeared to be sharply confined to the portion of the cell that underwent laser illumination over a few minutes. In sharp contrast, photoactivated cytosolic PAGFP


The authors declare no conflict of interest.

1To whom correspondence may be addressed. E-mail: zhengm@pku.edu.cn or armstroc@mail.med.upenn.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1300741110/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1300741110
diffusion process; alternatively, it may comprise intermittent, mitochondrial communication may occur via a slow but continuous

After photoactivating a 24-μm² rectangular area and surveying for 10 min in 94 cells, we successfully recorded 77 events of sudden intermitochondrial content transfer between two neighboring mitochondria, termed mitochondrial “kissing.”

The frequency of occurrence of kissing was estimated to be 0.23% s⁻¹ (assuming six mitochondria flanked a photoactivated region), and all these kissing events occurred within the same longitudinal mitochondrial bundle. Time course plots showed that a sudden decrease of fluorescence in a photoactivated mitochondrion occurred concomitantly with an abrupt increase of fluorescence in a neighboring nonactivated mitochondrion (Fig. 2A and B, and Movie S1), with their sum of fluorescence unchanged. The stepwise transition was completed within 49 ± 3.4 s (n = 24) for mtPAGFP, whereas conventional mitochondrial fusion has a briefer transition time (14). Spatiotemporal analysis showed that content exchange was restricted to the pair of kissing mitochondria (mito-1 and mito-2) because no change in the mtPAGFP signal was evident in their immediate neighbors (mito-3, mito-4, and mito-5) (Fig. 2D). Importantly, differing from conventional mitochondrial fusion, kissing was not associated with any detectable movement of the mitochondrial pair (Fig. 2E) and

Mitochondrial Kissing in Cardiomyocytes. In principle, cardiac mitochondrial communication may occur via a slow but continuous diffusion process; alternatively, it may comprise intermittent, discrete elemental steps analogous to fusion and fission. To discriminate between these possibilities, a large number of cells expressing mtPAGFP were monitored at a finer time resolution (one frame per 3 s) to catch any rare events, and simultaneous TMRM staining was used to optically locate individual mitochondria. After photoactivating a 24-μm² rectangular area and

Fig. 1. Intermitochondrial communication in adult rat cardiomyocytes. (A and D) Confocal images of cardiomyocytes transfected with adenovo-mtPAGFP (A) or adenovo-PAGFP-OMP25 (D), immediately after photoactivation (Upper) or 12 h later (Lower). (Scale bars: 5 μm.) (B and E) Regional loss (∆F) and gain of fluorescence (∆F) at 12 h after photoactivation. N, nucleus. (C and F) Distribution of PAGFP fluorescence along the cell length at indicated time points. i, photoactivated area; ii, nonactivated area.

To determine whether mitochondrial membrane components exchange as well, we opted to use PAGFP-OMP25 (14) in which PAGFP was fused with the membrane-targeting sequence mitochondrial outer membrane protein 25 (OMP25). Upon photoactivation of PAGFP-OMP25, we detected a sharply confined initial PAGFP-OMP25 signal that showed no immediate bulk propagation (Fig. 1D, Upper). At 12 h, however, maps of local gain (∆F) and loss (∆F) of fluorescence, as well as the spatial profile plot, showed clear propagation of PAGFP-OMP25 from the photoactivated region toward the nonactivated portion of the cell (Fig. 1D–F). Taken together, these data suggest that, despite the intersarcomere physical discontinuity and the lack of bulk movement, all mitochondria in an adult cardiomyocyte form one dynamically continuous network, exchanging both matrix and membranous components over a timescale of ∼10 h.
simultaneous TMRM staining showed no detectable changes in the mitochondrial membrane potential either (Fig. 2C).

If the content exchange was at equilibrium, no fluorescence intensity difference between donor (F1) and acceptor (F2) mitochondria would be expected after the transition. However, the donor was often still brighter than the acceptor even after the transfer (Fig. 2B), indicating that the content exchange was incomplete. We defined the transfer index as $\delta = 1 - D2/D1$, where D1 and D2 refer to the fluorescence difference ($F1 - F2$) before and after the transfer, and found that the transfer index varied from 0.35 to 1, with an average value of 0.57 ($n = 27$), far from complete mixing ($\delta = 1$) (Fig. 2G). Our data suggested that Kiss is too brief to allow for equilibrium between kissing mitochondria. In this scenario, kissing reflects a transient physical connectivity of otherwise segregated mitochondria and the aforementioned transition time provides a good estimate of the kiss duration.

**Mitochondrion-to-Mitochondrion Nanotunneling.** To our further surprise, we found that intermitochondrial communication occurred between pairs of well-separated mitochondria in a saltatory fashion, bypassing their intermediate neighbors. In Fig. 3A and Movie S2, sequential confocal images showed a sudden increase of the mtPAGFP signal in a remote mitochondrion (mito-5) about 8 μm away from a photoactivated mitochondrion (mito-1) in an adjacent interfibrillar mitochondrial bundle. With enhanced image contrast, we showed that a slender thin structure emerged and protruded from mito-1, moved across three intermediate mitochondria, mito-2, mito-3, and mito-4, and connected with mito-5, which showed an abruptly augmenting mtPAGFP signal (Fig. 3B) before this structure disappeared soon after the content transfer. We named this phenomenon “nanotunneling.” It is noteworthy that the synchronous changes of the mtPAGFP signal were confined to mito-1 and mito-5 without affecting the fluorescence intensity in the three intermediate mitochondria (Fig. 3C and D). A total of 21 nanotunneling events was observed in 298 cells over a 10-min time window, suggesting its incidence is about 14 times less frequent than that of mitochondrial kissing. Together, our findings suggest that kissing and nanotunneling constitute elemental steps of mitochondrial dynamics in adult cardiomyocytes.

**Ultrastructural Basis for Kissing and Nanotunneling.** To elucidate the basis of mitochondrial kissing and nanotunneling, we performed transmission electron microscopy (TEM) and found two directly relevant structural details. The first was the presence of very intimate contacts ("kissing junctions") between adjacent mitochondria both at their ends and where they abutted laterally. Most mitochondria, even where restrained between the myofibrils, had several opportunities for such contacts (Fig. 4A). Each contact extended over up to several hundred nanometers and within it the outer membranes had repeated sites where the two membranes seemed to actually touch with no detectable intervening space. Such contacts are highly probable candidates for the exchange of either outer membrane components or matrix proteins. Similar kissing junctions were recently described in skeletal muscle (15).

The second detail was a form of mitochondrial structure that seemed to be directly relevant to nanotunneling. As shown in Fig. 4B, a double-membrane, tubular structure extended from mitochondrion i, bypassed three mitochondria in the same bundle, and was finally connected with mitochondrion ii. These mitochondrial nanotubules were delimited by a double membrane and displayed diameters ranging from 90 to 210 nm. Nanotubules connecting two adjacent mitochondria in adult cardiomyocytes were rarely detected (Fig. 4C), as may be expected because the probability of following one nanotubule for its entire course in a thin section was low. Most frequently, we observed nanotubules connected with only one mitochondrial and possibly projecting from it, giving rise to a "tadpole" appearance (Fig. 4D), and also apparently disconnected nanotubules (Table S1). Because the tubules were often seen to extend over several sarcomeres, they were the most likely basis for the nanotunneling events that we observed. It was not clear whether the nanotubules established a direct continuity between the matrices of two mitochondria or whether they connected to the exchange partner via the equivalent of a kissing junction.

Because the experiments were performed in isolated cardiac myocytes that were maintained for several days in culture, and because this is likely to encourage some loosening of the myofibrils and liberation of mitochondria, we investigated whether the presence of kissing junctions and nanotubules is dependent on the time spent in culture. An examination of freshly fixed hearts and of isolated myocytes between 0 and 3 d in culture revealed that kissing junctions were very numerous and nanotubules were also present in the in situ heart. The frequency of nanotubules was slightly higher in cells that were kept in culture for 3 d relative to freshly plated cells, but the increase was very small (Table S1). This means that the structures necessary for kissing and nanotunneling communication are present in the normal intact heart.

**Quantitative Modeling of Mitochondrial Kissing and Nanotunneling.** At first glance, the kissing and nanotunneling events appeared to be relatively infrequent, occurring once every ~1 h for a mitochondrial pair (~5,000 events per h in a cardiomyocyte). So, we investigated whether these elemental events fully account for the cell-wide propagation of a local signal through the mitochondrial population. We devised a mathematical model for computer simulation and compared it with the spatiotemporal profiles of mtPAGFP spread. In the computer model, 50 units of mitochondria, each 2 μm long,
were positioned linearly in tandem (Fig. 5C). Kissing (between two adjacent mitochondria) and nanotunneling (between a mitochondrial drion and its second or third neighbor on either side) occurred at different rates (p1, p2) but with a constant $\delta$. We experimentally measured the mtPAGFP signal along mitochondrial bundles at 2, 4, and 6 h after photoactivation of a 10.6 $\mu$m x 5.9 $\mu$m rectangular area (Fig. 5A and B). The model parameters were then obtained by nonlinear least-square fitting of the Monte Carlo-simulated results to the experimental data, giving p1 a value of 1% s$^{-1}$; p2, 0.05% s$^{-1}$; and $\delta$, 0.33. Remarkably, this simple model closely reproduced the mtPAGFP spatial profiles at all three time points after photoactivation (Fig. 5D). In the simulation, there were totals of 1,048 kissing and 52 nanotunneling events in 6 h among the 50-mitochondrion bundle, with no fluorescence change when kissing and nanotunneling pairs contained no or equal photo-activated mtPAGFP. Taken together, these experimental and modeling results strongly suggest that kissing and nanotunneling are forms of mitochondrial dynamics that may quantitatively account for the kinetics of local intermitochondrial communication in adult cardiomyocytes.

**Communication Among Perinuclear Mitochondria.** The data in Fig. 1 showed that mtPAGFP and PAGFP-OMP25 propagated farther along the mitochondrial bundle connecting two nuclei of binucleate cardiomyocytes, suggesting a more efficient communication among perinuclear as well as internuclear mitochondria. Morphologically, more round mitochondria are densely packed in the perinuclear areas and along the internuclear mitochondrial band (8, 16). We thus measured the propagation rate of mitochondrial content in these areas. Compared with interfibrillar mitochondria, both matrix mtPAGFP and outer membrane PAGFP-OMP25 showed a faster propagation rate in perinuclear mitochondria: the farthest front of the PAGFP signal 2 h after local photoactivation spread about 19 $\mu$m in the perinuclear area, and 6.5 $\mu$m in the interfibrillar area (Fig. 5A). Mitochondrial kissing was also detected among irregularly organized perinuclear mitochondria (Fig. 5B and C), without motion and collision of the kissing mitochondrial pair. In electron micrographs (Fig. 5D and E), we also documented more frequent nanotubules extending from round mitochondria (i.e., tadpole-shaped mitochondria), as if they were the physical conduit underlying mitochondrial content transfer. Together, our data suggest that the irregularly organized perinuclear mitochondria have more active dynamics than the strictly organized interfibrillar mitochondria.

In a subset of cells (~30%), photoactivation from one side of a nucleus led to a rapid (<2 min), gradual (no stepwise change) increase of fluorescence signal of all of the apparently segregated mitochondria encompassing the nucleus (~12-$\mu$m long axis, 5-$\mu$m short axis) (Fig. S3.A and B). This strongly suggests that perinuclear mitochondria sometimes form a single physically interconnected 3D network. Indeed, we observed synchronous mitochondrial “flashes” in the perinuclear mitochondrial network in adult cardiomyocytes expressing the superoxide biosensor mt-cpYFP (Fig. S3E). The flashes initiated uniformly in the highly extended perinuclear mitochondrial network, rose to a peak amplitude at about the same time, and then dissipated with similar kinetics (Fig. S3E). Flash events that partially enveloped the nucleus were also observed (Fig. S3F). Likewise, synchronous perinuclear mitochondrial membrane potential oscillation was also detected with TMRM (Fig. S3C and D). This suggests that mitochondria in these
nanotunneling to be 0.23. Using a mathematical model, we estimated the rate of occurrence of kissing and nanotunneling events, overlaid with experimentally measured fluorescence distributions. 

2, 4, and 6 h (with 1,048 kissing and 52 nanotunneling events), overlaid with experimentally measured fluorescence distributions.

Discussion

In the present study, we have demonstrated two forms of dynamic mitochondrial communication in adult cardiomyocytes—kissing between adjacent mitochondria, and nanotunneling for a mitochondrion to reach a partner over a long range. Differing from the prevalent mitochondrial fusion model in which mitochondrial motility is a prerequisite (17–19), these types of intermitochondrial communication involve structurally restricted, motionless mitochondria in cardiomyocytes. Moreover, we uncovered several prominent features of these modes of intermitochondrial communication. First of all, the transfer of membranous and matrix contents does not occur as simple diffusion through a pre-connected reticular mitochondrial network. Rather, it is dynamic, manifesting as intermittent, sudden, and brief (<1 min) content transfer between two discrete mitochondria (with exceptions in the perinuclear population of mitochondria). By direct quantification and by parameter fitting the experimental data to a simple mathematical model, we estimated the rate of occurrence of kissing and nanotunneling to be 0.23–1.0‰, and the transfer index, 0.3–0.6. The latter indicates only a partial mix of the contents of the mitochondrial pair. Importantly, ultrastructural analysis revealed kissing junctions between adjacent mitochondria, as well as double-membrane nanotubes (90–210 nm in diameter) extending from mitochondria to physically connect with neighboring mitochondria or remote partners. Furthermore, time-lapse confocal imaging, together with the observation of incomplete content mixing, provides evidence for dynamic inter mitochondrial connectivity. Through kissing and nanotunneling, the population of ~6,000 stationary mitochondria is dynamically connected into one continuous functional network, where a local matrix and membranous content traverses the 120-μm length of a typical cardiomyocyte over the timescales of hours and days.

Interestingly, we found remarkably enhanced inter mitochondrial communication among the perinuclear and internuclear populations compared with interfibrillar mitochondria. The average transfer distance at 2 h after photoactivation was threefold greater. Consistent with this, TEM data showed densely packed mitochondria, more frequently with tadpole shapes and nanotubular projections in this special region. In addition, perinuclear mitochondria appear to form a functionally interconnected network on some occasions, evidenced by synchronous membrane potential oscillation and mitochondrial flash production that engulf the entire nucleus. It is also tempting to speculate that the dynamic perinuclear mitochondrial network is involved in mitochondria-to-nucleus signal transduction and coordination. Furthermore, our preliminary observations found dynamic, long and thin nanotube-like structures in cultured neonatal cardiomyocytes and in HeLa cells (Fig. S4 A and B), suggesting that nanotunneling coexists with conventional mitochondrial fusion. Taken together, mitochondrial nanotunneling is likely an intrinsic regions may be interconnected to form large reticular functional networks. The more active mitochondrial dynamics as well as the higher interconnectivity of perinuclear and interfibrillar mitochondria plays an important role in promoting content exchange over the entire mitochondrial population (Fig. 1).

**Fig. 5.** Model simulation of mitochondrial communication. (A) Confocal images of the mtPAFGP signal at indicated time points after photoactivation of a 10.6 μm × 5.9 μm rectangular region. (Scale bar: 5 μm.) (B) Spatial profiles of mtPAFGP distribution corresponding to A. (C) A model of dynamic content transfer in a linear mitochondrial bundle (Materials and Methods). (D) Monte Carlo simulations at 0 h (after 50 kissing and 2 nanotunneling events), overlaid with experimentally measured fluorescence distributions.

**Fig. 6.** Perinuclear mitochondrial communication. (A) Average travel distance of mtPAFGP and PAGFP-OMP25 signals 2 h after photoactivation, in mitochondria in the perinuclear (PN) and interfibrillar (IF) areas. (B) Confocal images showing mitochondrial kissing in the PN area. Region surrounded by brown dashed line indicates photoactivated area. (Scale bar: 2 μm.) (C) Fluorescence intensity changes in donor (mito 2) and acceptor mitochondria (mito 3) marked in B. Note the lack of change in mito 1 next to the kissing pair. (D and E) Electron micrographs of perinuclear mitochondria. The arrowheads track nanotubular structures of mitochondria. (Scale bars: 500 nm.)
property common to all mitochondria, whereas its phenotypic expression and relative contribution to intermitochondrial communication may vary in a cell type-specific manner.

The questions arise whether nanotunneling is a new property acquired only in highly specialized cells (e.g., heart cells) or a property common to all mitochondria that is best manifested in cardiomyocytes where mitochondrial fusion is ineffective due to lack of motility. An extensive literature search revealed that nanotubules may present an efficient way for organelle-to-organelle or even cell-to-cell communication. In plant cells, nanotubular structures termed stromules bridge separate chloroplasts, mediating the communication of contents between them (20, 21); similar nanotubules called plasmodesmata build routes for the intercellular transfer of nutrients and signals (22). Likewise, in various mammalian cells, intercellular nanotubules mediate long-distance cell-to-cell communication, and enable intercellular transfer of cytoplasmic contents and even organelles (23, 24). Nanotubules connecting bacteria provide superhighways for intercellular and interspecies exchange of cellular protein and DNA (25).

Notably, the mitochondrial nanotubules described in this study had diameters ranging from 90 to 210 nm, similar to those involved in mitochondrial fusion and fission (23). These observations raise the possibility that nanotubules may present an efficient way for organelle-to-organelle and even cell-to-cell communication (organism-to-organism for bacteria) and the underlying molecular mechanisms merit future investigation.

Recent studies have shown that normal mitochondrial dynamics are required to maintain the collective health and homogeneity among the mitochondrial population (1, 5, 27). As shown by us and others, defects of cardiac mitochondrial morphology are associated with a variety of heart diseases (11, 12, 28, 29), and in vivo treatment with a pharmacological inhibitor of the mitochondrial fission protein Drp1 has been suggested to provide cardioprotection against ischemia–reperfusion injury (13). Our preliminary data showed impaired mitochondrial communication in cardiomyocytes from spontaneously hypertensive rats (Fig. S5 A–D), a well-established model for hypertrophic heart disease. The concurrent enhancement of mtDNA damage, as reflected by a decrease in the proportion of integrated mtDNA to nuclear DNA (Fig. S5E), is consistent with a crucial role of mitochondrial dynamics in the maintenance of mtDNA integrity.

In summary, we have provided functional and structural evidence for kissing and nanotunneling as mechanisms allowing for slow but robust communication among immobile mitochondria in adult cardiomyocytes. With kissing and nanotunneling as the elemental steps, the entire mitochondrial population of ∼6,000 units is dynamically interconnected to form one continuous mitochondrial network. Hampering such intermitochondrial communication and the unity of the network may be associated with mitochondrial dysfunction in cardiovascular diseases. The idea that nanotunneling serves as a fundamental mechanism that participates universally in organelle-to-organelle and even cell-to-cell communication (organism-to-organism for bacteria) and the underlying molecular mechanisms merit future investigation.

Materials and Methods

All animal experiments were carried out in accordance with the protocols approved by the Institutional Animal Care and Use Committee of Peking University accredited by Association for Assessment and Accreditation of Laboratory Animal Care International. Adenovirus containing mtPAGFP or PAGFP-OMP25 gene was used to infect adult rat cardiomyocytes. Detailed methods of cardiomyocyte isolation and culture, confocal microscopic and TEM analyses, simulation model, mitochondrial DNA analyses, and statistics are described in SI Materials and Methods.

ACKNOWLEDGMENTS. We thank Dr. Mariusz Karbowiak for providing mtPAGFP plasmid, Dr. David C. Chan for providing PAGFP-OMP25 plasmid, Dr. Yan Zhang for valuable discussion, and Dr. Iain C. Bruce for editing the manuscript. This work was supported by 973 Program Grants 2013CB531103 and 2011CB809102; National Science Foundation of China Grants 30971062, 31130067, and 31221002; and National Institutes of Health Grant R01 HL 48093 (to C.F.-A.).

Transmission Electron Microscopy. Rat hearts were washed with saline and then perfused on a Langendorff apparatus with 3.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for fixation. These hearts were from previous experiments. Papillary muscles were excised and further treated as below. Isolated cardiomyocytes were fixed either in suspension immediately after isolation, or as soon as plated, or, after washing with PBS, incubation for 2–4 d with 2.5% glutaraldehyde in 0.1 M cacodylate (pH 7.2). All samples were postfixed with 2% osmium tetroxide in the same buffer for 1 h at 4 °C, treated en bloc with saturated uranyl acetate at room temperature, dehydrated, and embedded in Epon 812. Thin sections were either stained with lead citrate or double-stained with uranyl acetate and lead citrate and examined in a Philips 410 electron microscope (Philips Electron Optics) equipped with a Hamamatsu C4742-95 digital camera (Advanced Microscopy Techniques) or in a Tecnai 20 transmission electron microscope (FEI).

Simulation of Kissing and Nanotunneling in the Cardiomyocyte. One-dimensional numerical model consisting of 50 mitochondrial units 2 μm in length were devised to represent an array of mitochondria along the longitudinal direction in an adult cardiomyocyte. In this model, each mitochondrion had a rate of p1 to kiss with its forward or backward neighbor. During kissing, these two mitochondria exchanged contents with a transfer index of δ. In an event of nanotunneling, occurring at rate of p2, content exchange occurred between a mitochondrion (at position 0) and any of its neighbors at positions −3, −2, 2, and 3 with equal probability, bypassing its immediate neighbors at positions −1 and 1. The optimal values for the three model parameters, p1, p2, and δ, were determined by nonlinear (least-square error) fitting of Monte Carlo simulation results to the experimental data shown in Fig. 5D.

Statistics. Digital image processing and analysis were performed with IDL software (Research Systems) and custom-devised algorithms. Statistical data are expressed as mean ± SEM, and Student t test was applied to determine the significance of the difference. A value of P < 0.05 was considered statistically significant.

Supporting Information

Huang et al. 10.1073/pnas.1300741110

SI Materials and Methods

Isolation, Culture, and Adenovirus Infection of Adult Rat Cardiomyocytes. Male Sprague Dawley rats and spontaneously hypertensive rats (SHRs) (14 wk) were provided by the Centre for Experimental Animals at Peking University, China. Adult rat cardiomyocytes were enzymatically isolated. Cells were then cultured on dishes precoated with laminin (Sigma) in M199 medium (Sigma). After 1 h culture, cardiomyocytes were infected with adenovirus containing mitochondrial matrix-targeted photoactivatable green fluorescent protein (mtPAGFP) or outer membrane-targeted PAGFP-OMP25 or cytosolic PAGFP. Adenoviral vectors were packaged with the AdEasy XL Adenoviral System (Stratagene). Images were taken 60–72 h after infection.

Confocal Microscopic Analyses. Confocal imaging was carried out with a Zeiss LSM710 microscope with a 40× oil-immersion objective. Cells were kept in a Heating Inser P S1 incubator at a constant temperature of 33–34 °C under 5% CO2 (vol/vol). Regions of interest (ROIs) in cells expressing mtPAGFP or PAGFP-OMP25 or cytosolic PAGFP were photoactivated with an intense 405-nm laser scanning beam for designated durations. Time-lapse images were acquired by exciting at 488 nm and collecting the emission at >505 nm. Multitrack scanning was performed when cardiomyocytes were simultaneously loaded with tetramethyl rhodamine methyl ester (TMRM) (Invitrogen), when images were taken by exciting at 488 nm and collecting the emissions at 505–530 and >560 nm, respectively.

Mitochondrial DNA Integrity Assay. Mitochondrial DNA was quantified by the copy number ratio of integrated mtDNA with genomic DNA as previously reported (1). NADH dehydrogenase subunit 4 (ND4) was used to determine the integrated mtDNA, and the lipoprotein lipase (lpl) gene for determining the genomic DNA. Primers were as follows: ND4-5′, ATCGCACATGG-CCTCACATC; ND4-3′, TGTGTGTGAAGGTGGAGGT; Lpl-5′, GGATGGACGGTAAGAGTGATTC; Lpl-3′, ATCCAAAGGTTAGCAGCACAGGT. A value of δ < 0.05 was considered statistically significant.

Fig. S2. Fast diffusion of PAGFP in the cytosol. (A) Sequential confocal images of a cardiomyocyte transfected with adeno-PAGFP after photoactivation. (Scale bar: 5 μm.) (B) Time course of local PAGFP fluorescence from the photoactivated region in A. Inset shows total PAGFP fluorescence intensity from the whole cell. (C) Distribution of PAGFP fluorescence intensity along the cell length at different time points after photoactivation.
Fig. S3. Mitochondrial communication in the perinuclear area. (A) Rapid propagation of mtPAGFP signal in the mitochondrial network encircling the nucleus. The brown dashed box delimits the area of photoactivation. (B) Time course of local fluorescence intensity in boxed regions of interest (ROIs) in A. Data for the photoactivated region (ROI1) is shown as an Inset. (C) Synchronous mitochondrial membrane potential depolarization followed by uniform repolarization in the perinuclear zone as in A. The dashed lines delimit the boundary of the participating mitochondrial network (C). (Scale bar: 2 μm.) (D) Time course of the perinuclear mitochondrial membrane potential oscillation shown in C. Similar results were obtained from 13 cardiomyocytes. (E and F) Synchronous mitochondrial flashes that envelope the nucleus completely (E) or partially (F) in cardiomyocytes expressing the superoxide biosensor mt-cpYFP, suggestive of a continuum for the interconnectivity of perinuclear and internuclear mitochondria. (Scale bar: 5 μm.)
Fig. 54. Mitochondrial nanotunneling in neonatal cardiomyocytes and HeLa cells. (A) Mitochondrial nanotunneling in a neonatal cardiomyocyte. The arrows indicate the extending nanotubule-like structure. Note the transfer of mtPAGFP fluorescence. Region enclosed by the brown dashed line indicates photoactivated area. (Scale bar: 2 μm.) (B) Mitochondrial nanotunneling in a HeLa cell. Regions in white elliptical dashed line indicate the extending nanotubule-like structure. Region enclosed by the brown dashed line indicates photoactivated area. (Scale bar: 1 μm.) Green, mtPAGFP; red, TMRM.
Fig. S5. Impaired mitochondrial communication in cardiomyocytes from SHR heart. (A and B) Representative confocal images of mtPAFGP signal immediately or 2 h after photoactivation, in perinuclear (A) and interfibrillar mitochondria (B) in cardiomyocytes from SHR heart. (Scale bars: 5 μm.) (C) Travel distance of mtPAFGP signal 2 h after photoactivation, in mitochondria in the perinuclear (PN) and interfibrillar (IF) areas, from SHR and control cardiomyocytes. (D) Incidence of mitochondrial kissing in SHR and control hearts immediately after photoactivation of a 4.9 μm × 4.9 μm rectangular area recorded at one frame every 3 s for 10 min. (E) Change of integrated mtDNA to nuclear DNA ratio in SHR relative to control hearts. *P < 0.05; **P < 0.01 vs. control. n ≥ 4 pairs of control and SHR rats aged 14 wk.

Table S1. TEM analysis of mitochondrial nanotubules in cardiomyocytes

<table>
<thead>
<tr>
<th>Cell status</th>
<th>Mitochondria with nanotubules, %</th>
<th>Disconnected nanotubules, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly isolated and fixed cells</td>
<td>5</td>
<td>1.5</td>
</tr>
<tr>
<td>Freshly isolated and plated cells</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Cells cultured for 3 d</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

Numbers are percentage of total mitochondria without counting disconnected nanotubules. In total, 1,539–2,976 mitochondria from 18–24 cells in each group were counted.

Movie S1. Mitochondrial kissing.
Movie S2. Mitochondrial nanotunneling.