FGF13 modulates the gating properties of the cardiac sodium channel Na\textsubscript{v}1.5 in an isoform-specific manner

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\textbf{ABSTRACT}

FGF13 (FHF2), the major fibroblast growth factor homologous factor (FHF) in rodent heart, directly binds to the C-terminus of the main cardiac sodium channel, Na\textsubscript{v}1.5. Knockdown of FGF13 in cardiomyocytes induces slowed ventricular conduction by altering Na\textsubscript{v}1.5 function. FGF13 has five splice variants, each of which possess the same core region and C terminus but differing in their respective N termini. Whether and how these alternatively spliced N termini impart isoform-specific regulation of Na\textsubscript{v}1.5, however, has not been reported. Here, we exploited a heterologous expression to explore the specific modulatory effects of FGF13 splice variants FGF13S, FGF13U and FGF13YV on Na\textsubscript{v}1.5 function. We found these three splice variants differentially modulated Na\textsubscript{v}1.5 current density. Although steady-state activation was unaltered by any of the FGF13 isoforms (compared to control cells expressing Nav1.5 but not expressing FGF13), open-state fast inactivation and closed-state fast inactivation were markedly slowed, steady-state availability was significantly shifted toward the depolarizing direction, and the window current was increased by each of FGF13 isoforms. Most strikingly, FGF13S hastened the rate of Na\textsubscript{v}1.5 entry into the slow inactivation state and induced a dramatic slowing of recovery from inactivation, which caused a large decrease in current after either low or high frequency stimulation. Overall, these data showed the diversity of the roles of the FGF13 N-termini in Na\textsubscript{v}1.5 channel modulation and suggested the importance of isoform-specific regulation.

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\textbf{Introduction}

Voltage gated sodium channels (VGSCs) underlie the rapid upstroke and propagation of the action potential in most excitable cells.\textsuperscript{1} VGSC channels are large macromolecular complexes composed of a pore-forming \(\alpha\) subunit (Na\textsubscript{v}), auxiliary \(\beta\) subunits, and a growing list of channel interacting proteins (ChIPs).\textsuperscript{2,5} There are ten \(\alpha\) subunits and four \(\beta\) subunits encoded in the human genome.\textsuperscript{6,7} The individual \(\alpha\) subunits are expressed in different tissues and have different biophysical and pharmacological properties.\textsuperscript{8} Each \(\alpha\) subunit is composed of four homologous domains (DI-DIV) that contain six transmembrane spanning segments.\textsuperscript{7} Na\textsubscript{v}1.5 is expressed in heart and is the major cardiac VGSC.

The importance of ChIPs on Na\textsubscript{v}1.5 function is highlighted by multiples studies showing that when ChIPs are mutated or when their binding site on Na\textsubscript{v}1.5 is mutated to affect interaction an increasingly wide range of cardiac rhythm disorders results, including Long-QT syndrome, Brugada syndrome, cardiac conduction disorders, idiopathic ventricular fibrillation, and sinus node dysfunction.\textsuperscript{9-13} Recently, fibroblast growth factor homologous factors (FHFs) have emerged as a novel class of VGSC ChIP.\textsuperscript{14-16} There are four FHF genes: FGF11- FGF14, each with different splice variants. Some of these splice variants, e.g., FGF12B, FGF13S, FGF13B, FGF14A, FGF14B, have been reported to bind to and modulate cardiac or neuronal VGSC.\textsuperscript{17-19} The relevance of FHFs on VGSC function is highlighted by various human
disorders or mouse models. A human missense mutation in \textit{FGF14} or knockout in mice of \textit{Fgf14} causes ataxia and cognitive deficits.\textsuperscript{20,21} Conditional knockout of \textit{Fgf13} in mouse brain resulted in neuronal migration defects and altered learning and memory.\textsuperscript{22} \textit{Fgf13} knockdown in rat cardiomyocytes slowed cardiac conduction velocity by altering \textit{Nav1.5} function.\textsuperscript{23}

This study focuses on \textit{FGF13}. In rodent heart, \textit{Fgf13} is the main FHF expressed,\textsuperscript{23} so an understanding of how FGF13 proteins affect \textit{Nav1.5} channels provides essential information to understand the consequences of mouse models designed to study the effects of FHFs on cardiac VGSC currents. Moreover, \textit{FGF13}, which encodes five isoforms, each with a distinct N-terminal sequence generated through alternative promoter usage and 5' alternative splicing (Fig. 1), is expressed in human heart. So an understanding of the modulatory effects of FGF13 on \textit{Nav1.5} will provide context for unraveling certain human arrhythmias that result from variants in \textit{FGF13} or those within \textit{Nav1.5} that affect interaction with FGF13. The FGF-like core region, which forms the interaction domain for \textit{Nav1.5}, and C-terminal sequence of the FGF13 isoforms are identical for all FGF13 isoforms. How specific isoforms of FGF13 isoforms differing only in their N-termini modulate \textit{Nav1.5} channel have not been explored. To understand the influence of FGF13 on

\begin{figure}[h]
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\caption{Kinetics of \textit{Nav1.5} channel activation were not affected by FGF13 isoforms. (A) Diagram for FGF13 isoforms. Alternative splicing FGF13 isoforms are schematically shown with different color. (B) Representative families of currents recorded from tsA201 cells transiently co-transfected with \textit{Nav1.5} and either pIRE5-GFP (Control) or one of the FGF13 isoforms (FGF13S, FGF13U and FGF13VY) subcloned into pIRE5-GFP vector. (C) Summarized relative peak currents density for control or the FGF13 isoforms (**P < 0.01, compared to control). Numbers of cells tested are shown on the top of each column. (D) Steady-state conductance-voltage relationships of \textit{Nav1.5} channels in the absence and presence of FGF13 isoforms. Conductance was normalized to single Boltzmann fits, whose averages are represented by solid curves. (E) Normalized peak current-voltage relationships of \textit{Nav1.5} elicited from a holding potential of \(-120\) mV to the indicated test voltages.}
\end{figure}
cardiac VGSCs, we picked three specific FGF13 isoforms for detailed study: FGF13VY, which possess the longest N-terminus and is the isoform most highly expressed in rodent heart; FGF13U, which possess the shortest N-terminus and thereby mimics a FGF13 composed of only its FGF-like core and C-terminus; and FGF13S, which has a novel inactivation particle situated in the N-terminus, as reported in neurons. We found that the three splice variants differentially modulated Na\(_{v}\)1.5 current density and gating properties including activation, fast and slow inactivation, steady-state inactivation, window current, and the frequency dependence of Nav1.5 inactivation. These data provide structural insights into FGF13 modulation on Na\(_{v}\)1.5 currents and highlight the importance of FHF N-termini and their consequent isoform-specific regulation of VGSC.

**Results**

**Activation**

As shown in Figure 1A, all FGF13 isoforms share the same core domain and C-terminus, but have different N-termini, which vary in length and amino acid sequence (Fig. 1A). In tsA201 cells, Na\(_{v}\)1.5 was co-expressed with one of the three FGF13 isoforms chosen for study or IRES-GFP (control), and whole-cell currents were investigated by the voltage-clamp technique. To focus on the effects of the FGF13 isoforms, no \(\beta\) subunits were included in these studies. Exemplar traces are shown in Figure 1B. FGF13S did not altered Na\(_{v}\)1.5 current density, but FGF13U and FGF13VY significantly decreased current density, compared to control (Fig. 1C). Current density can be regulated either by channel trafficking to the plasma membrane and/or by effects upon channel gating, and FHF s are known to exert cell-type specific regulation of channel trafficking. We therefore tested whether the reduced current density observed when FGF13VY was expressed resulted from effects upon channel trafficking to the membrane. By immunoblotting and a cell surface biotinylation assay, we found that FGF13VY expression not only reduced the amount of Nav1.5 expression lysates, but also significantly decreased amount of Na\(_{v}\)1.5 at the cell surface in tsA201 cells (Supplemental Fig. 1). Thus, FGF13 isoforms can specifically affect trafficking of Na\(_{v}\)1.5 to the plasma membrane. Having established this isoform-specific effect on current density, we then turned our attention to how FGF13 isoforms exert specific modulation on Na\(_{v}\)1.5 channel gating.

We determined the voltage-dependence of channel activation by normalizing conductance and fitted the data with a Boltzmann function. The midpoint (V\(_{1/2}\)) and the slope factor (k) were not altered when any of the three FGF13 isoforms were co-expressed compared to control (Fig. 1D and Table 1). Further, normalized peak current-voltage relationship of Na\(_{v}\)1.5 elicited from a holding potential of −120 mV to the indicated test voltages were not different from control when any of the FGF13 isoforms were co-expressed (Fig. 1E).

**Fast inactivation**

We then measured the time constants of open-state fast inactivation by fitting the decay of current with a single exponential function. Cells were held at −120 mV to remove inactivation and then test pulses from −80 to +40 mV were applied in 5-mV increments. The normalized traces in Figure 2A and 2B emphasized the rate of decay of VGSC currents recorded at −20 mV (peak of the I-V curve) from cells expressing Nav1.5 in either the presence or absence of FGF13 isoforms. Compared to currents from cells not expressing FGF13, currents from cells expressing any one of the three FGF13 isoforms showed a slowed rate of decay. The time constants for open-state fast inactivation, measured between −60 mV and +40 mV are shown in Figure 2C. FGF13S significantly slowed the fast inactivation at all test potentials between +10 mV and −40 mV. However, FGF13U or FGF13VY only slowed the fast inactivation at test potentials more negative than −20 mV. We also examined the persistent current, measured at the end of a 100 ms test pulse to −20 mV and shown as percentage of peak current. There were no significant difference between control and FGF13 isoforms (Fig. 2D).

**Closed state inactivation**

To measure closed-state fast inactivation, cells were held at −120 mV, prepulsed to −70 mV or −90 mV for increasing amounts of time (1-200 ms) and then stepped

| V\(_{1/2}\) (mV) | k | n |
|----------------|---|---|
| Control        | −36.3 ± 0.4 | 5.8 ± 0.2 | 9 |
| FGF13S         | −39 ± 0.4   | 5.2 ± 0.2 | 9 |
| FGF13U         | −36.7 ± 0.5 | 5.3 ± 0.2 | 6 |
| FGF13VY        | −39.3 ± 0.45| 5.6 ± 0.2 | 8 |

V\(_{1/2}\) is the membrane voltage of half-maximal activation and k is the slope factor.

Table 1. Activation properties of Nav1.5 co-expression of pIRES-GFP or FGF13 isoforms.
to $-20 \text{ mV}$ and the fraction of current inactivated during the prepulse was determined. Compared to control, FGF13 isoforms markedly decreased the development of NaV1.5 close-state fast inactivation (Fig. 3 and Table 2).

**Steady state inactivation**

To measure the voltage dependence of steady-state fast inactivation in NaV1.5, we plotted the peak current amplitudes as a function of the prepulse potential (protocol as shown in Fig. 4A, inset) and fitted the data with a Boltzmann function as shown in Figure 4. FGF13 isoforms markedly shifted the $V_{1/2}$ of steady-state fast inactivation to the depolarizing direction compared to control (see also Table 3). Those changes are consistent with the effect observed in closed-state inactivation (Fig. 3). Window current was measured by calculating the area under the normalized activation curve and steady-state fast inactivation curve. Compared to control, FGF13 isoforms increased the relative window current by 463.5%, 879.5% and 968.4%, for FGF13S, FGF13U and FGF13VY, respectively (Fig. 4B).

**Slow inactivation**

We also examined whether FGF13 isoforms affected slower forms of inactivation that might be critical for channel availability on a time scale from several seconds to minutes. To measure slow inactivation, cells were depolarized to $-20 \text{ mV}$ for durations ranging from 500 ms to 10 s, allowed to recover from fast inactivation at $-120 \text{ mV}$ for 40 ms, and subjected to a 50 ms test pulse to $-20 \text{ mV}$. Compared with control, only FGF13S showed a significantly faster onset of slow inactivation (Fig. 5, Table 4).

**Recovery from inactivation and response to repetitive stimulation**

A previous study reported that FGF13S, but not FGF13U slowed the recovery of NaV1.6 from
To examine the effects of FGF13 isoforms on NaV1.5 recovery from inactivation, we used a two-pulse protocol, as shown in Fig. 6. Cells were held at $-120$ mV before a test depolarization to $-20$ mV for 30 ms followed by a variable (1-1000 ms) interval where the membrane potential was returned to $-120$ mV for recovery and then a test pulse at $-20$ mV (inset). Cells co-expressing Nav1.5 and FGF13 isoforms (FGF13S, FGF13U, FGF13VY) displayed an availability curve that was significantly depolarized shift comparing to cells expressing Nav1.5 and IRES-GFP (Control). (B) FGF13 isoforms increase window current.

**Table 2.** Development of inactivation properties of Nav1.5 co-expression of pIRES-GFP or FGF13 isoforms at $-70$ mV and $-90$ mV.

| Voltage (mV) | Offset (%) | tau (ms) | n |
|-------------|------------|----------|---|
| $-70$ mV Control | 0.3 ± 0.2 | 31.9 ± 1.1 | 4 |
| $-70$ mV FGF13S | 33.4 ± 0.1 | 84.4 ± 11** | 4 |
| $-70$ mV FGF13U | 40.9 ± 0.2** | 95.4 ± 7.6** | 4 |
| $-70$ mV FGF13VY | 26.3 ± 0.2 | 73.1 ± 7.3** | 4 |
| $-90$ mV Control | 42.7 ± 3 | 69.3 ± 11.8 | 4 |
| $-90$ mV FGF13S | 83 ± 8.7** | 164.9 ± 12.3** | 4 |
| $-90$ mV FGF13U | 90.8 ± 5.4** | 153.3 ± 12.9** | 4 |
| $-90$ mV FGF13VY | 73.2 ± 10.2** | 268.1 ± 28.4** | 4 |

Offset is the proportion of current that did not inactivate. **P < 0.01 compared to control at the same voltage.

**Table 3.** Inactivation properties of Nav1.5 co-expression of pIRES-GFP or FGF13 isoforms.

| Voltage (mV) | $V_{1/2}$ | k | n |
|-------------|-----------|---|---|
| $-70$ mV Control | $-89.8 ± 0.3$ | 4.5 ± 0.1 | 11 |
| FGF13S | $-75.6 ± 0.4^*$ | 4.2 ± 0.2 | 10 |
| FGF13U | $-79.1 ± 0.3^*$ | 4.2 ± 0.1 | 6 |
| FGF13VY | $-77.4 ± 0.3^*$ | 4.1 ± 0.1 | 7 |

$V_{1/2}$ is the membrane voltage of half-maximal activation and k is the slope factor. *P < 0.05, compared to control.
second test pulse for 30 ms to $-20$ mV. The peak current obtained during the second test pulse after a specific recovery interval was then normalized to the first and the data were fitted with double exponential functions. As shown in Figure 6, FGF13S cause a pronounced slower recovery from inactivation of NaV1.5 compared to control, but FGF13U and FGF13VY were without effect (Table 5). We then tested whether the FGF13 isoforms differentially affected NaV1.5 in response to repetitive stimulation. Cells were held at $-120$ mV before a series of 20 successive test potentials at $-20$ mV for 40 ms delivered over a range of frequencies (Fig. 7C, insert). Consistent with the effects upon recovery from inactivation, co-expression of FGF13S caused a larger decrement in peak current at higher stimulation frequencies (2 Hz, 5 Hz and 10 Hz) than that at lower stimulation frequencies (0.5 Hz and 1 Hz). There were no significant differences in peak currents at all frequencies tested for other FGF13 isoforms (Fig. 7).

Table 4. Entry into inactivation state properties of Nav1.5 co-expression of pIRES-GFP or FGF13 isoforms.

| Isoform       | Offset (%) | tau (ms)       | n  |
|---------------|------------|----------------|----|
| Control       | 31.9 ± 7.6 | 5851.6 ± 149.3 | 6  |
| FGF13S        | 18.9 ± 5.5 | 4477.2 ± 152.6 | 5  |
| FGF13U        | 23.5 ± 9.2 | 5401.8 ± 162   | 4  |
| FGF13VY       | 30.7 ± 10  | 5606.5 ± 189.5 | 4  |

Offset is the proportion of current that did not inactivate. *P<0.05 compared to control.

Discussion

In this study we focused on the functional effects of FGF13 isoforms on gating properties of cardiac NaV1.5 channels expressed in mammalian cells. We found that individual FGF13 isoforms did not affect channel activation but had multiple effects on channel inactivation. Overall, we observed that co-expression of all three FGF13 isoforms examined decreased channel availability (depolarized the $V_{1/2}$ of steady-state inactivation) and markedly slowed both the open-state and close-state fast inactivation. Among the FGF13 isoforms, the FGF13S splice variant was the most potent. Because the FGF13 splice variants share an identical core domain, which contains the major NaV1.5 interaction site, our data suggest that the alternatively spliced FGF13 N-termini exert the isoform-dependent effects on NaV1.5 gating properties.

Fast inactivation of sodium channel is a critical process that occurs within milliseconds of channel opening. A critical structure in open-state fast inactivation is the intracellular loop connecting domains III and IV, which serves as a hinged lid that occludes the inner pore. A triplet of hydrophobic residues (IFM) near the center of the III-IV linker may comprise a latch that holds the lid in a closed position over the inner pore. The docking site for the lid consists of multiple regions, including the cytoplasmic linkers connecting segments 4 and 5 in domains III and IV and the cytoplasmic end of the S6 segment in domain IV. The cardiac NaV1.5 channel exhibits faster close-state inactivation than skeletal muscle and neuronal sodium channels at potentials close to the normal cardiac resting potential. Inherited mutations associated with congenital arrhythmias have been shown to modify the close-state inactivation. Here, we showed that the development of closed-state inactivation at voltages of $-90$ mV or $-70$ mV (near the normal cardiomyocyte resting potential) was slowed when FGF13 isoforms were co-expressed. This suggests that mutations in FHFs or in the NaV1.5 channel at the site to which FHFs bind may lead to changes in closed-state inactivation and consequently to arrhythmogenesis.

The potency of the FGF13S isoform may have significant physiological and pathophysiological significance. Although FGF12 appears to be the main FHF in human heart, in which FGF13S expression appears relatively limited, any upregulation of FGF13S, such as during a disease state, would have important
consequences on Nav1.5 currents and subsequently upon arrhythmogenesis.

The molecular mechanism underlying the specific effects of FGF13S on NaV1.5 gating remains unclear. One possibility is that the N terminus of FGF13S might directly interact with some component of the NaV1.5 inactivation machinery (e.g., the III-IV intracellular linker) to promote inactivation or to prevent recovery from inactivation once the process is

![Figure 6](image.png)

**Figure 6.** FGF13S dramatically slow the recovery from fast inactivation of Nav1.5. (A) Recovery from fast inactivation was determined from a holding potential of −120 mV, using two 30 ms pulses separated by a recovery time from 1 to 1000 ms at −120 mV (B) Mean data was fitted by double exponential equation.

|                  | tau1   | tau2   | n  |
|------------------|--------|--------|----|
| Control          | 4.3 ± 0.3 | 20.6 ± 4.5 | 7  |
| FGF13S           | 9.7 ± 0.07* | 226.4 ± 7** | 6  |
| FGF13U           | 5.4 ± 0.9  | 20.4 ± 1.6  | 3  |
| FGF13VY          | 3.5 ± 0.1  | 15.3 ± 0.1  | 6  |

*P < 0.05, **P < 0.01 compared to control.
initiated. Further studies will be needed to clarify the structural basis underlying the specific interaction between the FGF13S N-terminus and NaV1.5 channels. In addition, calmodulin is known to interact with sodium channel and FHFs in a ternary complex. However, it is not known whether calmodulin contribute to the different modulation of FGF13 isoforms on Nav1.5 gating.

Besides modulation of Nav1.5 channel gating, we found that FGF13VY affected Nav1.5 current density in a heterologous expression system by reducing overall protein expression of Nav1.5 and by downregulating the amount of Nav1.5 at the cell surface. Together, these data suggest FGF13VY is an important regulator of VGSCs in heart through multiple mechanisms.

**Materials and methods**

**Plasmids and virus**

6xHis (His6) tagged human FGF13 isoforms (FGF13S, FGF13U and FGF13VY) were subcloned into pIRES2-AcGFP1 (Clontech).

**Cell culture and transfections**

tsA201 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 4 mM L-glutamine, 10% fetal bovine serum (Gibco), 100 U ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin and incubated at 37°C with 5% CO₂. tsA201 cells were transfected at 80%-90% confluency using Lipofectamine
2000 (Invitrogen) according to the manufacturer’s instructions. The total amount of DNA for all transfections was kept constant. All experiments were done 48-72 hours post-transfection.

Electrophysiology

Transfected cells were identified by GFP fluorescence. Na⁺ currents (Iₙa) were recorded using the whole-cell patch-clamp technique at room temperature (20-22 °C) 48–72 h after transfection. Electrode resistance ranged from 1-2 MΩ before series resistance compensation of 80%. Currents were filtered at 5 kHz. In tsA201 cells expressing Naᵥ1.5 and FGF13, Iₙa was recorded in solution containing (in mmol/L): NaCl 130, KCl 4, CaCl₂ 1.8, MgCl₂ 1, HEPES 10, glucose 10, pH 7.35 (adjusted with NaOH). The intracellular solution contained (in mmol/L): CsF 110, EGTA 10, NaF 10, CsCl 20, HEPES 10, pH 7.35 (adjusted with CsOH). 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 10 mV increments. To determine the voltage-dependence of steady-state activation, the sodium conductance (G) was calculated by dividing the peak current for each voltage step by the driving force (Vₘ-Vᵣₑᵥ) then normalized to the peak conductance (Gₘₐₓ). Data were fitted with the Boltzmann relationship, G/Gₘₐₓ=1/[1+exp((V₋Vₘ)/k)] in which Vₛ is the voltage at which half of Naᵥ1.5 channels is activated, k is the slope factor and Vₘ 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 −20 mV. Currents (I) were normalized to Iₘₐₓ and fit to a Boltzmann function of the form I/Iₘₐₓ=1/[1+exp((V₋Vₛ)/k)] in which Vₛ is the voltage at which half of Naᵥ1.5 channels is inactivated, k is the slope factor and Vₘ is the membrane potential. Data analysis was performed using Clampfit 10.2 software (Axon Instruments) and Origin 8 (Originlab Corporation). Development of closed state inactivation at −70mV was measured by holding cells at −120 mV, prepulsing to −70 mV for increasing amounts of time (1-200 ms), and then stepping to −20 mV to determine the fraction of current inactivated during the pre-pulse. For recovery from inactivation, two-pulse protocol was used. Cells were held at −120 mV and two depolarizations to −20 mV for 30 ms were applied, with an increasing interval of time (1-1000 ms) between them for recovery. Curves were fitted with a double rising exponential function.

Statistical analyses

Results are presented as means ± standard error; the statistical significance of differences between groups was assessed using either a two-tailed Student’s t test or one-way ANOVA and was set at P < 0.05.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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