SUPPLEMENTAL MATERIAL

EXPANDED MATERIALS AND METHODS

Generation of Cx43 Phospho-mutant Constructs

Site-directed mutagenesis was performed to introduce the S3E and S3A (residues 325,328,330) into the same gene-targeting vector previously used to conditionally inactivate Cx43 in the heart and other lineages.[1-3]

Animal models

All animal procedures were carried out in accordance with Public Health Service guidelines for the care and use of laboratory animals and approved by the New York University School of Medicine Institutional Animal Care and Use Committee. Cx43 phospho-mutant targeting vectors were introduced into the 129/Sv-derived ES cell line R1.[1, 4] Screening for correct recombinants was performed by Southern blot and PCR analysis, also as previously described.[5] For each construct, two correctly targeted ES clones were injected into C57BL/6 blastocysts. Highly chimeric male mice were crossed with wild-type (WT) CD1 females to generate F1 $Cx43^{S3A/WT}$ and $Cx43^{S3E/WT}$ heterozygous mutant mice. These were subsequently bred to generate homozygous $Cx43^{S3A/S3A}$ and $Cx43^{S3E/S3E}$ mice. Both heterozygous and homozygous intercrosses were maintained and for all experiments, WT littermates were used as controls.

Echocardiography

Left ventricular dimensions and function were assessed by echocardiography as previously described using an ATL 5000CV Ultrasound System (Philips Medical, Bothell, WA).[1]

Western Blot Analysis

Total protein lysates and Triton X-100 insoluble pellet fractions were prepared from the apical two-thirds of the ventricle as previously described.[6] Primary antibodies included a rabbit polyclonal anti-Cx43 (Sigma c6219), which recognizes all forms of Cx43; mouse monoclonal anti-Cx43 (Zymed 13-8300), which has been reported to bind selectively to non-phosphorylated Cx43.[7] Equivalency of protein loading was verified by probing for GAPDH. Signals were visualized and quantified using the Odyssey Imaging System (Li-Cor, Lincoln, NE).

Immunofluorescence, Confocal Microscopy, and Cx43 Quantification

Immunofluorescent staining was performed on formalin-fixed, paraffin embedded sections using rabbit polyclonal anti-Cx43 antibody (Sigma c6219) and mouse anti-N-cadherin antibody (Invitrogen). Secondary antibodies were FITC-conjugated goat anti-rabbit and Texas Red-conjugated goat anti-mouse antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Immunostained sections were examined using a Leica DMI6000B confocal laser-scanning microscope (Leica, Heidelberg, Germany). Gap junction remodeling was quantified by determining the extent of colocalization of Cx43 and N-cadherin at the intercalated discs using images collected at 63x magnification (CoLocalizer Pro software, CoLocalization Research Software). Calculations were performed on 7 – 9 sections from 3 hearts for each genotype.

Heart Isolation and Optical Mapping

High-resolution optical mapping experiments were performed as previously described, using a CMOS video camera (Ultima-L; SciMedia, Inc).[1, 8] Most studies were performed with the addition of blebbistatin (5 μM; BIOMOL International, LP) to reduce motion artifacts. Epicardial

conduction velocity (CV) and optical action potential duration (APD) measurements were obtained from the left ventricular surface at basic cycle length (BCL) of 100 ms with 3 ms stimuli at twice diastolic threshold. APD measurements were obtained using an S_1-S_2 protocol consisting of a 10 beat S_1 drive cycle at 100 ms BCL followed by a single S_2 extrastimulus. APD values were calculated from the single S_2 beat. APDs were measured for each pixel at 50% (APD_{50}) of repolarization. APD dispersion was calculated as the standard deviation of APD values for all pixels.

Electrophysiology Studies (EPS)

EPS was performed as previously described.[11] Briefly, male mice 3-4 months of age were anesthetized with isofluorane and intubated. A cut-down of the right internal jugular vein was performed, and a 2-French octapolar catheter was advanced into the right atrium and ventricle using electrocardiogram guidance and pacing capture to confirm intracardiac location.

Standard pacing protocols (single, double and triple extrastimulation and burst pacing (BP)) were performed to test for ventricular tachycardia (VT) inducibility. Briefly, drive trains of 8 paced beats (S_1 x 8) were delivered at rates of 140ms and 100ms and followed by a single, double and triple extrastimuli (S_2, S_3, or S_4) brought down to a minimum coupling interval of 30ms. Ventricular burst pacing was performed as eight 50ms and four 30ms cycle length trains applied once every 3s, up to a maximum 1min time limit of total stimulation. VT was defined as $\geq$ 4 consecutive beats.

Transverse aortic constriction

Transverse aortic constriction was performed on male mice at 3-4 months of age as previously described.[12]

No-Flow Ischemia Protocol

Langendorff global ischemia model was performed as previously described.[13] Briefly, hearts were excised, transferred to a Langendorff apparatus, and perfused via an aortic cannula. After a 10-minute stabilization interval of normoxic perfusion, hearts were made ischemic by cessation of perfusion for 30 minutes. Hearts were then flash frozen in liquid nitrogen and stored at -80° Celsius prior to protein isolation for Western blot analysis.

Statistical Analysis

Data are presented as mean ± SEM and analyzed by Student's t-test. For multiple groups, one way ANOVA was performed with post-hoc Newman-Keuls test. Arrhythmia inducibility was analyzed by Fisher's exact test. P values less than 0.05 were considered statistically significant.

References

1. Gutstein DE, Morley GE, Tamaddon H, Vaidya D, Schneider MD, Chen J, Chien KR, Stuhlmann H, Fishman GI. Conduction slowing and sudden arrhythmic death in mice with cardiac-restricted inactivation of connexin43. *Circ Res.* 2001;88:333-339. PMID 11179202.
2. Presley CA, Lee AW, Kastl B, Igbinosa I, Yamada Y, Fishman GI, Gutstein DE, Cancelas JA. Bone marrow connexin-43 expression is critical for hematopoietic regeneration after chemotherapy. *Cell Commun Adhes.* 2005;12:307-317. PMID 16531325.
3. Sridharan S, Simon L, Meling DD, Cyr DG, Gutstein DE, Fishman GI, Guillou F, Cooke PS. Proliferation of adult sertoli cells following conditional knockout of the Gap junctional protein GJA1 (connexin 43) in mice. *Biol Reprod.* 2007;76:804-812. PMID 17229929.

4. Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder JC. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc Natl Acad Sci U S A.* 1993;90:8424-8428. PMID 8378314.
5. Flenniken AM, Osborne LR, Anderson N, Ciliberti N, Fleming C, Gittens JE, Gong XQ, Kelsey LB, Lounsbury C, Moreno L, Nieman BJ, Peterson K, Qu D, Roscoe W, Shao Q, Tong D, Veitch GI, Voronina I, Vukobradovic I, Wood GA, Zhu Y, Zirngibl RA, Aubin JE, Bai D, Bruneau BG, Grynpas M, Henderson JE, Henkelman RM, McKerlie C, Sled JG, Stanford WL, Laird DW, Kidder GM, Adamson SL, Rossant J. A Gja1 missense mutation in a mouse model of oculodentodigital dysplasia. *Development.* 2005;132:4375-4386. PMID 16155213.
6. Nagy JI, Li WE, Roy C, Doble BW, Gilchrist JS, Kardami E, Hertzberg EL. Selective monoclonal antibody recognition and cellular localization of an unphosphorylated form of connexin43. *Exp Cell Res.* 1997;236:127-136. PMID 9344592.
7. Gutstein DE, Liu FY, Meyers MB, Choo A, Fishman GI. The organization of adherens junctions and desmosomes at the cardiac intercalated disc is independent of gap junctions. *J Cell Sci.* 2003;116:875-885. PMID 12571285.
8. Leaf DE, Feig JE, Vasquez C, Riva PL, Yu C, Lader JM, Kontogeorgis A, Baron EL, Peters NS, Fisher EA, Gutstein DE, Morley GE. Connexin40 imparts conduction heterogeneity to atrial tissue. *Circ Res.* 2008;103:1001-1008. PMID 18599871.
9. Kalcheva N, Qu J, Sandeep N, Garcia L, Zhang J, Wang Z, Lampe PD, Suadicani SO, Spray DC, Fishman GI. Gap junction remodeling and cardiac arrhythmogenesis in a murine model of oculodentodigital dysplasia. *Proc Natl Acad Sci U S A.* 2007;104:20512-20516. PMID 18077386.
10. Gutstein DE, Morley GE, Fishman GI. Conditional gene targeting of connexin43: exploring the consequences of gap junction remodeling in the heart. *Cell Commun Adhes.* 2001;8:345-348. PMID 12064616.
11. Maguire CT, Wakimoto H, Patel VV, Hammer PE, Gauvreau K, Berul CI. Implications of ventricular arrhythmia vulnerability during murine electrophysiology studies. *Physiol Genomics.* 2003;15:84-91. PMID 12888626.
12. Rockman HA, Wachhorst SP, Mao L, Ross J, Jr. ANG II receptor blockade prevents ventricular hypertrophy and ANF gene expression with pressure overload in mice. *Am J Physiol.* 1994;266:H2468-2475. PMID 8024008.
13. Beardslee MA, Lerner DL, Tadros PN, Laing JG, Beyer EC, Yamada KA, Kleber AG, Schuessler RB, Saffitz JE. Dephosphorylation and intracellular redistribution of ventricular connexin43 during electrical uncoupling induced by ischemia. *Circ Res.* 2000;87:656-662. PMID 11029400.

Supplemental Table I

Sample	LVAWd	LVAWs	LVIDd	LVIDs	LVPWd	LVPWs	Fractional Shortening (%)	Heart Wt (mg)/ Body Wt (g)
WT, Base (n=5)	0.07	0.11	0.38	0.22	0.074	0.104	41.84 +/- 0.75	5.537 +/- 0.142
WT, TAC (n=20)	0.07	0.11	0.40	0.31	0.077	0.115	23.22 +/- 0.87	8.488 +/- 0.179
S3A, Base (n=5)	0.06	0.10	0.39	0.23	0.066	0.100	39.32 +/- 0.83	5.935 +/- 0.148
S3A, TAC (n=17)	0.08	0.12	0.43	0.33	0.074	0.108	22.24 +/- 1.10	8.661 +/- 0.294
S3E, Base (n=5)	0.06	0.10	0.38	0.22	0.077	0.117	42.07 +/- 2.09	5.845 +/- 0.056
S3E, TAC (n=16)	0.07	0.11	0.39	0.30	0.074	0.111	24.91 +/- 0.88	8.086 +/- 0.118

Echocardiographic and Physiologic Comparison of WT and Mutant Mice at Baseline and Post-TAC. LVAW, LV anterior wall; LVID, LV internal dimension; LVPW, LV posterior wall; d, diastole; s, systole.