The targeting of cyclophilin D by RNAi as a novel cardioprotective therapy: evidence from two-photon imaging

Masashi Kato1, Masaharu Akao1*, Madoka Matsumoto-Ida1, Takeru Makiyama1, Moritake Iguchi1, Toshihiro Takeda1, Shigeomi Shimizu2, and Toru Kita1

1Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan; and 2Department of Pathological Cell Biology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku 113-8510, Tokyo, Japan

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Aims An opening of the mitochondrial permeability transition pore (MPTP), which leads to the loss of mitochondrial membrane potential (ΔΨm), is the earliest event that commits cells to death, and this process is potentially a prime target for therapeutic intervention against myocardial ischaemia/reperfusion. We aimed to investigate the protective effects of RNA interference (RNAi)-mediated gene silencing of cyclophilin D (CypD), one of the putative components of the MPTP, against myocardial ischaemia/reperfusion using two-photon laser scanning microscopy.

Methods and results We created an adenovirus carrying short-interfering RNA (siRNA) that inactivates CypD. Transduction of CypD-siRNA in rat cardiomyocytes achieved a 61% reduction in CypD mRNA and a 63% reduction in protein levels as well as protection against oxidant-induced ΔΨm loss and cytotoxicity. To further investigate the effects in vivo, we monitored the spatio-temporal changes of ΔΨm in perfused rat hearts subjected to ischaemia/reperfusion using two-photon imaging. Adult rats received direct intramyocardial injections of the adenovirus. Two to three days after injection, rat hearts were perfused by the Langendorff method and ΔΨm levels of individual cells were monitored. The progressive loss of ΔΨm during ischaemia/reperfusion was significantly suppressed in CypD-siRNA-transduced cells compared with non-transduced cells. Furthermore, the protective effect of CypD-siRNA was dose-dependent.

Conclusion Therapeutic interventions designed to inactivate CypD may be a promising strategy for reducing cardiac injury against myocardial ischaemia/reperfusion. The two-photon imaging technique provides deeper insight into cardioprotective therapy that targets mitochondria.

1. Introduction

Mitochondria are crucial modulators of life and death in a variety of cells1 and play pivotal roles in cardiomyocyte death in response to myocardial ischaemia/reperfusion.2 Some key molecules or protein complexes in mitochondria are involved in the mechanisms responsible for acute cell death during myocardial ischaemia/reperfusion injury and also in the mechanisms by which ischaemic preconditioning protects against it.3,4 An opening of the mitochondrial permeability transition pore (MPTP), which leads to loss of mitochondrial membrane potential (ΔΨm), mitochondrial swelling, and subsequent irreversible mitochondrial dysfunction, is the earliest event which commits cells to death.5,6 The MPTP has been hypothesized to consist of at least three fundamental proteins: the adenine nucleotide translocator in the inner membrane, the voltage-dependent anion channel in the outer membrane, and cyclophilin D (CypD) in the matrix.

CypD is a mitochondrial member of the cyclophilin family that has peptidyl-prolyl cis-trans isomerase activity and plays a crucial role in protein folding.7 Recently, we and other investigators demonstrated that CypD-deficient mice were remarkably protected from ischaemia/reperfusion-induced tissue injury in the heart and that mitochondria isolated from wild-type mice were resistant to MPTP opening in vitro.8,9 These data strongly implicate CypD and MPTP as the prime targets for therapeutic intervention against ischaemia/reperfusion injury. Indeed, cyclosporin A (CsA), a Prototype drug for the inhibition of CypD, has been reported to have cardioprotective effect against ischaemia/reperfusion tissue injury in both experimental10
RNA interference (RNAi) is a phenomenon whereby double-stranded RNA knocks down the expression of a gene in a sequence-specific manner.\(^\text{13}\) RNAi by synthetic short-interfering RNA (siRNA) 21–23 nucleotides in length silences cellular gene expression in mammalian cells,\(^\text{14}\) and its therapeutic potential is currently being evaluated in clinical trials.

We recently established a real-time imaging system to monitor mitochondrial function in perfused rat hearts using two-photon laser-scanning microscopy (TPLSM).\(^\text{15}\) TPLSM has unique advantages over conventional confocal microscopy (i.e. greater tissue penetration and lower tissue toxicity).\(^\text{16}\) These properties are quite suitable for the real-time imaging of living heart specimens, as the heart tissue is highly light-scattering and mitochondria are relatively susceptible to photodamage.

Thus, in the present study, we aimed to investigate the protective effects of RNAi-mediated gene silencing of CypD against myocardial ischaemia or ischaemia/reperfusion, using the TPLSM imaging.

2. Methods

All procedures were performed in accordance with the Kyoto University animal experimentation committee. The investigation conforms with 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).

2.1 Design of short-interfering RNA and generation of recombinant adenovirus

We created an adenovirus carrying siRNA which inactivates CypD. A pair of annealed 60-mer oligonucleotides that contain the target sequence of rat CypD mRNA was introduced into the pSUPER, a vector which directs the synthesis of siRNA, following the manufacturer’s instruction (Oligoengine: Seattle, USA). The sequence of the forward 60-mer oligonucleotide was the following: 5’-GATCCCCGGGGACACTTCTTCCTCATTAAGAGATGGAGGGAGAGTTCCCGCTTTTTT-3’. The nucleotides in bold correspond to nucleotides 139–157 of the rat CypD mRNA sequence. Non-targeting sequence was used for the control-siRNA. The recombinant adenovirus was generated using AdEasy system (Qbiogene: Irvine, USA). In brief, the H1-RNA promoter and siRNA-encoding insert were cut out from the pSUPER and subcloned into pAdTrack vector, a shuttle vector for construction of recombinant adenovirus, which is coding green fluorescent protein (GFP) under control of cytomegalovirus promoter. The recombinant adenoviral plasmids were generated by homologous recombination of pAdTrack with pAdEasy. Then PacI linearized adenoviral construct was transfected into QBI293A cells. Transfected cells were harvested when most of the cells peeled off. After three freeze-thaw cycles, the lysate was used for large-scale production of adenovirus in QBI293A cells. Virus was purified by double CsCl centrifugation and subsequently dialysed. Final concentration of virus product was assessed using reagents (Amersham Biosciences: Piscataway, NJ, USA).

2.2 Primary culture of neonatal rat cardiac myocytes

Primary culture of cardiac myocytes were prepared from 1-2-day-old Wistar rats (Shimizu Laboratory Supplies, Kyoto, Japan) and cultured as previously described.\(^\text{17}\) For all the in vitro studies, neonatal cardiac myocytes were transduced with the adenovirus on Day 4-6 in culture. Transductions were carried out in culture medium for 1 h at 37°C. The experiments were carried out at least 48 h after transduction.

2.3 Real-time RT-PCR

Forty-eight hours after transduction of the adenovirus, total RNA was extracted from neonatal rat cardiac myocytes using TRizol reagent (Invitrogen, Carlsbad, CA, USA). Measurement of CypD mRNA level was performed using real-time RT-PCR. In brief, purified RNA was treated with DNase and reverse-transcribed with SuperScript3 Reverse Transcriptase (Invitrogen) according to manufacturer’s protocol. Quantitative gene expression analysis was performed on an ABI prism 7700 Sequence Detection System (Applied Biosystems) using SYBR Green. We used the forward primer (5’-ACACCAATTGCCTCTCAGTC-3’) and the reverse primer (5’-AGGGCCCTTCCTCATACTCA-3’) to amplify CypD cDNA. Relative mRNA levels were normalized by the GAPDH level of each sample.

2.4 Immunoblot analysis

Either 48 h or 7 days after transduction of the adenovirus, neonatal rat cardiac myocytes were harvested and washed with phosphate-buffered saline (PBS) and lysed in a buffer containing 50 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulphate (SDS), 10% glycerol, and 0.01% bromophenol blue. After heating at 98°C for 5 min, equal protein concentrations of the cell lysates were subjected to SDS-polyacrylamide (10%) gel electrophoresis and electro-transferred onto nitrocellulose membranes. After pre-incubation with blocking reagent (PBS containing 0.1% Tween 20 and 5% non-fat dried milk) for 2 h, blotted membranes were incubated with a CypD monoclonal antibody\(^\text{9}\) for 2 h at room temperature, followed by washing with blocking reagent. Membranes were then incubated with a secondary antibody for 1 h at room temperature, washed in PBS containing 0.04% Tween 20, and visualized by ECL Western blotting detection reagents (Amersham Biosciences: Piscataway, NJ, USA).

2.5 Cell viability assay

Forty-eight hours after transduction of the adenovirus, cell viability was quantified on the basis of metabolic activity with the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) (MTS) assay (Promega, Madison, WI, USA), as described previously.\(^\text{18}\) MTS is reduced by metabolically active cells into a formazan product that is soluble in tissue culture medium. The quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture. Briefly, the cultures were incubated in serum-free medium containing 20 μL/well of the MTS tetrazolium compound for 3 h at 37°C. The absorbance of formazan products was photometrically measured at 490 nm with a microplate reader, ARVOx (Perkin-Elmer, Shelton, USA). The cell viability was expressed as the percentage of the absorbance measured in the control group.

2.6 Fluorescence-activated cell sorter analysis of ΔΨ\(_m\)

Cells plated on regular six-well plates (1.0–1.5 million cells per well) were used for the fluorescence-activated cell sorter (FACS) analysis of ΔΨ\(_m\). The tetratemethylrhodamine ethyl ester (TMRE) (Molecular Probes, Eugene, OR, USA)-loaded cells (100 nmol/L at 37°C for 20 min) were harvested by trypsinization at the end of the experimental protocols and analysed with FACSAnia (BD Biosciences, San Jose, CA, USA) (20 000 cells/sample). The fluorescence intensity of TMRE was monitored at 582 nm (FL-2). The results are representative ones from at least three independent experiments, and we have confirmed the reproducibility.
2.7 Confocal time-lapse analysis of $\Delta V_m$

To monitor $\Delta V_m$, cells were loaded with 100 nmol/L of TMRE at 37°C for 20 min. Cells plated on 35-mm glass-bottom dishes (1.0–1.5 million cells per dish) were maintained at 37°C in the presence of 5% CO2 with a heater platform installed on a microscope stage and were placed in serum-free DMEM. After the desired temperature was reached, time-lapse confocal microscopy was started with 2-min intervals, with a 20X objective lens. Images were taken with laser scanning confocal microscopy (LSM510, Zeiss). The TMRE was excited with a 543 nm line of a helium/neon laser. The results are representative ones from at least three independent experiments, and we have confirmed the reproducibility.

2.8 Intramyocardial injection of adenovirus in vivo

Adult male Wistar rats (weight 300–320 g) (Shimizu Laboratory Supplies) were used for all the in vivo studies. Rats were intubated and anesthetized with sevoflurane and left thoracotomy was performed. After incision of the pericardium, a total of 1.0 $\times$ 10^7 pfu adenovirus, in a final volume of 100 µL were delivered by a 30-gauge needle into six to eight sites at depth of 1–2 mm in the left ventricular free wall. After the operation, rats were allowed to have free access of standard rat chow, and water was provided ad libitum.

2.9 Two-photon laser-scanning microscopy imaging of Langendorff-perfused rat heart

After 2–3 days of operation, the heart was excised, the ascending aorta was cannulated with a customized needle, and hearts were perfused in the Langendorff mode. Perfusion was carried out at a constant mean perfusion pressure with oxygenated (95% O2) Tyrode’s solution containing (in mmol/L) 134 NaCl, 4 KCl, 1.2 MgSO4, 1.2 NaH2PO4, 10 HEPES, 11 D-glucose, and 2 CaCl2 (pH 7.4 adjusted with 1 mol/L NaOH). After an initial perfusion period of approximately 10 min, the buffer was switched to oxygenated Tyrode’s solution containing 100 nmol/L TMRE and this was followed by a 10 min washout with dye-free solution. The temperature was maintained at 37°C using a solution heater and a platform heater (Warner Instruments; Hamden, USA) installed on the microscope stage. After dye loading and washout periods, the hearts were placed in a circular glass-bottom dish (35-mm diameter) and were subjected to myocardial ischemia (60–80 min of global ischemia) or myocardial ischemia/reperfusion (30 min of global ischemia followed by 35 min of reperfusion). Ischemia was achieved by clamping the perfusion line and reperfusion by releasing the clamp. During reperfusion period, the hearts were perfused with Tyrode’s solution containing 10 mmol/L 2,3-butanedione monoxime to eliminate contraction-induced movement of the heart during image acquisition. Images were recorded with a Zeiss LSM510 laser scanning microscope modified for TPLSM. Illumination for two-photon excitation was provided by a mode-locked Ti-Sapphire laser (Spectra-Physics, Irvine, USA); the excitation wavelength was 810 nm. Hearts were imaged through a Zeiss 40X 1.30 numerical aperture oil-immersion objective with a working distance of 200 µm. Emitted light was collected by two photo-multiplier tubes fitted with bandpass filters for 500–550 nm (for GFP) and 565–615 nm (for TMRE), respectively. The images are representative ones from at least three independent experiments, and we have confirmed the reproducibility of the responses.

2.10 Image analysis

Post-acquisition image analysis was performed using a software (ImageJ; http://rsb.info.nih.gov/ij/). From the image sequences, regions of interest were drawn over a part of an individual cell, and fluorescence signals within these regions were collected over time. $\Delta V_m$ was monitored using mean TMRE brightness within the regions.

2.11 Statistical analysis

Quantitative data are presented as the mean ± SEM. Comparisons were carried out using either the unpaired Student’s t-test or one-way analysis of variance (ANOVA) with Bonferroni’s least significant difference as the post hoc test. A level of $P < 0.05$ was accepted as statistically significant.

3. Results

3.1 Gene silencing of cyclophilin D by adenovirally mediated RNAi

First, we verified the silencing of CypD in cultured cardiomyocytes by adenovirally mediated delivery of siRNA. We confirmed that the adenovirus achieved nearly 100% transduction efficiency in cardiomyocytes as determined by GFP expression (not shown). In real-time RT-PCR, mRNA of CypD was reduced by 61% in the CypD-siRNA group compared with the control-siRNA group, at 48 h after virus transduction ($P < 0.05$; Figure 1A). Immunoblot analysis also demonstrated the sufficient reduction of the CypD protein by 63% in the CypD-siRNA group at 48 h after virus transduction ($P < 0.05$), and the effect of gene silencing persisted for at least 7 days (Figure 1B).

3.2 Protective effects of cyclophilin D-short-interfering RNA against oxidative stress in vitro

First, we examined whether gene silencing of CypD affects the overall viability of neonatal rat cardiomyocytes exposed to oxidative stress (H2O2 100 µmol/L). Cell viability assays revealed that cells of the CypD-siRNA group were more resistant to oxidant injury compared with the control-siRNA group ($P < 0.05$; Figure 1C).

To examine whether the preservation of $\Delta V_m$ is associated with the cardioprotective effects of CypD-siRNA, we assessed the change of an indicator of $\Delta V_m$, TMRE fluorescence, by H2O2 stimulation in each group with FACS analysis (Figure 1D, E). Without H2O2 exposure, the majority of cells expressed high levels of TMRE fluorescence (above the vertical dashed line in Figure 1D). Exposure to H2O2 shifted most of the population to a lower TMRE fluorescence in the control-siRNA group. Silencing of CypD protected against the H2O2-induced loss of $\Delta V_m$, preserving a population of cells with a high $\Delta V_m$ level. These observations were rendered quantitatively by plotting the percentage of cells with high TMRE, as shown in Figure 1E.

We next examined the time-dependent changes of $\Delta V_m$ on a single-cell basis, using time-lapse confocal analysis of cardiomyocytes loaded with TMRE (Figure 2; see Supplementary material online, Movie S1, S2). Time-lapse scanning began immediately after the application of 100 µmol/L H2O2. In the control-siRNA group, cells treated with H2O2 progressively lost their red fluorescence intensity in about 40 min, indicating an irreversible loss of $\Delta V_m$. In contrast, the TMRE fluorescence was remarkably preserved in the CypD-siRNA group (Figure 2A). Fifteen cells were randomly selected in each group, and the TMRE fluorescence intensity from each individual cell was plotted. The CypD-siRNA group remarkably preserved $\Delta V_m$ after H2O2 exposure compared with the control-siRNA group (Figure 2B). Figure 2C shows the average of TMRE fluorescence from 15 cells in each group. Mean fluorescence of the CypD-siRNA group after...
60 min of H₂O₂ exposure was significantly higher than that of the control-siRNA group (P < 0.05; Figure 2D).

3.3 In vivo transduction of the adenovirus and two-photon laser-scanning microscopy imaging of Langendorff-perfused rat heart

To further investigate the protective effects in vivo, we transduced the adenovirus carrying CypD-siRNA into the rat hearts. Two to three days after intramyocardial injections of the adenovirus, the hearts were perfused in Langendorff mode, and underwent TPLSM imaging (as shown in Figure 3A), which showed a 30-50% transduction efficiency of the adenovirus within the region of interest (Figure 3B). Successfully transduced cells can be distinguished from non-transduced cells by GFP fluorescence as shown in Figure 3B.

To confirm whether TMRE fluorescence reflects changes in ΔΨᵢ, we monitored the fluorescence in hearts perfused with mitochondrial uncoupler, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (1 μmol/L; Figure 3C). Time courses of TMRE fluorescence from individual cells (n = 10) were plotted in Figure 3D. After the administration of FCCP, the TMRE fluorescence underwent rapid dissipation within a couple of minutes, excluding the possibility that TMRE loading exceeds the potential-dependent mitochondrial uptake.
results suggest that residual oxygen molecules that flow through the respiratory chain as well as the inverse operation of F_{0}F_{1}ATPase are likely to contribute to the maintenance of $\Delta \Psi_{m}$ during the early phases of ischaemia.

### 3.4 Protective effects of cyclophilin D-short-interfering RNA against myocardial ischaemia or ischaemia/reperfusion

To investigate the effect of CypD-siRNA against myocardial ischaemia, we monitored $\Delta \Psi_{m}$ changes in perfused hearts subjected to no-flow ischaemia. Fifty to sixty minutes after global ischaemia was induced, the vast majority of cells underwent dissipation of TMRE fluorescence. In the control-siRNA-transduced heart, the TMRE fluorescence of...
each individual cell was progressively lost, irrespective of GFP-positive and GFP-negative cells (Figure 4A; see Supplementary material online, Movies S3, S4). Meanwhile, in the CypD-siRNA-transduced heart, the GFP-positive cells appeared to maintain steady levels of $\Delta \psi_m$ during ischaemia, compared with the more remarkable reduction of TMRE fluorescence in the GFP-negative cells. Six cells were randomly selected from GFP-positive cells and GFP-negative cells in each group. Time courses of TMRE fluorescence from each individual cell and the average of six cells were plotted (Figure 4B). In the CypD-siRNA group, the GFP-positive cells significantly preserved $\Delta \psi_m$ during ischaemia while the GFP-negative cells did not. However, in the control-siRNA group, there was no difference between GFP-positive and -negative cells. Figure 4C shows mean TMRE fluorescence intensity from six cells at 80 min of global ischaemia. In the CypD-siRNA group, mean TMRE fluorescence was significantly higher in GFP-positive cells than in GFP-negative cells; the same difference could not be observed in the control siRNA group. Based on the assumption that the GFP fluorescence level in a cell is proportional to the amount of siRNA transduced, we analysed

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**Figure 3** In vivo transduction of the adenovirus and two-photon laser-scanning microscopy (TPLSM) imaging of Langendorff-perfused rat heart. (A) Schematic drawing of the perfused rat heart and optical slice. (B) Images of cardiomyocytes in perfused rat hearts. Two to three days after transduction of adenovirus, the perfused hearts were loaded with tetramethylrhodamine ethyl ester (TMRE) and observed with TPLSM. Transduction of adenovirus was verified with the green fluorescence protein (GFP) fluorescence. Scale bar: 50 $\mu$m. (C) Representative images of the TMRE fluorescence after administration of 1 $\mu$mol/L FCCP. Scale bar: 50 $\mu$m. (D) Time course of the TMRE fluorescence of 10 cells randomly selected after FCCP. (E) Representative images during ischaemia with or without 2 $\mu$g/mL oligomycin and 10 mmol/L cyanide. Scale bar: 50 $\mu$m. (F) Time course of the TMRE fluorescence of 10 cells randomly selected from each group. (G) Mean TMRE fluorescence intensity of 10 cells in each group.
the dose-response of siRNA (Figure 4D). In these figures, each symbol represents an individual GFP-positive cell. The x-axis illustrates GFP fluorescence at the beginning of ischaemia, and represents the levels of siRNA expression. The TMRE fluorescence at the end of ischaemia, represented by the y-axis, demonstrates the preserving effect of ΔΨm. In the control-siRNA group, there was no correlation between GFP and TMRE. In contrast, in the CypD-siRNA group, there was a strong correlation between the two, which leads us to believe that the more siRNA is expressed, the more protection cells receive.

We further confirmed the protective effects of CypD-siRNA against myocardial ischaemia/reperfusion. After 30 min of global ischaemia, the hearts were reperfused and the levels of ΔΨm were monitored (Figure 5A). Time courses of TMRE fluorescence from each individual cell and the average of six cells were plotted (Figure 5B). Ten to twenty minutes after reperfusion, the TMRE
fluorescence was progressively lost in both GFP-positive and GFP-negative cells in the control-siRNA-transduced heart. Meanwhile, in the CypD-siRNA-transduced heart, the GFP-positive cells appeared to maintain steady levels of $\Delta \Psi_m$ after reperfusion compared with the GFP-negative cells. In the CypD-siRNA group, mean TMRE fluorescence was significantly higher in GFP-positive cells than in GFP-negative cells (Figure 5C). The TMRE fluorescence in CypD-siRNA-treated hearts disappeared after the infusion of FCCP, indicating that the residual TMRE fluorescence after ischaemia/reperfusion also reflected $\Delta \Psi_m$, but did not reflect non-specific binding of the probe to lipophilic cell components (Figure 5D). In addition, we tested whether CypD-siRNA could demonstrate protective effects against the $\Delta \Psi_m$ loss induced by respiratory chain inhibition (continuous perfusion with 10 mmol/L cyanide; Figure 5E and F). $\Delta \Psi_m$ started to decline at around 20–30 min, and CypD-siRNA failed to prevent the loss of $\Delta \Psi_m$ induced by respiratory chain inhibition.

4. Discussion
In this study, we successfully visualized the real-time spatio-temporal changes of mitochondrial function in CypD-inactivated cardiomyocytes subjected to ischaemia/reperfusion, using TPLSM. We demonstrated that CypD-siRNA transduction in cardiomyocytes remarkably protected against progressive mitochondrial dysfunction during ischaemia/reperfusion in a dose-dependent manner.

4.1 Advantage of real-time two-photon laser-scanning microscopy imaging in assessing cardioprotective therapy
Studies from CypD-deficient mice demonstrated the protective effects of gene silencing of CypD against necrotic stimulation in the heart, but they are all based on rough endpoints such as macroscopic measurement of infarct size in excised fixed heart specimens. The TPLSM imaging is a valuable new tool to test the effects of anti-ischaemia therapy that targets mitochondria; it enables us to monitor dynamic changes in mitochondrial function in the living heart with high spatio-temporal resolution. Taking advantage of this system, we successfully visualized cellular responses of CypD-inactivated cardiomyocytes in perfused rat hearts subjected to ischaemia/reperfusion, and strongly implicated the potential usefulness of RNAi-mediated CypD silencing as a novel therapeutic strategy for cardioprotection.

4.2 Cyclophilin D as a therapeutic target for cardioprotection
Although the issue of elucidating the molecular composition of the MPTP in its entirety remains unresolved, recent data from CypD-deficient mice strongly implicate CypD as a key component of the MPTP. Along with other investigators, we have shown that CypD-deficient mice were developmentally normal, however CypD-deficient mitochondria did not undergo the calcium-induced MPTP opening. CypD-deficient cells died normally in response to various apoptotic stimuli, but showed resistance to necrotic cell death induced by reactive oxygen species (ROS) and calcium overload. Moreover, CypD-deficient mice showed a high level of resistance to ischaemia/reperfusion-induced tissue injury in the heart. Our results are in good agreement with these observations. Whereas the CypD knockout mouse presents with a congenital anomaly implicating CypD as a potential target for therapeutic intervention, the present study goes beyond this capacity and successfully negotiates RNAi to transiently inactivate CypD as a therapeutic intervention strategy against ischaemia/reperfusion. By doing so, this study not only confirms the veracity of RNAi-induced CypD knockdown as a promising form of therapy, but also erects the framework for future studies employing differing strategies for an established therapeutic target.

As shown by TPLSM images, $\Delta \Psi_m$ did not result in immediate collapse after the onset of ischaemia, but were maintained for approximately 40 min during ischaemia. In contrast, in cultured cardiomyocytes, simulated ischaemia induces an immediate collapse of $\Delta \Psi_m$. The mechanism responsible for the prolonged maintenance of $\Delta \Psi_m$ during ischaemia in the perfused heart remains elusive, but our results with oligomycin and cyanide suggest the contribution of the inverse operation of F$_0$F$_1$ATPase and residual oxygen molecules that flow through the respiratory chain complexes. $\Delta \Psi_m$ was maintained for several minutes even in the presence of both oligomycin and cyanide; however, this may be owing to a slow cardiomyocyte uptake of these drugs in perfused heart. Regardless of what the underlying mechanisms may be, $\Delta \Psi_m$ can be maintained for a considerable length of time after the onset of ischaemia. During such a period, accumulating ROS during ischaemia can as well as the burst of ROS upon reperfusion can trigger the MPTP opening and result in the consequent loss of $\Delta \Psi_m$. CypD silencing could exert protective effects by decreasing the likelihood of MPTP opening. However, CypD silencing offered no protection against cyanide-induced $\Delta \Psi_m$ loss, as it may not be mediated by the MPTP opening.

4.3 Cyclosporin A and other blockers of mitochondrial permeability transition pore
CsA has been reported to prevent MPTP opening and have cardioprotective effects in experimental studies. Recent report from Ovize group proved the efficacy of CsA against ischaemia/reperfusion injury even in clinical situations. CsA is an immunosuppressive drug, which has high affinity for cyclophilins and is widely used as an inhibitor of MPTP and cell death caused by various cellular stimuli. However, the use of CsA is often open to criticism as this drug has pleiotropic effects that affect multiple pathways. For example, CsA inhibits calcineurin, which also plays a crucial role in modulating mitochondrial death signals. Moreover, nephrotoxicity, microvascular dysfunction, a narrow therapeutic window, reduction in left ventricular function in the long-term are major drawbacks associated with the clinical use of this drug. NIM811, a non-immunosuppressive CsA derivative, which does not inhibit calcineurin, also inhibited MPTP opening and protected against ischaemia/reperfusion injury, as did sanguifehrin A, which exerts its immunosuppressive action by binding to CypD. Despite the reported efficacy of these drugs in animal models, the clinical use of these agents was only limited as CypD is widely expressed in other organs as well as the heart. Therefore, a targeted disruption of the heart CypD is highly preferred.
4.4 RNAi as a novel therapeutic strategy

RNAi is a powerful tool for specific gene silencing and now being evaluated in clinical trials as a potentially novel therapeutic strategy for a wide array of diseases. But there still remains a number of obstacles to be negotiated before the therapeutic application of RNAi can be realized. For specific delivery of siRNA into the hearts, we used direct...
intramyocardial injections of the adenovirus, which provided us with sufficient transduction efficiency (approximately 30–50% cells within the region of interest) that enabled us to successfully monitor and compare mitochondrial function in both siRNA-transduced and non-transduced cardiomyocytes. To apply this RNAi-mediated gene silencing to clinical situations, we need to step in before ischaemic events occur, thus it is not practical to adopt this method into ongoing sudden myocardial ischaemic events such as acute coronary syndrome. In some other clinical situations where the ischaemic events can be highly predicted, such as cardiac surgery, severe chronic ischaemia, or high-risk patients with multiple coronary risk factors, this method could be a feasible therapeutic intervention for cardioprotection.

4.5 Study limitation
Measurements with TPLSM are limited to myocytes in superficial layers of ventricle, approximately 0–50 μm. As only a very small number of cells are transduced in our experiment (the infected areas are limited to the epicardial injection sites), we could not examine global cardiac function or infarct size. We verified the silencing of CypD in neonatal cardiomyocytes for at least 7 days after the virus infection. Cultured cardiomyocytes infected with virus did not survive any longer, and we do not know how long the effect can last. In clinical use of our methods, more sophisticated and less invasive, hopefully virus-free, siRNA delivery methods would be required.

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