Supplemental Material

Maruyama M, et al. FKBP12 is a Critical Regulator of the Cardiac Voltage-Gated Sodium Current and Cardiac Arrhythmogenicity

Expanded Methods

Generation of Cardiomyocyte-specific FKBP12 Transgenic and Knockout Mice

All animal studies were performed according to NIH and Institutional Animal care and Use Guidelines. α-Myosin heavy chain (αMyHC) promoter (gift from Dr. Jeffery Robbins) was placed 5' of a human FKBP12 cDNA (coding region), followed by SV40 early region transcription terminator/polyadenylation site. A flag epitope tag was added in frame to the 5' of FKBP12 cDNA (Figure 1A). The flag tag allows us to distinguish the transgenic FKBP12 from endogenous mouse FKBP12. Transgene was microinjected into inbred C3HeB/FeJ (The Jackson Laboratories, Bar Harbor, ME) zygotes, which were then implanted into the oviducts of pseudopregnant Swiss Webster mice. The procedures that generated αMyHC-FKBP12 transgenic mice were carried out at mouse transgenic core of Indiana University Cancer Center as previously described.[1] From 12 αMyHC-FKBP12 transgenic positive founders, we were able to maintain three αMyHC-FKBP12 transgenic lines (#4, #9, #12). Transgenic mice were maintained in the DBA/2J inbred strain. Western blot analyses demonstrated that the overexpressed protein levels of FKBP12 in αMyHC-FKBP12 hearts were about 9-fold higher than endogenous FKBP12 levels in all three transgenic lines used in this study. All three transgenic lines gave rise to identical abnormal cardiac arrhythmia phenotypes and cardiac histology. Therefore, transgenic line #4 was used for subsequent detailed electrophysiological analyses.

To achieve spatially and temporally-regulated FKBP12 knockout via the Cre-loxP system, we first generated FKBP12 floxed ($FKBP12^{flox}$) mice: two loxP sites were placed flanking exon 3 in the mouse FKBP12 gene (Figure 5A). From 200 ES cell clones, we obtained 14 correctly targeted clones and two of them were used successfully to generate $FKBP12^{flox}$ mice. Sequencing analysis confirmed that the two loxP sites were correctly positioned. $FKBP12^{flox}$ mice have normal FKBP12 expression via Western blot analysis, and do not exhibit any noticeable abnormality. By crossing to cardiomyocyte specific cre transgenic mice (αMyHC-cre), we are able to generate cardiomyocyte-restricted FKBP12-deficient mice ($FKBP12^{cmko}$).

qRT-PCR, Western Blot, Histological, and Immunofluorescence Analyses

Total RNA was prepared from the hearts using TRIzol (Invitrogen). First strand cDNA was synthesized by Transcriptor First Strand cDNA kit (Roche) and qRT-PCRs were performed on Roche Lightcycler480 using SYBR Green Mix (Roche). The relative expression was normalized to the reference gene ribosomal protein L7 (RPL7) as previously described.[1] Western blots were performed as shown elsewhere.[1] Antibodies against FKBP12 and flag were Cell Signaling Technology. The hearts were fixed with 10% neutral buffered formalin. The tissues were processed for paraffin section, and stained with hematoxylin & eosin (H&E) or fast green/sirius red for cardiac histology examination. To evaluate Nav1.5 expression at cellular level, we used immunofluorescence staining and confocal imaging. Mouse hearts (2-month old) were fixed in 4% PFA in PBS overnight. Fixed hearts were cryoprotected in 30% sucrose, and frozen in OCT. Ten-μm cryosections were incubated with primary antibody against Nav1.5 (ASC-005 or ASC-013, Alomone Labs) or control rabbit IgG (for negative control),

followed by a rhodamine-conjugated anti-rabbit IgG secondary antibody (Fl-1200, Vector). Images were obtained with a confocal laser scanning microscope (Olympus FV-1000) using an excitation wavelength of 559 nm. The pinhole diameter was set to 1 Airy unit. Emission was collected between 560 nm and 650nm. The subcellular distribution pattern for Nav1.5 demonstrated in our study (Figure 4 and Figure 7) is similar to that previously published[2].

Surface and Telemetric Electrocardiographical Analyses

Mice were lightly anesthetized with 1.5% isoflurane. Anesthetized mouse was placed on a warm platform. Three-lead surface ECGs was measured by using limb electrodes. Data were amplified and recorded with a Gould 148 amplifier (Gould Instrument Systems Inc., Valey View, OH) and Axotape program (Axon Instrument Inc., Union City, CA). Telemetric analysis of ECGs was performed on the PhysioTel Telemetry system (Data Sciences International, St. Paul, MN). The data were collected using an implantable radiofrequency micro-transmitter (TA1010ETA-F20). The subcutaneously leads were surgically placed over the scapula in a lead II configuration. The continuously data recording were allowed by using hardware modification in the waveform format from multiple instrumented mice. The data analyses were done by using Dataquest A.R.T. version 4.1 (Data Sciences International).

Electrophysiologic and Optical Mapping Study in Langendorff-perfused Hearts

After intraperitoneal injection of heparin (0.5 U/g), mice were killed by cervical dislocation. The heart was rapidly excised and perfused with a Langendorff perfusion system with 37°C Tyrode's solution (NaCl 125, KCl 4.5, NaH_2PO4 1.8, $NaHCO_3$ 24, $CaCl_2$ 1.8, $MgCl_2$ 0.5, and glucose 5.5 mmol/L) with pH equilibrated to 7.40±0.05 at a flow rate of ≈2 mL/min. The heart was

immersed into the water-jacketed bath and maintained at 37°C. Intracardiac bipolar electrograms were recorded with a 2Fr octapolar electrode catheter with 0.5-mm interelectrode distance (NuMed Inc., Hopkinton, NY, USA). The cycle length at which Wenckebach AH block occurred was assessed by incremental atrial burst pacing. Effective refractory periods of the AV node were evaluated with the standard S1-S2 protocol. The S1 (baseline) pacing train includes 20 beats at a pacing cycle length 20 ms shorter than the sinus cycle length. The S2 (single premature extrastimulus) was then given. The S1-S2 coupling interval was decreased by 10 ms each time until the premature beat failed to propagate to the ventricle. That S1-S2 interval was used as AV node effective refractory period. Transmembrane action potentials were recorded from the left ventricle with standard glass microelectrodes at the digitization rate of 20 kHz.[3] Action potential duration (APD) was determined from the onset of the upstroke to the time of 90% repolarization (APD_{90}) during ventricular pacing at a cycle length of 150 ms. Optical mapping of voltage-dependent signals was performed so that ventricular conduction velocities in the longitudinal (CV_{max}) and transverse (CV_{min}) directions were measured. The hearts were stained for 10 min with the 5 μmol/L of di-4-ANEPPS (Molecular Probes, Carlsbad, CA) and excited with laser light at 532 nm (Verdi, Coherent Inc.). The emitted fluorescence was collected using a high-speed CMOS camera (MiCAM Ultima, BrainVision, Tokyo, Japan) at 0.2 ms/frame and 100x100 pixels with spatial resolution of 0.07 x 0.07 mm^2 per pixels. The center of the left ventricular lateral wall was paced with a platinum unipolar electrode at a cycle length of 100 ms using pulses 2 ms in duration at 1.5 times diastolic threshold, and the epicardial activation pattern was recorded. Activation time was defined as the timing when the AP amplitude reached 50% of the maximum value. Conduction velocities were measured, as described previously.[4]

Whole-Cell Patch Techniques

Cardiac myocytes were isolated from the ventricles of Langendorff-perfused mouse hearts. Sodium currents were recorded using standard whole-cell patch clamp techniques and voltage-clamp experiments were performed with an Axopatch 700A amplifier (Axon Instruments). The recording electrodes (Borosilicate glass, Sutter) were pulled (P-87, Sutter Instruments) and polished (F-83, Narishige) down to 1.2 – 1.8 MΩ when filled with pipette solution (in mmol/L: CsOH 125; Aspartic acid 35; Tetraethylammonium chloride 30; HEPES 11; Mg-ATP 5.0; EGTA 10; Phosphocreatine 3.6, pH adjusted with 1 N CsOH to 7.30). After the formation of the gigaohm-seal, the capacitance was electronically compensated and the cell membrane under the pipette tip was then ruptured by a brief increase in suction, forming the whole-cell recording configuration. After 2-5 min period for intracellular dialysis, the Tyrode solution was changed by bath perfusion of extracellular recording solution designed for Na^+ current recordings (in mmol/L: NaCl 50; $MgCl_2$-$6H_2O$ 1.2; $CaCl_2$ 1.8; Tetraethylammonium chloride 125; CsCl 5.0; HEPES 20; Glucose 11; 4-AP 3.0; $MnCl_2$ 2.0; pH adjusted with 1 N CsOH to 7.30). All cells were recorded at room temperature (22 – 23 °C). Current amplitude data of each cell were normalized to its cell capacitance (current density, pA/pF). Current-voltage relationship (I-V curve) was presented by the currents normalized by the peak currents. Voltage-dependent activation and steady-state inactivation profiles were calculated by Boltzmann fitting function. The activation curves were obtained by fitting the data points to a Boltzmann equation of the form $I_{Na} = \frac{G_{\max}(V - V_{rev})}{1 + \exp\left[-\left(V - V_{act\,1/2}\right)/k_{act}\right]}$, where G_{max} is the maximum

conductance of the voltage-gated Na^+ channels, V_{rev} is the extrapolated reversal potential of I_{Na}, $V_{½}$ is the potential for half – maximal conductance, and k is the slope.). The curve for voltage dependence of steady state I_{Na} inactivation was obtained by fitting the data to a Boltzmann distribution of the form $\frac{I_{Na}}{I_{Na,\max}} = \frac{1}{1+\exp\left[-\left(V - V_{inact\,½}\right)/k_{inact}\right]}$.

Confocal $[Ca^{2+}]_i$ Imaging

Simultaneous recording of I_{Ca} and $[Ca^{2+}]_i$ transients were performed using Zeiss LSM 510 laser scanning confocal microscope and Axon-200B patch-clamp system, as described previously.[5]

Statistical Analysis

Continuous variables are presented as mean ± SD. Student's t tests were used to compare the means between groups. $P \leq 0.05$ was considered statistically significant.

References

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Supplemental Figure I. Assessment of other ionic currents. A, **A**, **A**, FKBP12-overexression had no effect on the densities of the inward rectifier K^+ current (I_{K1}, NTG, n=23; TG, n=15 cells) and the transient outward K+ current (I_{to}; NTG, n=21; TG, n=17 cells), but significantly increased sustained outward K^+ current density (I_{Ksus}; NTG, n=21; TG, n=17 cells). **B**, The density of the L-type Ca^{2+} current was significantly reduced at test potential, which was associated with a proportional reduction in the amplitude of the peak intracellular calcium ($[Ca^{2+}]_i$) transient. ($[Ca^{2+}]_i$ transients were measured as F/F_0, where F is fluorescence intensity, and F_0 is resting fluorescence intensity (NTG, n=28; TG, n=32 cells).

Supplemental Table I.

Maximum I_{Na}, TTX sensitivity, steady-state activation and inactivation

	FKBP12$^{f/+}$/αMHC-Cre^{+}	FKBP12$^{f/f}$/αMHC-Cre^{+}	Non-transgenic	αMyHC - FKBP12
Number (cells/animals)	6/3	8/3	14/4	14/4
Max I_{Na} (A/F)	102.9 ± 32.2	255.5 ± 39.4*	92.8 ± 25.9	19.7 ± 14.3†
Hill factor of TTX sensitivity	ND	ND	1.2	1.4
$V_{1/2,\ act}$ (mV)	-43.5 ± 6.91	-44.87 ± 6.31	-44.49 ± 8.50	-26.91 ± 10.56†
$K_{1/2,\ act}$ (mV)	5.94 ± 1.34	5.22 ± 1.09	4.17 ± 1.40	6.31 ± 1.19†
$V_{1/2,\ inact}$ (mV)	-70.7 ± 8.32	-71.95 ± 7.76	-74.49 ± 8.62	-64.13 ± 5.64†
$K_{1/2,\ inact}$ (mV)	5.42 ± 0.56	5.19 ± 0.42	5.18 ± 0.38	5.56 ± 1.12

TTX indicates tetrodotoxin. Values are mean ± 1 SD. *P<0.01 versus FKBP12$^{f/+}$/αMHC-Cre^{+}. $^{\dagger}P$<0.01 versus non-transgenic. ND, not determined.

Supplemental Table II.

Open-state inactivation

	FKBP12$^{f/+}$/αMHC-Cre^{+}	FKBP12$^{f/f}$/αMHC-Cre^{+}	Non-transgenic	αMyHC-FKBP12
A_{slow} (pA)	-729 ± 389	-1003 ± 425	-1070 ± 694	-163 ± 151$^{\#}$
A_{fast} (pA)	-7887 ± 4071	-16677 ± 7112	-7218 ± 3746	-2851 ± 1856$^{\#\#}$
τ_{slow} (ms)	8.11 ± 0.93	4.54 ± 0.46**	7.57 ± 2.37	13.98 ± 1.88$^{\#\#}$
τ_{fast} (ms)	1.62 ± 0.38	1.05 ± 0.16**	1.42 ± 0.28	2.47 ± 1.01$^{\#\#}$
y_0	-41.2 ± 15.98	-46.3 ± 16.81	-34.2 ± 19.3	-109.6 ± 68.4$^{\#}$

Current traces were fitted with a double exponential: $y(t) = A_{slow} \exp(-t/\tau_{slow}) + A_{fast} \exp(-t/\tau_{fast}) + y_0$

Values are mean ± 1 SD. **P<0.01 versus FKBP12$^{f/+}$/αMHC-Cre^{+}, and $^{\#}P$<0.05, $^{\#\#}P$<0.01 versus non-transgenic.

Supplemental Table III.

Steady-state activation and inactivation in FKBP12$^{f/f}$/αMHC-Cre^{+} cardiomyocytes dialyzed with FKBP12 protein

	+FKBP12	
	At 5 min	**At 45 min**
$V_{1/2,\ act}$ (mV)	-44.1 ± 7.04	-37.2 ± 6.86**
$K_{1/2,\ act}$ (mV)	5.43 ± 1.26	5.69 ± 1.19
$V_{1/2,\ inact}$ (mV)	-71.3 ± 7.25	-65.0 ± 6.71**
$K_{1/2,\ inact}$ (mV)	5.29 ± 1.28	5.87 ± 1.44

Values mean ± 1 SD from 4 ventricular cardiomyocytes. ***P*<0.01 versus values at 5 min.

Supplemental Table VI.

Open-state inactivation in FKBP12$^{f/f}$/αMHC-Cre^{+} cardiomyocytes dialyzed with FKBP12 protein

	Negative Control		+ FKBP12	
	At 5 min	**At 45 min**	**At 5 min**	**At 45 min**
A_{slow}	-987 ± 406	-1003 ± 425	-973 ± 588	-284 ± 195*
A_{fast}	-17037 ± 5681	-17289 ± 6405	-16942 ± 4371	-5733 ± 3329*
τ_{slow} (ms)	4.93 ± 1.36	5.21 ± 0.99	5.45 ± 1.82	11.86 ± 3.72**
τ_{fast} (ms)	1.62 ± 0.67	1.54 ± 0.39	1.67 ± 0.58	3.87 ± 0.88*
y_0	-38.7 ± 8.24	-42.0 ± 11.5	-45.7 ± 15.1	-89.4 ± 24.4*

Current traces were fitted with a double exponential: $y(t) = A_{slow} \exp(-t/\tau_{slow}) + A_{fast} \exp(-t/\tau_{fast}) + y_0$

Values mean ± 1 SD from 4 ventricular cardiomyocytes. *P<0.05, **P<0.01 versus values at 5 min.

Supplemental Figure I