Fibroblast Growth Factor Homologous Factors Modulate Cardiac Calcium Channels

Jessica A. Hennessey, Eric Q. Wei, Geoffrey S. Pitt

**Rationale:** Fibroblast growth factor (FGF) homologous factors (FHFs; FGF11–14) are intracellular modulators of voltage-gated Na⁺ channels, but their cellular distribution in cardiomyocytes indicated that they performed other functions.

**Objective:** We aimed to uncover novel roles for FHFs in cardiomyocytes, starting with a proteomic approach to identify novel interacting proteins.

**Methods and Results:** Affinity purification of FGF13 from rodent ventricular lysates followed by mass spectroscopy revealed an interaction with junctophilin-2, a protein that organizes the close apposition of the L-type Ca²⁺ channel Ca₁.2 and the ryanodine receptor 2 in the dyad. Immunocytochemical analysis revealed that overall T-tubule structure and localization of ryanodine receptor 2 were unaffected by FGF13 knockdown in adult ventricular cardiomyocytes but localization of Ca₁.2 was affected. FGF13 knockdown decreased Ca₁.2 current density and reduced the amount of Ca₁.2 at the surface as a result of aberrant localization of the channels. Ca₁.2 current density and channel localization were rescued by expression of an shRNA-insensitive FGF13, indicating a specific role for FGF13. Consistent with these newly discovered effects on Ca₁.2, we demonstrated that FGF13 also regulated Ca²⁺-induced Ca²⁺ release, indicated by a smaller Ca²⁺ transient after FGF13 knockdown. Furthermore, FGF13 knockdown caused a profound decrease in the cardiac action potential half-width.

**Conclusions:** This study demonstrates that FHFs not only are potent modulators of voltage-gated Na⁺ channels but also affect Ca²⁺ and Ca²⁺ channels and their function. We predict that FHF loss-of-function mutations would adversely affect currents through both Na⁺ and Ca²⁺ channels, suggesting that FHFs may be arrhythmogenic loci, leading to arrhythmias through a novel, dual-ion channel mechanism.

Key Words: arrhythmias, cardiac ▪ cardiac electrophysiology ▪ fibroblast growth factors ▪ heart ▪ ion channels/membrane transport ▪ junctophilin

Despite an ever-growing understanding of ion channel function, structure, and regulation, many components of the macromolecular complexes anchored by ion channels are not yet known or well characterized. Identification of these channel-interacting proteins and discovery of their functions within the channelsome provide important insight into physiological and pathological function. Mutations in newly defined channel-interacting proteins often explain genetic causes of arrhythmias in cases in which mutations in known arrhythmia loci are not found. Fibroblast growth factor (FGF) homologous factors (FHFs), a subfamily of FGF proteins (FGF11–14) expressed predominantly in excitable cells, are prime examples of channel-interacting proteins for which cardiac functions are not well understood. Although part of the FGF superfamily, FHFs do not function as growth factors and are incapable of activating FGF receptors. Rather, FHFs remain intracellular and have been shown to bind and modulate voltage-gated Na⁺ channels. Their roles as Na⁺ channel regulators have been studied most extensively in the brain, driven in large part by the identification of FGF14 as the locus for spinocerebellar ataxia and by observations that FGF14−/− mice display an ataxia phenotype that correlates with decreased Na⁺ channel function and diminished neuronal excitability.

FHFs are also expressed in cardiomyocytes, but their roles in regulating cardiac function have heretofore received less attention. We showed that FGF13, the most highly expressed FHF in murine heart, directly binds Na₁.5, the predominant cardiac Na⁺ channel, and participates in trafficking Na₁.5 to the sarcosomal membrane and modulating Na⁺ channel kinetics. Consistent with these regulatory roles for Na⁺ channels, knockdown of FGF13 led to a reduction in conduction velocity and maximum capture rate (the ability of the cells to recover from a stimulus of a specific speed) in a neonatal rat ventricular cardiomyocyte monolayer.
Several lines of evidence suggest that the effects of FHFs in excitable cells extend beyond Na$^+$ channel modulation. For example, the complex changes in synaptic physiology in Fgf14$^{-/-}$ mice are not consistent with a defect limited to Na$^+$ channel dysfunction. Indeed, we recently found that FGF14 knockdown in cerebellar granule cells reduced presynaptic Ca$^{2+}$ currents and synaptic transmission at the granule cell to Purkinje cell synapse. Moreover, as we reported, the cellular distribution of FHFs in ventricular cardiomyocytes extended beyond the distribution of Na$_v$1.5 channels. We therefore aimed to determine novel roles for FHFs in cardiomyocytes by looking for new FHF interactors. Here, we report the discovery that junctophilin-2 (JPH2) interacts with FGF13 in rodent ventricular myocytes. JPH2 is a protein responsible for coordinating the interaction of the sarcolemma and sarcoplasmic reticulum in the dyad. We therefore investigated the role of FGF13 in regulating ionic currents in the dyad and showed that FGF13 has essential roles in regulating the L-type, voltage-gated Ca$^{2+}$ channel (Ca$_{1.2}$) currents in adult ventricular myocytes. These results provide, to the best of our knowledge, the first evidence that FHFs modulate ion channels other than voltage-gated Na$^+$ channels in cardiomyocytes and highlight previously unknown modulatory roles for FHFs in cardiac physiology such as regulation of Ca$^{2+}$-induced Ca$^{2+}$ release (CICR) and the integrated electric activity of the ventricular action potential. Our results lead to the hypothesis that loss-of-function mutations in FHFs could underlie inherited cardiac arrhythmias.

Methods

Detailed Methods are provided in the Online Data Supplement.

Adenovirus

The adenoviruses expressing FGF13 shRNA or scrambled shRNA with green fluorescent protein (GFP) have been previously described. FGF13 rescue viruses and the shRNA virus with the GFP removed were generated similarly using the AdEasy System (Agilent).

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- to 8-week-old C57/Bl6 mice or Sprague-Dawley rats and cultured as previously described.

Electrophysiology

Ca$^{2+}$ currents ($I_{Ca}$) were recorded using the whole-cell voltage-clamp technique as previously described. Cardiac action potentials were recorded in current clamp as previously described.

Immunocytochemistry and T-Tubule Staining

Immunocytochemistry methods have been previously described. Imaging was blinded to the manipulation, and all cells imaged were used for analysis. For T-tubule staining, cells were plated onto glass-bottom plates (MatTek Corp) and cultured as above. Cells were then incubated in 0.5 μmol/L di-8-butyl-amino-naphthyl-ethylenepyridinium-propyl-sulfonate (di-8-ANEPPS, Life Technologies) and were imaged live.

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. Whole-cell patch clamping was performed as above with the addition of 150 μmol/L Fura-2-penta-potassium salt (Life Technologies) and the removal of EGTA in the pipette solution. For Ca$^{2+}$ transient peak measurement, autofluorescence and background emissions were first subtracted and the peak was measured as the difference from the baseline using IonWizard software (IonOptix). Excitation-contraction coupling gain was defined as the Ca$^{2+}$ transient peak divided by the Ca$^{2+}$ current peak.

Sarcoplasmic Reticulum Load Measurements

Sarcoplasmic reticulum (SR) load was measured using 0.25 μmol/L Fura-2 AM–loaded cardiomyocytes as previously described.

Immunoprecipitation

Fresh adult mouse ventricular heart lysate was prepared by homogenizing tissue and immunoprecipitating FGF13 as previously described. After immunoprecipitation, samples were subjected to SDS-PAGE, and immunoprecipitation was verified by Western blot.

Proteomics

Antibody (20 μg anti-FGF13 or control IgG) was irreversibly cross-linked to protein A/G agarose beads (Santa Cruz Biotechnology), and ventricular tissue lysate (23 mg total protein) was added to cross-linked beads. The bound proteins were eluted in 400 μL of 0.2% Rapigest SF Surfactant (Waters) in 50 mmol/L ammonium bicarbonate and subjected to an in-solution tryptic digestion at the Duke Proteomics Core Peptide. Identifications were determined using liquid chromatography/tandem mass spectrometry; after 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.

Statistical Analyses

Results are presented as mean±SE of the mean. Statistical significance of differences between groups was assessed using 1-way ANOVA and was set at $P<0.05$.

Results

JPH2 Is in Complex With FGF13

Although we previously showed that FGF13 is an intracellular modulator of voltage-gated Na$^+$ channels, close analysis of FGF13 immunocytochemistry in adult rat cardiomyocytes revealed an overall distribution that extended beyond what has been reported for Na$_v$1.5 in heart. Specifically, we observed...
a striated pattern similar to a T-tubule distribution (Figure 1A, inset), in addition to the sarcolemmal and nuclear distribution we previously reported. This led us to hypothesize that FGF13 may have roles in cardiomyocyte physiology beyond Na\textsubscript{v}1.5 regulation. To identify other potential FGF13 interactors, we performed immunoprecipitation using a previously validated FGF13 antibody or an IgG (as a control) from adult ventricular tissue and liquid chromatography/mass spectrometry analysis of the immunoprecipitated FGF13 protein complex. One interesting candidate we identified was JPH2. As shown in Figure 1B, we identified 4 unique peptides spanning all soluble domains of JPH2. We found this candidate to be of interest because it is a protein responsible for properly juxtaposing Ca\textsubscript{v}1.2 and the transmembrane domain in the SR, and its N terminus interacts with the inner leaflet of the sarcolemma, providing a means to juxtapose the sarcolemma and SR so as to promote efficient CICR by apposing Ca\textsubscript{v}1.2 and RyR2.16 On the basis of our discovery that FGF13 was a component of the JPH2 macromolecular complex, we tested whether FGF13 participated in dyad ion channel targeting. We performed immunocytochemistry on mouse ventricular myocytes that were uninfected (control) or had been infected with a FGF13 shRNA adenovirus targeting all FGF13 splice variants (FGF13 knockdown) or a scrambled shRNA adenovirus (SCR; Figure 2). As we previously showed, repeated (in Figure 4), the knockdown adenovirus reduced FGF13 protein by >90%, and the SCR virus had no effect. In the control and SCR, we observed \( \alpha \text{c}_{\text{IC}} \) the C\textsubscript{a},1.2 pore-forming subunit, colocalized with RyR2 in a striated pattern. Little \( \alpha \text{c}_{\text{IC}} \) or RyR2 was observed between the striations. After FGF13 knockdown, however, we saw a loss of colocalization, and a large portion of \( \alpha \text{c}_{\text{IC}} \) was now present between the RyR2 striations (Figure 2A; Pearson correlation coefficient of 0.52±0.03 versus 0.65±0.03 and 0.62±0.03 in control and SCR cardiomyocytes, respectively; \( P<0.05 \) for FGF13 knockdown versus control or SCR cardiomyocytes; \( n=8 \) cells per group). We then performed quantitative analysis on the pattern of C\textsubscript{a},1.2. In control adult mouse cardiomyocytes (Figure 2A) or after infection with the SCR virus (Figure 2B), \( \alpha \text{c}_{\text{IC}} \) displayed the expected striated pattern with a periodicity of \( \approx 2 \) \( \mu \)m, consistent with a T-tubular distribution.17 In contrast, the pattern in FGF13 knockdown cells was discontinuous, with multiple punctae found between the residual striations (Figure 2B). With the analyzer blinded to treatment status, we quantified the change in distribution with intensity profiles of \( \alpha \text{c}_{\text{IC}} \) in confoal z stacks of nonnuclear areas, as shown in Figure 2B. In SCR cardiomyocytes, we detected a regular pattern of intense \( \alpha \text{c}_{\text{IC}} \) staining at \( \approx 2 \) \( \mu \)m intervals (analyzed by fast Fourier transform; Figure 2D), with almost no signal in the intervening intervals. In contrast, after FGF13 knockdown, the peak amplitude of \( \alpha \text{c}_{\text{IC}} \) staining at the \( \approx 2-\mu \)m interval was reduced by

Figure 1. Fibroblast growth factor (FGF13) associates with junctophilin-2 (JPH2) as part of the dyad macromolecular complex.
A. Immunocytochemical analysis shows FGF13 (green) in the nucleus, sarcolemma, and T tubules, enlarged in the inset. 4',6-diamidino-2-phenylindole is in blue to indicate nuclei. Scale bar 50 \( \mu \)m.
B. Schematic of JPH2 indicating the putative protein motifs and the location of the unique peptides identified by mass spectroscopy in red. Membrane occupation and recognition nexus (MORN) motifs are indicated in yellow. JPH2 is found in the dyad in which voltage-gated Ca\textsuperscript{2+} channel (Ca\textsubscript{v},1.2) is juxtaposed to ryanodine receptor 2 (RyR2).
C. Representative coimmunoprecipitation and Western blot to validate the interaction of JPH2 and FGF13, repeated 3 times. \( \alpha \)Helix indicates alpha helical domain; divergent, divergent region; SR, sarcoplasmic reticulum; and TMD, transmembrane domain.
FGF13 Modulates Ca\textsubscript{v1.2} Trafficking to the Surface and Current Density

We hypothesized that this aberrant Ca\textsubscript{v1.2} localization would lead to a decrease in Ca\textsubscript{v1.2} at the surface. We therefore quantified the relative amount of Ca\textsubscript{v1.2} at the sarcolemma after FGF13 knockdown by labeling surface proteins with biotin and capturing them with avidin beads after cell lysis. Both the surface fraction and the whole-cell lysate of adult mouse ventricular cardiomyocytes were then probed with an antibody against the pore-forming subunit of Ca\textsubscript{v1.2}, with an antibody to JPH2 but not a control IgG (Figure 2F). These data are consistent with a previous observation that JPH2 interacts with Cav1.1 in skeletal muscle\textsuperscript{16} but are, to the best of our knowledge, the first demonstration of an interaction between JPH2 and an L-type Ca\textsuperscript{2+} channel in cardiac muscle. Together, these data indicated that FGF13 knockdown did not affect sodium-calcium exchanger distribution (Figure 2C and 2D). Second, the membrane-binding dye di-8-butyl-amino-naphthyl-ethylene-pyridinium-propylsulfonate revealed that T-tubular distribution was grossly unaffected by FGF13 knockdown (Figure 2E).

The specific change in Ca\textsubscript{v1.2} localization after FGF13 knockdown led us to hypothesize that FGF13, as a member of the JPH2 macromolecular complex anchored by JPH2, affected Ca\textsubscript{v1.2} targeting T tubules. We therefore tested whether Ca\textsubscript{v1.2} was a component of the JPH2 complex. Indeed, by coimmunoprecipitation, we were able to pull down the α\textsubscript{1C}, the pore-forming subunit of Ca\textsubscript{v1.2}, with an antibody to JPH2 but not a control IgG (Figure 2F). These data are consistent with a previous observation that JPH2 interacts with Cav1.1 in skeletal muscle\textsuperscript{16} but are, to the best of our knowledge, the first demonstration of an interaction between JPH2 and an L-type Ca\textsuperscript{2+} channel in cardiac muscle. Together, these data indicated that FGF13 knockdown did not affect T-tubular or SR architecture, but FGF13 has a specific role in targeting Ca\textsubscript{v1.2} to its proper T-tubular location.

Human FGF13-VY Can Rescue Ca\textsubscript{v1.2} Current Density and Localization

To confirm a role for FGF13 in targeting Ca\textsubscript{v1.2} to the T tubules and regulating L-type Ca\textsuperscript{2+} channel current density, we performed rescue experiments on adult rat ventricular cardiomyocytes treated with FGF13 shRNA by coexpressing human
red fluorescent protein, allowing us to identify cells in which hFGF13-VY was expressed in the context of endogenous FGF13 knockdown (Figure 4A). Immunostaining for endogenous FGF13 (with an anti-FGF13 antibody) and hFGF13-VY (with an anti-Hisx6 antibody) demonstrated not only effective knockdown of endogenous FGF13 but also that the expressed hFGF13-VY recapitulated the overall cellular distribution of endogenous FGF13 (Figure 4A), although the striated pattern was even more obvious when compared with endogenous FGF13. We suspected that the subtle differences in pattern reflected the specific distribution of hFGF13-VY compared with the distribution of all endogenous splice variants recognized by the antibody in control cells.

Having established the efficacy of the hFGF13-VY rescue, we examined Ca\(_{\text{a,2}}\) current density in adult rat ventricular myocytes using whole-cell patch clamp. As in mouse cardiomyocytes (Figure 3C), FGF13 knockdown reduced Ca\(_{\text{a,2}}\) channel current density (Figure 4B; Table). Expression of hFGF13-VY restored Ca\(_{\text{a,2}}\) current density to wild-type levels (Figure 4B). We also noted a hyperpolarizing shift in steady-state inactivation compared with control cells (Table). The reasons for this are unclear but may result from the overexpression of the specific FGF13-VY splice variant in the context of the knockdown of other FGF13 splice variants, thus altering any counterbalancing effects imparted by these absent variants. Additionally, the cellular distribution of \(\alpha_{\text{1C}}\) was restored with FGF13-VY overexpression, as indicated by the overall pattern and by Fourier transform (Figure 4C and 4D). This rescue strategy therefore firmly established a role for FGF13 in targeting CaV1.2 to T tubules and provided a potent confirmation of the specificity of the FGF13 knockdown virus.

**FGF13 Affects Ca\(_{\text{a,2}}\) Transients But Preserves Excitation-Contraction Coupling Gain**

The mislocalization of Ca\(_{\text{a,2}}\) after FGF13 knockdown, the newly described interaction between FGF13 and JPH2, and previous studies that defined clear roles for JPH2 in CICR\(^{14}\) prompted us to query whether FGF13 influenced excitation-contraction coupling gain. We, therefore, simultaneously recorded Ca\(_{\text{a,2}}\) currents and Ca\(_{\text{a,2}}\) transients in control, SCR, and FGF13 knockdown rat ventricular myocytes (Figure 5).

### Table. Summary of Electrophysiological Data

<table>
<thead>
<tr>
<th></th>
<th>(I_{\text{ca}}) Peak at 0 mV, pA/pF</th>
<th>(V_{1/2}) of Activation, mV</th>
<th>(k) of Activation, pA/mV</th>
<th>(V_{1/2}) of Inactivation, mV</th>
<th>(k) of Inactivation, pA/mV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mouse cardiomyocytes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>(-4.20\pm0.51) (11)</td>
<td>(-10.27\pm1.51) (11)</td>
<td>5.43\pm0.21 (11)</td>
<td>(-29.12\pm1.21) (6)</td>
<td>5.59\pm0.20 (6)</td>
</tr>
<tr>
<td>Scrambled shRNA</td>
<td>(-4.01\pm0.36) (6)</td>
<td>(-12.16\pm1.84) (6)</td>
<td>5.02\pm0.37 (6)</td>
<td>(-30.80\pm1.06) (11)</td>
<td>5.44\pm0.14 (11)</td>
</tr>
<tr>
<td>FGF13 shRNA</td>
<td>(-2.66\pm0.36) (14)*</td>
<td>(-11.50\pm1.14) (14)</td>
<td>5.46\pm0.15 (14)</td>
<td>(-28.73\pm1.74) (13)</td>
<td>5.39\pm0.25 (13)</td>
</tr>
<tr>
<td><strong>Rat cardiomyocytes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>(-11.61\pm1.34) (14)</td>
<td>(-12.01\pm1.56) (12)</td>
<td>4.48\pm0.36 (12)</td>
<td>(-27.24\pm1.23) (9)</td>
<td>4.09\pm0.16 (9)</td>
</tr>
<tr>
<td>FGF13 shRNA</td>
<td>(-7.44\pm1.38) (11)*</td>
<td>(-12.43\pm1.24) (9)</td>
<td>4.71\pm0.29 (9)</td>
<td>(-29.83\pm1.85) (9)</td>
<td>4.42\pm0.16 (9)</td>
</tr>
<tr>
<td>FGF13 shRNA+hFGF13-VY</td>
<td>(-12.57\pm1.15) (10)</td>
<td>(-11.65\pm1.81) (9)</td>
<td>4.42\pm0.41 (9)</td>
<td>(-35.98\pm1.91) (7)*</td>
<td>4.88\pm0.21 (7)*</td>
</tr>
</tbody>
</table>

The number of cells analyzed for each parameter is in parentheses. FGF indicates fibroblast growth factor; and \(I_{\text{ca}}\), calcium current.  

*P*<0.05 by ANOVA.
mV to induce influx of Ca\(^{2+}\) through Ca\(^{2+}\) channels, and recorded it via whole-cell patch clamp. We simultaneously recorded Ca\(^{2+}\) transients using Fura-2 in the pipette internal solution. Representative traces are shown in Figure 5A. Knockdown of FGF13 not only reduced Ca\(^{2+}\) current through Ca\(^{2+}\) channels (Figure 5B), as previously shown, but also led to decreased Ca\(^{2+}\) release from the SR (Figure 5C). Interestingly, it appeared that those channels that were at the surface were coupling with RyR2 appropriately because excitation-contraction coupling gain was not different between the groups. These changes were not due to decreased SR load as measured by rapid application of caffeine in Fura-2–loaded cells (peak height: 0.28±0.05 Δ340/380 [n=5]; FGF13 KD, 0.15±0.04 [n=6]; and FGF13 KD+FGF13-VY, 0.27±0.04 [n=5]; P<0.001 for FGF13 KD vs CON and FGF13 KD+FGF13-VY. *P<0.05 for FGF13 KD vs CON and FGF13 KD+FGF13-VY.\)

FGF13 Affects Multiple Phases of the Cardiac Action Potential

Having established that FGF13 modulates not only voltage-gated Na\(^{+}\) channels \(^{7}\) but also Ca\(^{2+}\) channels and Ca\(^{2+}\) transients, we hypothesized that loss of FGF13 would have measurable effects on the cardiac action potential. Therefore, using current clamp, we recorded evoked action potentials in control, SCR, or knockdown adult rat ventricular cardiomyocytes (Figure 6A–6D). In FGF13 knockdown cells, the action potential peak amplitude decreased by ≈20% (Figure 6A and 6B), consistent with the previously defined effects of FGF13 on the cardiac Na\(^{+}\) channel current.\(^{7}\) We also observed a shortening of the action potential half-width (Figure 6A and 6C; control, 18.60±3.34 milliseconds [n=9]; scrambled shRNA, 17.41±3.40 milliseconds [n=5]; FGF13 shRNA, 9.85±1.34 milliseconds [n=7]; P<0.05 for FGF13 knockdown versus SCR and control). These data are consistent with changes in phase 2 of the cardiac action potential that is mediated predominantly by Ca\(^{2+}\) channels and implicate FGF13 as a potent regulator of Na\(^{+}\) and Ca\(^{2+}\) channels in cardiac myocytes, leading to changes in the cardiac action potential.

Discussion

Since the initial identification of FHFs,\(^{1}\) their complete physiological roles have remained shrouded. Originally hypothesized to act as extracellular growth factors similar to canonical FGFs, these nonsecreted FHFs do not appear capable of activating FGF receptors.\(^{2}\) The subsequent discovery that FHFs are binding partners for the intracellular C terminus of voltage-gated Na\(^{+}\) channels\(^{3}\) provided context to appreciate how knockout of FGF14 might reduce neuronal excitability and cause ataxia in mice\(^{19}\) and to hypothesize mechanisms for spinocerebellar ataxia \(27\), for which FGF14 was identified as the genetic locus.\(^{5}\) Our recent identification of a role for FHFs in the regulation of cardiac Na\(^{+}\) channel function and conduction\(^{7}\) demonstrated cardiac-specific roles. Still, the cellular
distribution of FGF13 in cardiomyocytes hinted at additional functions distinct from Na+ channel regulation.

Here, we show for the first time that FHFs regulate cardiac ion channels other than voltage-gated Na+ channels. Thus, these results fit well with our recent demonstration that FGF14 regulates presynaptic Ca2+ channels in cerebellar neurons. Specifically, we have demonstrated here that FHFs are part of the dyad macromolecular complex with JPH2 and CaV1.2. Through electrophysiological and molecular biological methods, we have demonstrated that FGF13 affects Ca2+ current density and targeting of CaV1.2 to T tubules. This effect on targeting is specific to CaV1.2, not a general effect on T-tubule structure, because sodium-calcium exchanger and RyR localization were not altered, nor was overall T-tubule structure perturbed by FGF13 knockdown. We hypothesize that the abnormal localization of CaV1.2 after FGF13 knockdown represents a defect in intracellular sorting of these proteins in cardiomyocytes. Missorting of CaV1.2 was rescued by overexpression of a human FGF13 splice variant, demonstrating the specificity of the FGF13 knockdown shRNA. These data not only provide the first evidence that FGF13 is required for proper targeting of CaV1.2 to the T tubule in cardiac muscle but also show that FGF13 knockdown had profound physiological effects on Ca2+ current density and Ca2+ cycling, culminating in a decrease in cardiac action potential amplitude and duration and a reduced Ca2+ transient amplitude in parallel with its reduction in CaV1.2 Ca2+ channel current.

The identification of JPH2 as an FGF13-interacting protein provides mechanistic insight to this effect. JPH2 is a structural protein found in the cardiac dyad, where the T tubule is juxtaposed to 1 terminal cisterna of the SR. The structure of JPH2 fixes the distance between the plasma membrane and SR for efficient CICR (Figure 1C). JPH2 contains a cytosolic alpha helical domain that is capped on either side by membrane interaction motifs. On the N terminus are multiple membrane occupation and recognition nexus motifs that interact with the inner leaflet of the sarcolemma. At the C terminus is a transmembrane domain that anchors JPH2 to the SR. Reminiscent of our results after FGF13 knockdown, JPH2 knockdown in cardiomyocytes affects CICR. This provides further support that the interaction between JPH2 and FGF13 that we observed is functionally relevant. Moreover, analogous to loss-of-function mutations in JPH2, we hypothesize that FGF13 mutations may lead to orphaned ryanodine receptors that are no longer apposed to CaV1.2 and thus may be associated with heart failure. Further studies in the appropriate model are necessary to test that hypothesis.

Nevertheless, the phenotypes after FGF13 and JPH2 knockdown are not identical. JPH2 knockdown did not decrease CaV1.2 Ca2+ current and has no reported effect on voltage-gated Na+ currents. Moreover, JPH2 is observed only in a striated pattern in cardiomyocytes, whereas we observed additional FGF13 throughout the cytoplasm and in the nucleus. Thus, the fraction of FGF13 that interacts with JPH2 likely represents only 1 component of the overall FGF13 pool and is likely also distinct from the fraction interacting with NaV1.5. Other potential FGF13 interactors such as microtubules in neurons have previously been reported, likely indicating additional FGF13 pools. The tubulin interaction site on FGF13 maps to a region in close proximity to where NaV1.5 interacts in our recent crystal structure. Thus, we predict that any FGF13 interacting with microtubules would be unable to bind NaV1.5 simultaneously, further underlining the concept of distinct FGF13 pools. Although we have so far been unable to observe high affinity binding between tubulin and FGF13, the possibility of such an interaction in cardiomyocytes is attractive, particularly in light of the demonstration that CaV1.2 is trafficked along microtubules via BIN1 in ventricular myocytes.

In summary, this study identified FHFs as novel modulators of the cardiac L-type CaV1.2 Ca2+ channel and thereby significantly expands our understanding of the roles of these proteins. Our data have important clinical implications. FGF12, the dominant FHF in human heart (data not shown), is >60% homologous with mouse FGF13. Our data suggest that FGF12 loss-of-function mutations would decrease both Ca2+ and Na+ channel currents. Because loss-of-function mutations in NaV1.5 or CaV1.2 have been reported in Brugada syndrome, we specifically hypothesize that FGF12 should be explored as a candidate locus for Brugada syndrome. Only ~30% of Brugada syndrome patients have an identified mutation, so loss-of-function mutations in FGF12 may underlie the mechanism in at least some of the remaining cases by nature of its ability to affect both Na+ and Ca2+ currents.

Acknowledgments

We thank the staff at the Duke Proteomics Core Facility for their help with the affinity purification/mass spectroscopy and G. Vann Bennett (Duke University) for the antibody to sodium-calcium exchanger.
Disclosures

This work was supported by National Heart, Lung, and Blood Institute (NHLBI) RO1 HL71165 and RO1 HL112918 (G.S. Pitt), by the Gertrude Eliion Mentored Medical Student Award and NHLBI F30 HL112540-01 (J.A. Hennessey), and by an American Heart Association predoctoral fellowship award (E.Q.W.).

References


Novelty and Significance

What Is Known?

- Fibroblast growth factor (FGF) homologous factors bind to and modulate the voltage-gated Na+ channels.
- FGF13 is the predominant adult murine cardiac FGF homologous factor that modulates Nav1.5 channel current density and kinetics.
- FGF13 has widespread subcellular distribution within ventricular cardiac myocytes.

What New Information Does This Article Contribute?

- FGF13 knockdown reduces peak amplitude and half-width of the action potential in adult rat ventricular cardiac myocytes.

FGF13 modulates voltage-gated Na+ channels in cardiac myocytes, but its widespread distribution, specifically in a striated pattern, indicates that it might also regulate other ion channels. We discovered that FGF13 resides in the cardiac dyad in complex with the structural protein junctophilin-2. Without disturbing the T-tubule structure or other ion channels in the dyad, FGF13 knockdown specifically reduces Ca1.2 channel density at the sarcolemma as a result of mislocalization, leading to reduced ICa,L and decreased Ca2+-induced Ca2+ release. Cardiac myocytes from FGF13 knockdown show a decrease in the peak amplitude and the half-width of the action potential. These findings reveal a novel modular pathway through which FGF13 regulates Ca2+ channel function and suggest that mutations in fibroblast growth factor homologous factors could underlie cardiac arrhythmias characterized by the loss of function of Na+ or Ca2+ channels.
Fibroblast Growth Factor Homologous Factors Modulate Cardiac Calcium Channels
Jessica A. Hennessey, Eric Q. Wei and Geoffrey S. Pitt

Circ Res. 2013;113:381-388; originally published online June 26, 2013;
doi: 10.1161/CIRCRESAHA.113.301215
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/113/4/381

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2013/06/26/CIRCRESAHA.113.301215.DC1.html

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published
in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the
Editorial Office. Once the online version of the published article for which permission is being requested is
located, click Request Permissions in the middle column of the Web page under Services. Further information
about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
SUPPLEMENTAL MATERIAL

Fibroblast Growth Factor Homologous Factors Modulate Cardiac Calcium Channels

Short title: Hennessey et al., FGF13 regulates Ca\textsubscript{V}1.2

Jessica A. Hennessey, BA; Eric Q. Wei, BSe; and Geoffrey S. Pitt, MD, PhD
Detailed Methods

Adenovirus
The adenoviruses expressing FGF13 shRNA or scrambled shRNA with GFP has been previously described. 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. 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, NaH2PO4•H2O 1.7, NaHCO3 4.2, MgCl•6H2O 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% O2/5% CO2 for at least 30 minutes. Cells were then analyzed for electrophysiology, immunocytochemistry and Ca2+ transient recording 36-48 hours later.

Electrophysiology
Ca2+ currents (ICa) were recorded using the whole-cell patch-clamp technique as previously described. 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, CaCl2 1, MgCl2 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, CaCl2 2, MgCl2 1.2, 4-aminopyridine 2, D-glucose 5.5, pH 7.3 adjusted with CsOH. Internal solution contained (in mM, from Sigma): CsOH•H2O 70, aspartic acid 80, CsCl 40, NaCl 10, HEPES 10, EGTA 10, MgATP 5,
Na$_2$GTP 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$_2$GTP 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-estearify 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$_{1}$C 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$_{1}$C 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$_V$1.2, 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 Ca²⁺ 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•H₂O 70, aspartic acid 80, CsCl 40, NaCl 10, HEPES 10, Fura-2 pentapotassium salt 0.150 (Life Technologies), MgATP 5, Na₂GTP 0.2, Na₂-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 [Ca²⁺] 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 Ca²⁺ 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 Ca²⁺ transient peak divided by the Ca²⁺ 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-α₁C 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. 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 dithiothreitol. 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-α1C 1:1000 (Alomone), anti-transferrin receptor 1:1000 (Life Technologies), anti-β-actin 1:5000 (Sigma) and anti-FGF13 1:200. 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

<table>
<thead>
<tr>
<th>Protein name</th>
<th># of Peptides</th>
<th>FGF13 IgG</th>
<th>Control IgG</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblast growth factor 13 FGF13_MOUSE</td>
<td>3</td>
<td>0</td>
<td></td>
<td>NKPAAHFLPKPLK SVSGVLNGGK VVAIQGVQTK</td>
</tr>
<tr>
<td>Sodium channel type 5 subunit alpha SCN5A_MOUSE</td>
<td>8</td>
<td>0</td>
<td></td>
<td>ALNQLSLTHGLSR ALSAVSVLTSALEELEESHR DQGSEADFADDENNSTAGEESESHR EGLPEEEARPRQDLQASK ESLAAIEKR HASFLFR KPAALATHSQLPSCIAAPR TSLLVPWPLR</td>
</tr>
<tr>
<td>Junctophilin-2 JPH2_MOUSE</td>
<td>4</td>
<td>0</td>
<td></td>
<td>ELAPDFYQPGPEYQK LLQIEIENSESILLEPPER RSDAPPSPVSATVPEEEPPAPR YEWEWLDNLR</td>
</tr>
</tbody>
</table>

* Protein accession numbers from SwissProt 2011
Supplemental References


