SUPPLEMENTAL MATERIAL

Methods

Differentiation and culture of human induced pluripotent stem cells-derived cardiomyocytes (hiPSC-CMs)

Human iPS cells were cultured on Matrigel (growth factor reduced, BD Bioscience, Bedford, MA) in mTeSR1 medium (WiCell Institute, WI) as a monolayer. Cells were overlaid with a thin layer of Matrigel (0.5 mg /6-well plate) in mTeSR1 medium when reaching 80-90% confluency and grown for another 1-2 days in mTeSR1 medium until reaching 100% confluency, defined as day 0. Then, the mTeSR1 medium was replaced with RPMI 1640 medium (Life Technologies, Carlsbad, CA) plus B27 without insulin supplementation (Life Technologies) and containing Activin A (100 ng/mL; R&D Systems, Minneapolis, MN) and Matrigel for 24 h. After which, the medium was changed to RPMI1640 plus B27 without insulin supplemented with bone morphogenetic protein 4 (R&D Systems; Minneapolis, MN 5 ng/mL) and basic fibroblast growth factor (Invitrogen, Grand Island, NY; 10 ng/mL) for 4 days. After the growth factor treatment, the cells were maintained in RPMI plus B27 complete supplement medium. Confluent contracting cells were observed at day 7. The method we used to differentiate hiPSC-CMs generates > 90% cardiomyocytes.[1,2] Since the endpoint measurement was cardiac sodium channels, expressed almost exclusively in cardiac myocytes, it is unlikely that other cell types present after differentiation would affect the results significantly.

Human heart tissue samples

Among 10 end-stage cardiomyopathic heart samples, six samples had ischemic myopathy, four samples had nonischemic myopathy. Seven and three samples were from males and females, respectively. The age range of sample donors was is 34 to 75

years old, and their average age was 59 years old. End-stage cardiomyopathic heart samples were obtained at the time of left ventricular assist device (LVAD) placement or cardiac transplantation. Specimens were obtained under Advocate Christ Medical Center Institutional Review Board (IRB) approval (Protocol 3184) from samples that would have been otherwise discarded. Subjects with end-stage cardiomyopathy exhibited severely reduced ejection fraction, left ventricular dilation, elevated pulmonary arterial and wedge pressures, and a reduced cardiac index. The control samples were obtained from donor hearts unused for transplantation. Control subjects were younger (median age 42 years with a range of 24–50 years) but with a similar gender profile (66% male). Samples were analyzed under University of Illinois at Chicago IRB approval (Protocol 2009-0881). The investigations conformed to the principles outlined in the Declaration of Helsinki.

Real-Time PCR quantification

Total RNA was isolated from cultured cells and human ventricular tissue using the RNeasy Mini Kit and RNeasy Lipid Tissue Mini Kit, respectively (Qiagen, Valencia, CA). Primers for target genes were: SCN5A (5'-TTACGCACCTTCCGAGTCCTCC-3'; 5'-GATGAGGGCAAAGACGCTGAGG-3');HSCN5AE28C/R(5'-TCTCTTCTCCCCTCCTG-CTGGTCA-3'); HSCN5AE28D/R (5'-GGAAGAGCGTCGGGGAGAAGAAGTA-3'); PERK (5'-AGTCTCTGCTGGAGTCTTCATGACACTGTGTCTCAGACTCTT-3'); Kv4.3(5'-GCG-CGCGAATTCCCTTTTGCCCGGGCTGCG-3;5'-GCACACGGATCCCAAAGAAGTACT-CCTTGG-3'); CHOP (5'-CCTAGCTTGGCCTGACAGA GA-3'; 5'-CTGCTCCTTCTCC-TTCATGC-3'); calreticulin (5′-CATGATGGAC ATGATGATG ACAC-3′; 5′-GGTCTTCAG-ACTTGCATCTGGC-3′); β-actin (5'-GGATCGGCGGCTCC AT-3'; 5'-CATACTCCTGCT-

TGCTGATCCA-3'). TRPM7 primers were obtained from the All-in-One™ qPCR kit (Genecopoeia, Rockville, MD). The amplification conditions were a holding stage of 95°C for 20 min and 40 cycles at 95°C for 30 s and 60°C for 60 s.

Transfection and infection assays

Human cardiomyocytes were placed in a 24-well plate at the density of 200,000/well. PERK short hairpin RNA (shRNA; 5 μL for each well based on pre-titer results) was pre-incubated with polybrene (Sigma, Milwaukee, WI) at final concentration 8 μg/mL for 1 h and aliquoted to each well. Fugene 6 reagents from Roche (Madison, WI) were used for transfection assays following the manufacturer's instructions. Human pGIPZ lentiviral short hairpin RNAmir anti-PERK particles were purchased from Open Biosystems (Huntsville, AL). Human cardiomyocytes were placed in a 24-well plate at the density of 200,000/well. PERK short hairpin RNA (shRNA; 5 μL for each well based on pre-titer results) was pre-incubated with polybrene (Sigma, Milwaukee, WI) at final concentration 8 μg/mL for 1 h and aliquoted to each well. The scrambled shRNA group followed the same protocol. The media was replaced by regular culture media after 5 h. The infection rates and PERK knockdown rates were evaluated by Western blotting and quantitative PCR (qPCR) respectively on day 2 and day 3. C-terminal GFP-tagged E28C variant, E28D variant, or full-length SCN5A constructs were transduced into cells. Previously, we have shown that full-length, C-terminal GFP tagged channels have normal targeting and function.[1] An empty vector and a vector expressing only the fluorescent marker were used as controls. The transfection assays followed the manufacturer's instructions.

Immunofluorescence staining and confocal microscopy

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Transfected hiPSC-CMs on coverslips were fixed with 4% paraformaldehyde (with 4% sucrose in phosphate buffered saline (PBS)) for 15 min, permeabilized with 0.2% Triton X-100 for 5 min, and blocked with 5% bovine serum albumin for 1 h. Fixed cardiomyocytes were then incubated with the primary antibody, anti-calreticulin (Abcam, Cambridge, MA), at room temperature for 1 h. After washing with PBS (3 X 5 min), the cells were incubated with Alexa Fluor 594-labeled secondary antibody (1:1000, room temperature 1 h). The cells were then washed with PBS (3 X 5 min) and mounted with ProLong Gold and stained with DAPI (4',6-diamidino-2-phenylindole) (Life Technologies). CellMask Plasma Membrane Stains (Invitrogen) was used for cell plasma membrane staining. The detailed method followed manufactory protocol. Fluorescent imaging was acquired using a Carl Zeiss LSM 510 Meta confocal microscope.

Electrophysiology

hiPSC-CMs were trypsinized (0.25% trypsin-EDTA, Invitrogen) for 10 min and plated in 35 mm culture dishes at a cell density of ~100 cells/dish on the day before the experiments. Na^+ channel currents were measured by using the whole-cell patch-clamp technique in the voltage-clamp configuration at room temperature. hiPSC-CMs were not selected by action potential morphology, but the differentiation technique used results in predominantly ventricular-like cells.[2] To measure Na^+ channel currents, pipettes (3 to 4 MΩ) were filled with a pipette solution containing (in mmol/L): CsCl 80, cesium aspartate 80, EGTA 11, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 10, and Na_2ATP 5 (adjusted to pH 7.4 with CsOH). The bath solution consisted of (in mmol/L): NaCl 20, NMDG 90, TEA-Cl 20, CsCl 5, $CaCl_2$ 2, $MgCl_2$ 1.2, HEPES 10, and glucose 5 (adjusted to pH 7.4

with CsOH). The holding potential was -100 mV. A voltage step protocol ranging from -80 to +70 mV with steps of 10 mV was applied to establish the presence of Na^+ channel currents. The peak current density was used to plot current-voltage (I-V) curves. As before, nifedipine (10 μM, Sigma) was added in the bath solution to block L-type Ca^{2+} channel currents.[3] Steady state activation and inactivation were characterized by Boltzmann functions. Macroscopic inactivation was fitted to a single exponential function.

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Supplemental Results

Overexpression of full-length SCN5A has no effect on the expression of PERK expression

hiPSC-CMs were tranfected with full-length SCN5A constructs at two different doses (2 μg and 3 μg). The expression of PERK was examined by Western blot. The results showed that the overerexpressed full-length SCN5A had no effect on the expression of PERK (**Fig. 1**; P = 0.093).

Downregulation efficiency of PERK by pGIPZ lentiviral anti-PERK shRNAmir

hiPSC-CMs were divided into control (untreated) and experimental groups. Cells were pre-infected by pGIPZ lentiviral anti-PERK shRNAmir. The expression of PERK were examined at 48h by Western blot. The results showed the PERK knockdown efficiency was 60.0% (95% CI 55.3%, 64.7%; P = 0.024) at 48 h. A respresentative Western blot was shown in **Fig. 2**.

Membrane localization of full-length Na^+ channels

To examine the cellular localization of full-length Na^+ channels, a SCN5A construct tagged on the carboxyl terminal end with green fluorescent protein (SCN5A-GFP) was overexpressed in hiPSC-CMs. The detailed characteristics of this construct have been reported previously.[1] These characteristics include comparable gating and cellular distribution to that of wild-type channels. As has been previously reported, a portion of the full-length cardiac sodium channel colocalizes with the cell membrane (**Fig. 3**).

Sodium channel gating is unchanged by treatments

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Fig. 4 and **Table 1** show that there were only minor changes in Na^+ channel gating with AngII, PERK shRNA, or scrambled shRNA. These changes are not likely to be sufficient to explain the large effects of these treatments on peak Na^+ current.

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Supplemental Table 1. Steady state activation and inactivation parameters of sodium currents.

	Activation				Inactivation			
	$V_{1/2}$, mV	***p***	**k, mV**	***p***	**$V_{1/2}$, mV**	***p***	**k, mV**	***p***
Control	**-12.3 ± 0.5**		**2.9 ± 0.5**		**-74.2 ± 0.8**		**6.0 ± 0.5**	
Ang II-treated	**-11.6 ± 0.6**	**0.47**	**2.8 ± 0.8**	**0.16**	**-78.5 ± 1.1**	**0.46**	**6.4 ± 0.7**	**0.50**
Pre-infected by PERK shRNA	**-12.6 ± 1.0**	**0.47**	**2.9 ± 0.9**	**0.45**	**-83.5 ± 0.8**	**0.40**	**6.9 ± 0.5**	**0.49**
Pre-infected by scrambled shRNA	**-12.3 ± 1.3**	**0.38**	**3.0 ± 1.2**	**0.18**	**-80.7 ± 1.6**	**0.41**	**7.7 ± 1.0**	**0.50**

$V_{1/2}$ is midpoint of the Boltzmann function. The value, k, is the slope factor of the Boltzmann function. n=5 for each group.

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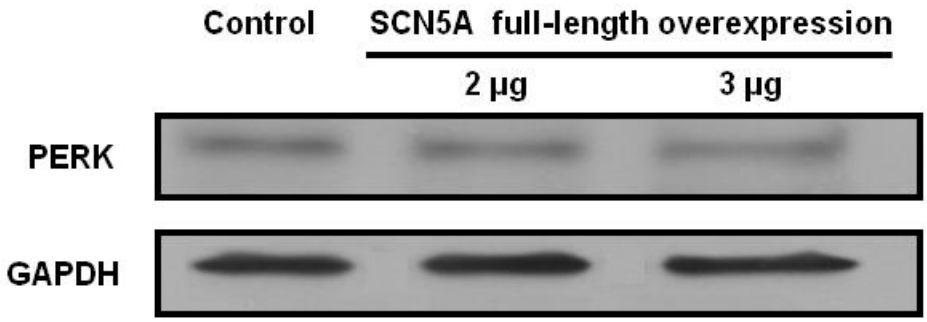
Supplemental Figures



Supplemental Figure 1. The effect of overexpression of full-length SCN5A on the PERK expression level. hiPSC-CMs were tranfected with full-length SCN5A constructs at two different dose (2 µg and 3 µg). The expression of PERK were examed by Western blot at 48 h.

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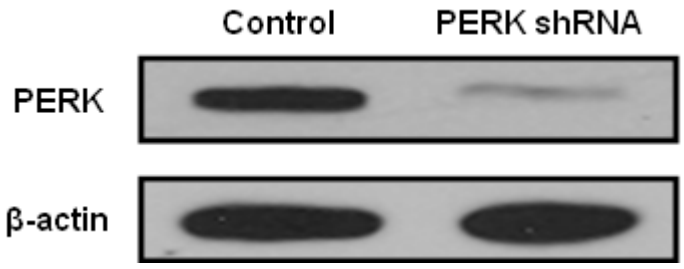

Supplemental Figure 2. The downregulation efficiency of PERK by pGIPZ lentiviral anti-PERK shRNAmir. Cells were pre-infected by pGIPZ lentiviral anti-PERK shRNAmir. The expression of PERK was examined at 48 h.

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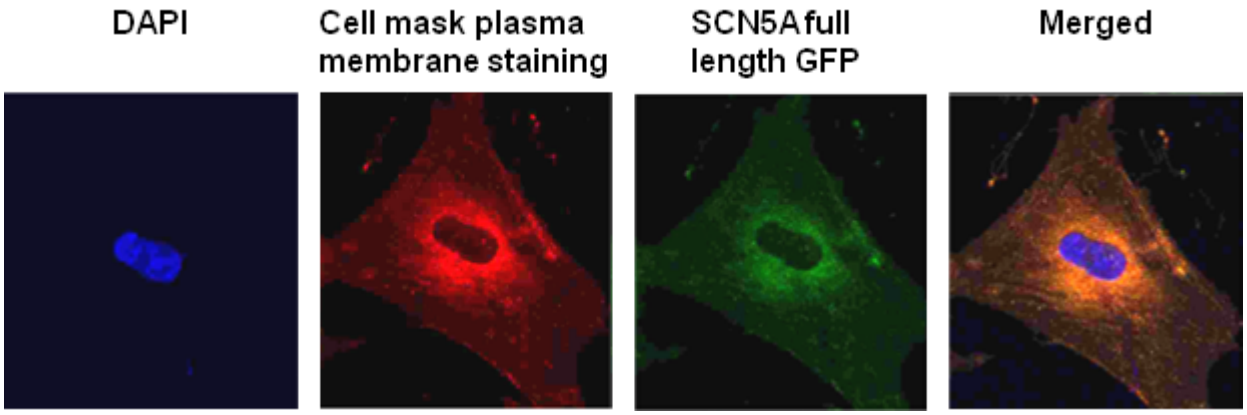

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Supplemental Figure 3. The distribution of Na^+ channels encoded by full-length SCN5A labeled with GFP. hiPSC-CMs were transfected with a GFP-labeled, full-length SCN5A construct. The full-length Na^+ channel protein is shown in green. The cell membrane and nuclei are shown in red and blue, respectively. The co-localization of full-length Na^+ channels is shown in the merged image and indicates Na^+ channels at the membrane surface consistent with cellular electrophysiology.

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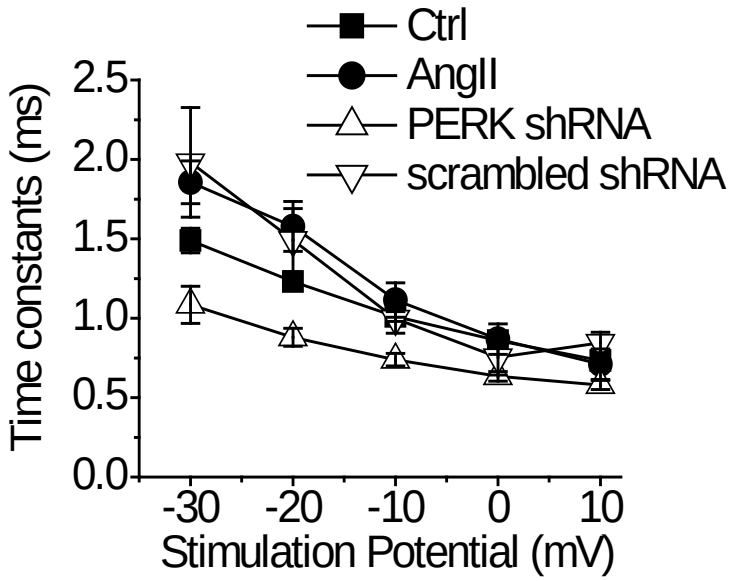

Supplemental Figure 4. The Na^+ current macroscopic time constants of inactivation as a function of voltage for control iPS-hCMs and those treated with AngII, PERK shRNA, or scrambled shRNA (P = 0.085).

SUPPLEMENTAL MATERIAL

References

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3. Gao G, Xie A, Huang SC, Zhou A, Zhang J, Herman AM, Ghassemzadeh S, Jeong EM, Kasturirangan S, Raicu M, Sobieski MA, Bhat G, Tatooles A, Benz EJ, Jr., Kamp TJ, Dudley SC, Jr. Role of RBM25/LUC7L3 in abnormal cardiac sodium channel splicing regulation in human heart failure. *Circulation.* 2011;124:1124-1131.