Supplemental Methods and Data

FGF12 is a candiate Brugada syndrome locus

Hennessey, et. al., FGF12 and Brugada Syndrome

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Detailed Methods

Study Population

The study population consisted of 102 unrelated patients with BrS who were referred to either the Molecular Cardiology Laboratory, Fondazione IRCCS Policlinico San Matteo, Pavia Italy or to the Windland Smith Rice Sudden Death Genomics Laboratory at Mayo Clinic, Rochester, Minnesota for laboratory-based genetic testing. All BrS patients included in this study remained genotype negative after comprehensive genotyping for mutations in the fourteen known BrS-susceptibility genes listed here: *SCN5A*, *GPD1L, CACNA1C, CACNB2B, SCN1B* [including the alternatively spliced exon 3A; *SCN1Bb*], *SCN3B*, *KCNE3*, *KCNJ8, KCND3*, *CACNA2D1, MOG1* and *HCN4, KCNE1L* and *SLMAP*. This study was approved by both the Mayo Foundation Institutional Review Board and the Medical Ethical Committee of Fondazione IRCCS Policlinico San Matteo. Informed consent was obtained for all patients.

Mutational Analysis and Control Population

Comprehensive open reading frame/splice site mutational analysis of all amino acid coding exons and intron borders of *FGF12* was performed using polymerase chain reaction (PCR), denaturing high performance liquid chromatography (DHPLC), and DNA sequencing as previously described.[1] PCR primer sequences and PCR / DHPLC conditions are available upon request.

In order to be considered as a putative pathogenic mutation, any *FGF12* variant had to be i) non-synonymous and ii) absent among at least 1000 ethnically-matched controls obtained from the European Collection of Cell Cultures (HPA Culture Collections, UK), the Human Genetic Cell Repository sponsored by the National Institute of General Medical Sciences and the Coriell Institute for Medical Research (Camden, New Jersey),

and from the Blood Transfusional Centre in IRCCS Policlinico San Matteo of Pavia (Italy) and all available online databases, including the 1000 Human Genome Project database[2, 3] (www.1000genomes.org/ensembl-browser, n=1094 individuals; 381 Caucasian; 246 African-American, 286 Asian, and 181 Hispanic), the NHLBI GO Exome Sequencing Project[4] (http://evs.gs.washington.edu/EVS/, n=5379 individuals; 3510 Caucasian and 1869 African-American), and the Exome Chip Design[5] (http://genome.sph.umich.edu/wiki/Exome_Chip_Design, n=12000 individuals). Mutations were annotated using the single letter nomenclature whereby F45M for example denotes a non-synonymous variant producing a missense mutation involving a substitution of Phenylalanine (F) by a methionine (M) at amino acid position 45.

Subcloning and adenovirus production

Human FGF12-B (accession no. NM_004113.5) in pIRES2-AcGFP[6] was mutated using Quickchange II Site-directed Mutagenesis (Agilent Technologies) to form Q7R-FGF12 and then both were subcloned into the pAdRFP adenovirus shuttle vector. The adenoviruses expressing FGF13 shRNA with GFP has been previously described.[7] WT-FGF12 and Q7R viruses were generated similarly using the AdEasy System (Agilent). The adenoviral plasmid was packaged in HEK293 cells. The recombinant virus was isolated by multiple freeze/thaw cycles, further amplified and then purified and concentrated using Vivapure Adenopack 20 (Sartorius Stedim Biotech). The viral titer was determined using optical density. All constructs were confirmed by sequencing.

HEK293T cell transfection and co-immunoprecipitation

Transfection, $Na_V1.5$ Na^+ current recording with FGF12-B and immunoprecipitation techniques have been previously described in HEK293T cells.[6] The construct encoding

wild-type human JPH2 was generously provided by Xander Wehrens (Baylor College of Medicine, Houston, TX). For immunoprecipitation, HEK293T cells were plated to 60% confluency and transfected with Lipofectamine 2000 (Invitrogen), according to manufacturers instructions with 2 μg WT-FGF12 or Q7R-FGF12 (subcloned in pIRES-AcGFP, Invitrogen) and 4 μg of plasmid encoding JPH2 or $Na_V1.5$ (both subcloned in pCDNA3.1, Invitrogen). After 36 to 48 hours, transfected cells were washed with ice-cold PBS 24 h after transfection, and cell lysates were prepared with the addition of lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Triton with protease inhibitor mixture (Roche). The pelleted cells were pipetted up and down 20 times with lysis buffer and then passed 20 times through a 22 gauge needle, incubated at 4 °C for 1 h and then centrifuged at 16,000 × g for 10 min at 4 °C. The lysates were precleared by exposure to 20 μl of protein A/G-agarose beads (Santa Cruz Biotechnology) for 30 min at 4 °C. The protein concentration was determined using the BCA Protein Assay kit. Immunoprecipitation was performed with 1 μg of anti-His6 (Qiagen) or anti-JPH2 (Santa Cruz) antibody added to 100 μg of precleared lysates. The samples were rocked gently at 4 °C for 1 h followed by addition of 30 μl of protein A/G-agarose slurry. The samples were rotated overnight at 4 °C and centrifuged at 7000 rpm for 2 min. After washing with lysis buffer three times, 40 μl of loading buffer was added to the pellet, and protein was eluted from the beads by heating at 70 °C for 20 min. The samples were subjected to NuPAGE 8–16% Bis-Tris gels (Invitrogen). As a negative control, parallel reactions were performed with mouse IgG. The proteins were transferred to nitrocellulose membranes and subsequently immunoblotted with the anti-His antibody or an anti-pan NaV antibody (Sigma). The blots were visualized by enhanced chemiluminescence, and images were captured with a Kodak Image Station 4000 R.

HEK293T cell electrophysiology

HEK293T cells were transfected at 60% confluence using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The total amount of DNA for all transfections was kept constant. Transfected cells were identified by GFP fluorescence. Na^+ currents were recorded using the whole-cell patch-clamp technique at room temperature (20–22° C) 48–72 hr after transfection. Electrode resistance ranged from 2–4 MΩ. Currents were filtered at 5 kHz and digitized using an analog-to-digital interface (Digidata 1322A, Axon Instruments). Capacitance and series resistance were adjusted (70% to 85%) to obtain minimal contribution of the capacitive transients. The bath solution contained (in mM): NaCl 130, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 10, glucose 10, pH 7.35 (adjusted with NaOH). The intracellular solution contained (in mM): CsF 110, EGTA 10, NaF 10, CsCl 20, HEPES 10, pH 7.35 (adjusted with CsOH). Osmolarity was adjusted to 310 mOsm with sucrose for all solutions. Standard two-pulse protocols were used to generate the steady-state inactivation curves: from a holding potential of −120 mV, cells were stepped to 500-ms preconditioning potentials varying between −130 mV and −10 mV (prepulse), followed by a 20 ms test pulse to −20 mV. Currents (I) were normalized to I_{max} and fit to a Boltzmann function of the form $I/I_{max} = 1/\{1 + \exp[(V_m - V_{1/2})/k]\}$ in which $V_{1/2}$ is the voltage at which half of $Na_V1.5$ channels are inactivated, k is the slope factor, and V_m is the membrane potential. Data analysis was performed using Clampfit 10.2 software (Axon Instruments) and Origin 8. Results are presented as means ± SEM; the statistical significance of differences between groups was assessed using a two-tailed Student's t test and was set at $p < 0.05$.

Isothermal titration calorimetry

ITC of the $Na_V1.5$ CTD with FHFs has been previously described.[8] Experiments were performed with an ITC-200 (MicroCal) at 20° C. Solution containing $Na_V1.5$ (20–51 μM) were titrated with 20–30 10-μl injections of solution containing WT-FGF12 or Q7R-FGF12 (240–510 μM). ITC experiments were repeated with different preparations and different concentrations at least three times to confirm thermodynamic parameters and stoichiometry values. The binding isotherms were analyzed with a single site binding model using the Microcal Origin version 7.0 software package (Originlab Corporation), yielding binding enthalpy (ΔH), stoichiometry (n), entropy (ΔS), and association constant (K_a). Results are presented as mean ± SEM; statistical significance was assessed using a two-tailed Student's t test and was set at $p < 0.05$.

Cardiomyocyte isolation

Animals were handled according to National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study was approved by Duke University Animal Care and Welfare Committee. Cardiomyocytes were isolated from 6-8 week old Sprague Dawley rats and cultured as previously described.[7] Animals were anesthetized with Avertin and anti-coagulated with heparin. Hearts were removed and the aorta was cannulated to perfuse the heart using a Langendorff apparatus. The hearts were first perfused with basal solution containing (in mM, from Sigma unless otherwise specified): NaCl 112, KCl 5.4, $NaH_2PO_4 \bullet H_2O$ 1.7, $NaHCO_3$ 4.2, $MgCl \bullet 6H_2O$ 1.63, HEPES 20, glucose 5.4, taurine 30, L-carnitine 2, creatine 2.3, 2,3-butanedione monoxime (BDM) 10. After five minutes, the solution was switched to basal solution plus 150 u/ml Collagenase Type II (Worthington) and the heart was perfused until it was soft and boggy. The heart was then taken down from the Langendorff, minced, and triturated in

enzyme solution until all cell clumps were broken. Calcium tolerance was performed in basal solution plus 5 mg/ml bovine serum albumin to quench the enzyme. For culture, cells were plated on laminin coated coverslips or glass bottom plates (MatTek) in plating medium of Minimal Essential Medium (MEM) with Earle's Salts and L-glutamine (Mediatech), 10 mM BDM, 5% fetal bovine serum (Life Technologies) and 1% penicillin/streptomycin (Sigma). After cells had adhered to the plates, the cells were washed once and the medium changed to culture medium into which the proper adenovirus had been added. Culture medium contained MEM with Earle's Salts and L-glutamine, bovine serum albumin 0.5 mg/ml, BDM 10 mM, 1X insulin-selenium-transferrin supplement (Life Technologies), creatine 5 mM, taurine 5 mM, L-carnitine 2 mM, and blebbisatin 25 µM (Toronto Reseach Chemicals). All solutions were oxygenated in 95% O_2/5% CO_2 for at least 30 minutes. Cells were then analyzed for electrophysiology, immunocytochemistry and Ca^{2+} transient recording 36-48 hours later.

Cardiomyocyte electrophysiology

Ca^{2+} currents (I_{Ca}) and Na^{+} currents (I_{Na}) were recorded using the whole-cell voltage-clamp technique in cardiomyocytes after 36 to 48 hours as previously described.[7, 9] Cardiac action potentials were recorded in current clamp as previously described.[9] Ca^{2+} currents (I_{Ca}) were recorded using the whole-cell patch-clamp technique as previously described.[10] Voltage-clamp experiments were performed at room temperature (22-24 °C), 36-48 hours after infection of adult cardiomyocytes with adenovirus. Bath (Tyrode) solution contained (in mM, from Sigma): NaCl 140, KCl 5.4, $CaCl_2$ 1, $MgCl_2$ 1, HEPES 5, glucose 10, pH 7.3 adjusted with NaOH. Once the cell was ruptured, solution was quickly changed to recording solution containing (in mM, from Sigma): N-Methyl-D-glucamine 150, HEPES 10, CsCl 2, $CaCl_2$ 2, $MgCl_2$ 1.2, 4-aminopyridine 2, D-glucose

5.5, pH 7.3 adjusted with CsOH. Internal solution contained (in mM, from Sigma): CsOH•H_2O 70, aspartic acid 80, CsCl 40, NaCl 10, HEPES 10, EGTA 10, MgATP 5, Na_2GTP 0.2, Na_2-phosphocreatine 5, pH 7.3 adjusted with CsOH. Osmolarity was adjusted to ~300 mOsm with sucrose for all solutions. Recordings were filtered at 5 kHz and digitally sampled at 25 kHz. Amplitude was normalized to cell capacitance (pA/pF). Na^+ currents (I_{Na}) were recorded using the whole-cell patch-clamp technique as previously described.[11-13] I_{Na} was recorded in bath solution containing (in mmol/L): NaCl 20, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 20, CsCl 55, CsOH 10, Glucose 10, 4-AP 2, $CdCl_2$ 0.5, TEA-Cl 50, pH 7.35 (adjusted with HCl). The pipette solution contained the following (in mmol/L): NaCl 5, CsF 135, EGTA 10, MgATP 5, HEPES 5, TEA-Cl 20, pH 7.35 (adjusted with CsOH). Osmolarity was adjusted to 310 mOsm with sucrose for all solutions. Recordings were filtered at 5 kHz and digitally sampled at 30 kHz. The pulse protocol cycle time was 3 seconds to ensure full Na^+ channel recovery. Whole-cell capacitance and series resistance were ~80% compensated to assure that the command potential was reached within microseconds with a voltage error < 2 mV. Current amplitude data for each cell were normalized to its cell capacitance (current density, pA/pF). To determine the voltage-dependence of steady-state activation, currents were elicited by a 40 ms pulse from a holding potential of -120 mV to test potentials between -100 mV and +60 mV in 5 mV increments. The sodium conductance (G) was calculated by dividing the peak current for each voltage step by the driving force (V_m-V_{rev}) then normalized to the peak conductance (G_{max}). Data were fitted with the Boltzmann relationship, $G/G_{max}=1/\{1+\exp[(V_{1/2}-V_m)/k]\}$ in which $V_{1/2}$ is the voltage at which half of $Na_V1.5$ channels is activated, k is the slope factor and V_m is the membrane potential. Standard two-pulse protocols were used to generate the steady-state inactivation curves: from the holding potential -120 mV, cells were stepped to 500-ms preconditioning potentials varying between -130 mV and -10 mV (prepulse), followed by

a 20 ms test pulse to -30 mV. Currents (I) were normalized to I_{max} and fit to a Boltzmann function of the form $I/I_{max}=1/\{1+\exp[(V_m-V_{1/2})/k]\}$ in which $V_{1/2}$ is the voltage at which half of $Na_V1.5$ channels is inactivated, k is the slope factor and V_m is the membrane potential. Curve fitting and data analysis were performed using Clampfit 10.2 software (Axon Instruments) and Origin 8 (Originlab Corporation). Data analysis was performed using Clampfit 10.2 software (Axon Instruments) and Origin 8 (Originlab Corporation). For current clamp, perforated patch with 400 nM amphotericin (Sigma) was performed using the following internal solution[14] (in mM, from Sigma) KCl 110, NaCl 5, MgATP 5, Na_2-phosphocreatine 5, Na_2GTP 1, HEPES 10, pH 7.3 and Tyrode extracellular solution. Cells were stimulated with current injections at 1 Hz at 1.5x threshold to induce action potentials recorded with 25 kHz sampling frequency. Input resistance was not different between the groups and junction potential was calculated to be 5.6 mV and not corrected.

Immunocytochemistry

Immunocytochemistry was performed as previously described.[9] Imager/analyzer was blinded to the manipulation and all cells imaged were used for analysis. Cardiomyocytes on glass coverslips were washed in PBS and fixed for 15 minutes in 2% paraformaldehyde in PBS. Fixation was quenched with 10 mM glycine in PBS and cells were permeabilized with 0.2% triton X-100 in PBS for 8 minutes. Non-specific binding was blocked with 10% goat serum for one hour at room temperature. Cells were then incubated in primary antibody dissolved in antibody dilution solution containing 3% goat serum, 1% bovine serum albumin and 0.1% triton X-100 in PBS overnight at 4 °C. Primary antibody concentrations were anti-FGF13 1:400,[7] anti-α_{1C} 1:1000 (Alomone), anti-RyR 1:1000 (Sigma), anti-H6 1:1000 (Qiagen). Cells were washed three times with PBS then incubated in secondary antibody in antibody dilution solution for 45 minutes at

room temperature. Secondary antibodies were conjugated to Alexa-fluor 488, 633 (Life Technologies) or Cy3 (Jackson Immunoresearch). Following three more washes, coverslips were mounted in Vectashield (Vector Labs). All images were collected on a Zeiss 510 inverted confocal microscope with a Zeiss 63x oil (NA, 1.4) lens at room temperature. The pinhole was set to 1.0 (Airy Disc) using Carl Zeiss Imaging software (version 4.0, SP1). For $Ca_V1.2$ and RyR localization, 0.5 µm stacks were taken at 512 x 512 resolution with 3x digital zoom. All cells were prepared identically, and imaged by using identical parameters (e.g. gain, offset, magnification, brightness, contrast, pinhole, scan time, resolution, etc.).

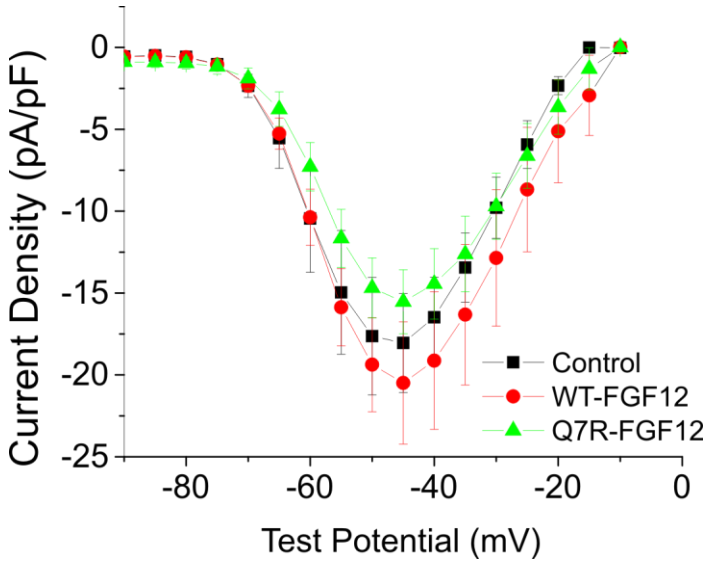
Statistical analysis

Results are presented as means ± standard error of the mean; statistical significance of differences between groups was assessed using one-way analysis of variance (ANOVA) unless otherwise stated in the specific methods and was set at $P < 0.05$.

Supplemental Table 1: qPCR primer pairs used for detecting FHF mRNA levels in adult human ventricle

FGF isoforms	Forward primer (5' – 3')	Reverse primer (5' – 3')	Product length (bp)
FGF11	CCAAGGTGCGACTGTGCG	CGACGCTGGCGGTAGAGAG	354
FGF12-A	CCGCAAGAGGCCGGTGAG	CACTACACGCAGGCCCACGG	177
FGF12-B	GGAGAGCAAAGAACCCCAG	CACTACACGCAGGCCCACGG	159
FGF13-S	CGAGAAATCCAACGCCTGC	CACCACTCGCAGACCCACAG	279
FGF13-U	GTTAAGGAAGTCGTATTCAGAGC	CACCACTCGCAGACCCACAG	155
FGF13-V	GCTTCTAAGGAGCCTCAGC	CACCACTCGCAGACCCACAG	158
FGF13-VY	GCTTCTAAGGTTCTGGATGAC	CACCACTCGCAGACCCACAG	326
FGF13-VY/Y	CACAGAGCCGGAAGAGCCTCAG	CACCACTCGCAGACCCACAG	266
FGF14-A	GAGCAGCCCCAGCAAGAAC	GTAGAATTAGTGCTGTCATC	215
FGF14-B	CCCAAATCAATGTGGTTTC	GTAGAATTAGTGCTGTCATC	211
GAPDH	CATCAGCAATGCCTCCTGCA	CCGTTCAGCTCAGGGATGAC	220

Supplemental Figure



Supplemental Figure. WT and Q7R FGF12 do not affect Na^+ channel current density without FGF13 KD. I-V curve depicting the current density for CON and those cells with overexpression of WT or Q7R FGF12 (peak current densities at -45 mV were -18.05 ± 3.02 pA/pF (n=5), -20.49 ± 3.73 pA/pF (n=10), -15.53 ± 1.96 pA/pF (n=7) for Control, WT-FGF12, and Q7R-FGF12, respectively, p = 0.55.

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