SUPPLEMENTAL MATERIAL

Fibroblast Growth Factor Homologous Factors Modulate Cardiac Calcium Channels

Short title: Hennessey et al., FGF13 regulates $Ca_V1.2$

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Detailed Methods

Adenovirus

The adenoviruses expressing FGF13 shRNA or scrambled shRNA with GFP has been previously described.[1] FGF13 rescue viruses and the shRNA virus with the GFP removed were generated similarly using the AdEasy System (Agilent). For rescue, human FGF13VY was mutated at the site of shRNA recognition to replace each third nucleotide, changing the DNA sequence but not the ultimate protein product. This construct was subcloned into pAdRFP (Addgene). 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.

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 C57/Bl6 mice or Sprague Dawley rats and cultured as previously described.[1] 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.

Electrophysiology

Ca^{2+} currents (I_{Ca}) were recorded using the whole-cell patch-clamp technique as previously described.[2] 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): $CsOH \bullet H_2O$ 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). 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[3] (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.

Sarcoplasmic reticulum load measurements

Cardiomyocytes were plated on glass bottom plates, cultured and infected with virus. After two days in culture, cells were washed twice with Tyrode solution and loaded with 0.25 μM Fura-2 AM for 15 minutes. Cells were then washed three times with Tyrode solution and allowed to de-esterify for 30 minutes. Cells were field stimulated at 1 Hz with a 50 V unipolar pulse for at least one minute prior to recording to allow them to reach steady state. Calcium transients were measured by excitation of Fura-2 with alternating 340 nm and 380 nm wavelengths of light (cycle time 4 ms) once the cells had reached steady state. After a 10 s pause, 10 mM caffeine was rapidly applied. Following recording, cells were moved out of the field of view and background fluorescence was measured for subtraction. Background subtracted SR load peak height was measured using IonWizard software (IonOptix).

Immunocytochemistry and T-tubule staining

Immunocytochemistry methods have been previously described.[1] 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,[1] anti-α_{1C} 1:1000 (Alomone), anti-RyR 1:1000 (Sigma), anti-NCX 1:1000 (generously provided by G. Vann Bennett, Duke University[4]). 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). For T-tubule staining, cells were plated onto glass bottom plates (MatTek Corp.) and cultured as above. Cells were then washed twice with Tyrode solution and incubated in 0.5 μM di-8-ANEPPS (Life Technologies) in Tyrode solution plus 25 μm blebbistatin to inhibit contraction. Cells were imaged live. For all image analysis, control plates were imaged first. To ensure there were no effects of culture on cells, only those cultures in which greater than 95% of the controls had proper α_{1C} localization were used for analysis. All images were collected on a Zeiss 510 inverted confocal microscope with a Zeiss 63x oil (NA, 1.4) or a Zeiss 40x oil (NA, 1.3) lens for immunocytochemistry or live imaging, respectively at room temperature. The pinhole was set to 1.0 (Airy Disc) using Carl Zeiss Imaging software (version 4.0, SP1). For $Ca_V1.2$, NCX and RyR localization, 0.5 μm stacks were taken at 512 x 512 resolution

with 3x digital zoom. For T-tubule staining, a single slice was imaged through the center of the cell. All cells were prepared identically, and imaged by using identical parameters (e.g. gain, offset, magnification, brightness, contrast, pinhole, scan time, resolution, etc.).

Image processing and Fast Fourier Transform

The experimenter analyzing images was blinded to treatment. Stacks were deconvolved using Hyugens software (Scientific Volume Imaging) and exported as Tiff files. Voxel colocalization was performed on deconvolved images using Pearson correlation coefficient. For channel localization, images were imported into ImageJ (NIH), and 15 μm by 5 μm, non-nuclear, non-sarcolemmal sections were selected and line scanned to create an average plot profile as in Figure 3B. The data was then imported into OriginLab software and a fast Fourier transform was performed at a 0.1 μm^{-1} sampling frequency. Peaks analyzed ranged from 1.8 μm to 2.1 μm intervals. The amplitude at the peak was then compared between the groups.

Simultaneous patch clamping and Ca^{2+} transient recording

Cardiomyocytes were plated onto glass bottom plates (MatTek Corp.) and cultured as above. Viruses expressing GFP were not used as GFP interferes with the Fura-2 emission. Therefore the cells were co-infected with a virus expressing mRFP to identify infected cells. Cells were washed twice with Tyrode solution. Whole cell patch clamping was performed as above with the following modifications. Internal solution contained (in mM, from Sigma): $CsOH•H_2O$ 70, aspartic acid 80, CsCl 40, NaCl 10, HEPES 10, Fura-2 pentapotassium salt 0.150 (Life Technologies), MgATP 5, Na_2GTP 0.2, Na_2-phosphocreatine 5, pH 7.3 adjusted with CsOH. Bath solution was a normal Tyrode solution. Prior to rupture, emission was recorded to account for autofluorescence. Upon rupture, cells were recorded at rest until a steady basal $[Ca^{2+}]_i$ was recorded for at least 30 seconds. Cells were then given a series of 50 ms voltage steps to 0 mV from holding at -40 mV (to inactivate Na^+ channels) at 0.5 hz to equalize SR contents and then one 500 ms pulse to 0 mV that was used for measurement. Following recording, the patch pipette was removed and the cell moved out of the field to account for background fluorescence. Current was normalized to cell capacitance (pA/pF). For Ca^{2+} transient peak measurement, background emissions were first subtracted and the peak was measured as the difference from the baseline using IonWizard software (IonOptix). EC-coupling gain was defined as the Ca^{2+} transient peak divided by the Ca^{2+} current peak.

Immunoprecipitation

Fresh adult mouse ventricular heart lysate was prepared by homogenizing tissue on ice in lysis buffer containing 150 mM NaCl, 50mM Tris, 1% Triton X, and protease inhibitor cocktail (Roche) as previously described.[1] 10 μg of anti-JPH2 (Santa Cruz), anti-FGF13, or anti-α_{1C} antibody, or control IgG rabbit/goat (Santa Cruz) were used. Samples were subjected to SDS-PAGE and co-immunoprecipitation was verified by western blot.

Proteomics

To crosslink anti-FGF13 antibody to agarose beads, 20 μg of FGF13 antibody or control rabbit IgG was coupled to 40 μl of protein A/G agarose beads in 1 ml PBS. After rocking overnight at 4 °C, beads were washed 3 times with 1 ml of 0.2M sodium borate (pH 9). Dimethyl pimelimidate (DMP) crosslinking reagent (Thermo Scientific) was dissolved in 0.2 M sodium borate (pH 9) to make 20mM DMP solution, and added to the coupled beads. After rocking at room temperature for 40 minutes, the sample was spun down and supernatant removed. The crosslinking reaction was quenched with 0.2M

ethanolamine (pH 8) and the antibody cross-linked beads were ready for use. Ventricular tissue lysate (~23 mg total protein) was added to cross-linked beads, and rocked overnight at 4 °C. Beads were washed 3 times with lysis buffer and eluted in 400 µl of 0.2% Rapigest SF Surfactant (Waters) in 50 mM ammonium bicarbonate. Samples were heated at 70 °C for 10 min, centrifuged, and the supernatant was subjected to an in-solution tryptic digestion. Peptide identifications were determined by the Duke Proteomics Core Facility using liquid chromatography/tandem mass spectrometry; following data acquisition, all spectra were searched against the SwissProt database with the mouse taxonomy selected.

Biotinylation and western blotting

Surface biotinylation and western blotting were performed as previously described.[1] Cardiomyocytes were plated on laminin coated 60 mm plates, infected with adenovirus and cultured. After two days, cells were washed twice with cold PBS and incubated with 1 mg/ml EZ-Link Sulfo NHS-SS Biotinylation (Pierce) in cold PBS for 30 minutes. Biotinylation was quenched with 100 mM glycine in PBS and cells were lysed. Biotinylated proteins were incubated with NeutrAvidin (Pierce) overnight, then washed three times and eluted in 2x LDS Sample Buffer (Life Technologies) plus 10 mM dithiothreiotol. The biotinylated proteins and the whole lysate were run on 8-16% tris-glycine SDS page gels, transferred to PVDF membrane and western blotted. Primary antibodies used were anti-α_{1C} 1:1000 (Alomone), anti-transferrin receptor 1:1000 (Life Technologies), anti-β-actin 1:5000 (Sigma) and anti-FGF13 1:200.[1] Blotting for β-actin demonstrated if intracellular proteins had been biotinylated and those replicates were not quantified if the biotinylated fraction was positive. Transferrin receptor was used as a surface loading control.

Statistical analyses

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) with Fisher's Least Significant Difference as a post-hoc test. The cut off for statistical significance was set at $P < 0.05$.

Supplemental Table

Supplemental Table. Proteomic Unique Peptide Data

Protein name	# of Peptides FGF13 IgG	# of Peptides Control IgG	Peptide Sequence
Fibroblast growth factor 13 FGF13_MOUSE*	3	0	NKPAAHFLPKPLK SVSGVLNGGK VVAIQGVQTK
Sodium channel type 5 subunit alpha SCN5A_MOUSE*	8	0	ALNQLSLTHGLSR ALSAVSVLTSALEELEESHR DQGSEADFADDENSTAGESESHR EGLPEEEAPRPQLDLQASK ESLAAIEKR HASFLFR KPAALATHSQLPSCIAAPR TSLLVPWPLR
Junctophilin-2 JPH2_MOUSE*	4	0	ELAPDFYQPGPEYQK LLQEILENSESLLEPPER RSDSAPPSPVSATVPEEEPPAPR YEGEWLDNLR

* Protein accession numbers from SwissProt 2011

Supplemental References

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