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First Full-Length Cloning of Human NaV1.7 in *S. Cerevisiae*-Based Novel D-Crypt™ Platform for its High-Scale Production

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Abstract

NaV1.7, a voltage-gated sodium channel, induces chronic pain. Current research on the discovery of inhibitors against NaV1.7 is advancing with the hope of treating chronic pain conditions in patients suffering from various diseases. However, to characterize NaV1.7 and inhibitor interactions, a higher yield of expression is required to obtain sufficient protein. Molecular cloning of hNaV1.7 was done using the homologous recombination method in our novel *S. cerevisiae*-based D-crypt™ platform. The platform was designed for the high-yield production of 'difficult-to-express' proteins (DTE-Ps) up to 500 L. The changes in the cell membrane potential of hNaV1.7 were determined using a fluorescence resonance energy transfer (FRET) assay with tetracaine (hNaV1.7 inhibitor). Expression analysis of hNaV1.7 showed 2 bands of size 250 and 280 kDa that were absent in untransfected yeast cells. Immunofluorescence images revealed the presence of hNaV1.7 on the membrane of hNaV1.7-expressing yeast cells. Tetracaine exhibited concentration-dependent inhibition of the FRET ratio, with the order of potency (IC_{50} = 0.46 μM) being approximately the same as previously reported, suggesting the functionality of the channel protein expressed in the D-crypt™ platform. Cloning of NaV1.7 in the D-crypt™ platform will help to scale up the production of channel proteins, which will ultimately help in the structural and functional characterization of the binding interactions between toxins and the NaV1.7 channel to identify more specific NaV1.7 inhibitors.

Keywords: NaV1.7 Voltage-gated sodium channel, Fluorescence resonance energy transfer, *Saccharomyces cerevisiae*, Sodium channel blockers

Introduction

Voltage-gated sodium channels (VGSC) are transmembrane proteins responsible for propagating electrical signals in neurons and cardiac, skeletal, and neuroendocrine cells. NaV1.7 is preferentially expressed in the dorsal root ganglion (DRG) and sympathetic neurons [1]. It has been reported that loss-of-function mutations in the *SCN9A* gene in families lead to a complete inability to sense pain [2], whereas gain-of-function mutations lead to painful inherited human neuropathy known as erythromelalgia [3]. There are other pain-related diseases, such as diabetic peripheral neuropathy [4], paroxysmal extreme pain disorders [5], postoperative dental pain [6], and osteoarthritis [7], where NaV1.7 inhibitors play an important role in relieving pain. These findings, in addition to animal knockout models, have pointed to NaV1.7 as a primary drug target [8], and screening for many NaV1.7 inhibitors is essential to identify more specific NaV1.7 antagonists.

The *SCN9A* gene encodes the NaV1.7 protein, which consists of a large pore-forming α-subunit that is an essential component of a VGSC. Each alpha subunit contains 4 voltage-sensing domains (VSD1-4). Each domain has 6 membrane-spanning segments (S1 - S6). The sodium channel ion-conducting pore is formed by the P-loop region between helical segments S5 and S6 from each of the repeated domains. Owing to the presence of multiple positively charged amino acid residues in the S4 segment of all 4 VSD, it senses changes in voltage during depolarization of the cell membrane potential. The generated current passes through the channel pores and constitutes a depolarizing state of the action potential [9]. The discovery of novel NaV1.7-modulating compounds requires the structural characterization of channel and inhibitor interactions. To study channel and inhibitor interactions, large-scale mammalian cell growth is required to obtain an adequate amount of protein, which is not cost effective. Therefore, there is an unmet need to develop a cost-effective platform for cloning and expressing the NaV1.7 channel that can be used to characterize channel inhibitor interactions.

There are 2 major challenges in the screening of ion channel inhibitors: 1) The expression of ion channels in mammalian vectors is time-consuming and expensive [10]. 2) The electrophysiological patch-clamp technique, which is the gold standard for studying ion channels, requires technical expertise and is not amenable to screening large numbers of compounds [11]. Although an automated patch clamp can overcome limitations, such as low throughput, the operation of the instrument requires technical expertise. Yeasts have continuously served as "screening platforms" for the analysis of various bioactive compounds owing to their straightforward cultivation, genetic manipulation, and exceptionally well-characterized molecular biology and biochemistry. In this study, for the first time, we cloned the full length hNaV1.7 gene in *S. cerevisiae*-based D-crypt™ platform [12]. The D-Crypt™ platform combines a yeast expression host with more than 20 custom-made expression vectors. This protease-deficient strain of Baker's yeast, *S. cerevisiae*, provides an ideal eukaryotic environment for expressing recombinant proteins that retain the correct structure, glycosylation, and localization of membrane proteins. Fluorescence resonance energy transfer (FRET) assay suggested that the channel was functionally active when expressed in *S. cerevisiae*-based D-crypt™ platform [11,13].

Materials and methods

Molecular cloning of full-length hNaV1.7

To clone full-length human NaV1.7 (hNaV1.7), the ORF was synthesized in 2 parts of 2 and 4.1 kb fragments (GeneArt cloning vector, Invitrogen). Briefly, a 2.0 kb fragment was digested with EcoR1/KpnI restriction enzymes, followed by ligation and transformation in DH5α cells to generate pYRE100-2kb. Cloning of 4.1 kb fragment was further divided into the amplification of 2 fragments (1.3 and 2.8 kb) due to its toxic nature. Next, pYRE100-2kb and 1.3 kb PCR products were digested with KpnI/SmaI, and ligation was performed to generate pYRE100-3.3kb. However, plasmid propagation and cloning of a 2.8 kb fragment in *E. coli* DH5α were toxic to the cells, as confirmed by sequencing analysis, which showed rearrangements in the *N*-terminal region of the 2.8 kb fragment. Therefore, plasmid propagation of a 2.8kb fragment was performed using SURE-competent cells to generate the initial material for PCR amplification and cloning. Following this, *in vivo* homologous recombination method was used to generate full-length hNaV1.7 ORF using the following PCR primers, which add homology tails at the 5' end of 3.3 kb fragment and the 3' end of 2.8 kb fragment:

Forward:

5'CTTTAACGTCAAGGAGAAAAAACCGATATCGAATTCATGGCTATGTTGCCACCACCA GGTC3'

Reverse:

5'GGAAGCTTGCGGCCGCTGGATCCTACGTAGCATGCTTATCACTTCTTGGATTCTTTGG AATCCTTACCC3'

Transformation in D-Crypt™

A reaction mix consisting of EcoR1/Xho1 digested linearized pYRE100 expression vector and PCR-amplified 3.3 and 2.8 kb amplicons was prepared. The reaction mixture was transformed into protease-deficient *S. cerevisiae* (*PYPD*) cells. The transformation was performed using a lithium acetate/ssDNA/PEG-mediated protocol [12]. Positive clones were confirmed using sequence-specific overlapping PCR primers (forward: 5'TTGATTGCTATGGACCCATACG3'; reverse:5'TCCTTCCTTTTCGGTTAGAGCGG3'). The positive clones obtained were used for expression analysis.

Expression analysis of the cultures

Cultures were grown in YNB glucose agar plates without URA at 28 °C for 24 h and were induced with galactose YNB minimal medium (2 %) for 24 h. Following induction, harvested cultures were used for expression analysis [12].

Immunoblotting analysis

Immunoblot analysis was performed using a human NaV1.7-specific antibody (Cat# ab85015, Abcam), as described previously [12].

Immunofluorescence microscopy

Spheroplasts were prepared by incubating yeast cells at 1 OD with lyticase (Sigma; 50 U/mL) for 30 min at 30 °C. Next, the cells were fixed, permeabilized, and incubated with NaV1.7-specific antibody

(Abcam) for 3 h, followed by incubation with Alexa Fluor 488 anti-mouse IgG-conjugated secondary antibody (Invitrogen). Images were captured using a fluorescence microscope.

Fluorescence resonance energy transfer (FRET) Assay

One OD of hNaV1.7-expressing yeast cells was incubated with PTS_{18} donor dye (Abcam; ab274880) for 30 min, washed with Hanks' HEPES buffer, and centrifuged at 5,000 rpm. The cells were then incubated with 20 µM DiSBaC2(3) dye (Invitrogen; B413) for 20 min at 37 °C. Four different concentrations (0.02, 0.2, 1 and 10 µM) of tetracaine (Sigma-Aldrich) were added to the cells for 10 min. The cells were excited at 387 nm, and the ratio of the fluorescence intensity signals (FRET ratio) emitted at 435 and 570 nm was measured every 5 min using an FLS900 spectrometer. After the baseline emission was obtained, 400µM veratridine (Sigma) was added to measure the maximal depolarizing response.

Statistical analysis

Each experiment, including western blotting, immunofluorescence microscopy, and FRET assays, was repeated 3 times. The results of the FRET assay are presented as the mean ± standard error of the mean. Student's t-test was used to compare the maximal depolarizing response in control and hNaV1.7 overexpressing cells. All statistical analyses were performed using OriginPro software (version 2020b). Statistical significance was set at $p < 0.05$.

Results and discussion

In this study, we generated a *S. cerevisiae* cell line expressing the full-length hNaV1.7 protein, for the first time. Positive clones were selected using sequence-specific overlapping primers, confirming full-length NaV1.7 in the host *PYPD* strain of *Saccharomyces cerevisiae* (**Figure 1**).

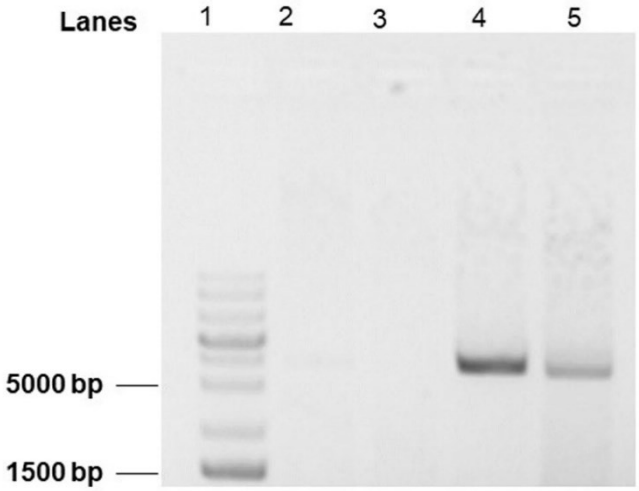

Figure 1 Colony PCR for screening positive clones. Lane 1: Gene ruler 1 kb plus (DNA Ladder); lanes 2 - 3; pYRE100 vector; Lane 4 - 5; full-length human NaV1.7-coding gene.

Expression analysis of hNaV1.7 showed 2 bands (~250 and ~280 kDa) that were not present in the membranes of untransfected yeast cells (**Figure 2(A)**). These bands corresponded to the different glycosylated states of NaV1.7, as previously reported by Laedermann *et al.* [14]. Immunofluorescence images revealed the presence of hNaV1.7 on the membrane of hNaV1.7-expressing yeast cells (**Figure 2(B)**).

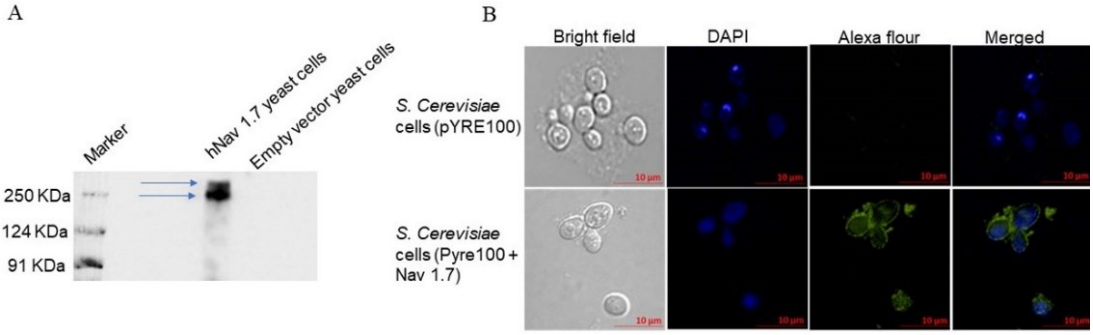

Figure 2 A) Immunoblot showing expression of human NaV1.7 protein and B) Immunofluorescence imaging depicting localization of the protein.

Full-length cloning of recombinant voltage-gated sodium (NaV) ion channels is problematic because of difficulties arising during bacterial plasmid amplification, random mutations in the coding sequences of the channels, and stable cell line development. Although multiple factors make cloning of these channels difficult, genome instability is a major concern [15,16]. Nucleotide sequences within the NaV1.1-coding region, which resemble prokaryotic promoter-like elements and are presumed to drive the transcription of translationally toxic mRNAs in bacteria, may cause instability [16]. Furthermore, there is evidence proving that NaV channel-coding genes are toxic to the bacterial host, since random mutation events are not limited to full-length NaV channel-coding sequences, but also occur during bacterial propagation involving NaV channel-coding DNA fragments. These findings support previous studies, which reported that cell lines such as JM101, DH5α, and One-Shot Top10® are susceptible to production issues and have a high chance of producing a negative outcome when cloning ion channel proteins [15]. Nav channels are notoriously difficult subjects when it comes to bacterial plasmid amplification, mutagenesis, and stable cell line development. The basis for this behavior is unknown, and no study investigating the same has been published [15]. Therefore, careful planning during cloning is necessary to reduce or eliminate the instability or toxicity. We showed that the homologous recombination technique used for cloning full-length hNaV1.7 in our novel *S. cerevisiae*-based D-crypt™ platform maintains the correct reading frame, allowing reassembly in a single transformation, thereby making the entire cloning process straightforward. We also observed a toxic effect due to the rearrangement in the first 500 bp region when cloning the 2.8 kb fragment in DH5α cells. These results suggest a deletion or rearrangement of a 2.8 kb fragment by the DH5α repair system. Therefore, SURE-competent cells were used to clone this fragment. The genotype of SURE cells used in this study favors cloning of the 2.8 kb fragment of the hNaV1.7 channel owing to their recombination-deficient nature. SURE-competent cells are deficient in *E. coli* genes involved in the rearrangement and deletion of DNA, thus reducing toxicity, and improving cloning efficiency.

Membrane potential high-throughput FRET-based assays for hNaV1.7 have been established on a VIPR™ (Aurora Discovery, San Diego, CA) [13], FDSS6000 instrument [11], and PHERAstar FSX (BMG Labtech) [17] to identify novel state-dependent NaV1 inhibitors. However, these assays have been developed using HEK-NaV1.7 cells or CCD-1064sk cells, which makes these assays cost-ineffective. To show the activity of the NaV1.7 channel expressed in the D-crypt™ platform, a high concentration of veratridine (400 µM), a NaV1.7 agonist, was used to investigate the FRET ratio of yeast cells expressing hNaV1.7 [18-20]. The FRET ratio was examined at 5, 10, 15 and 20 min to determine its ability to initiate channel-dependent depolarization in hNaV1.7-expressing yeast cells. An increase in FRET ratio was observed with veratridine (400 µM) in hNaV1.7 cells till 20 min (equilibrium attained). Upon depolarization of the cells, the donor dye remained on the outer surface, but the mobile acceptor dye rapidly translocated to the inner surface of the cell membrane, resulting in diminished FRET and an increase in the 435/570 nm emission ratio. These results indicate the activity of the NaV1.7 channel in our yeast-based platform, making this assay cost-effective. However, a higher concentration of the activator was required for the depolarization of yeast cells (**Figure 3**). This phenomenon may be due to the barrier presented by the cell wall and the presence of numerous active efflux pumps and detoxification mechanisms, as previously reported [21].

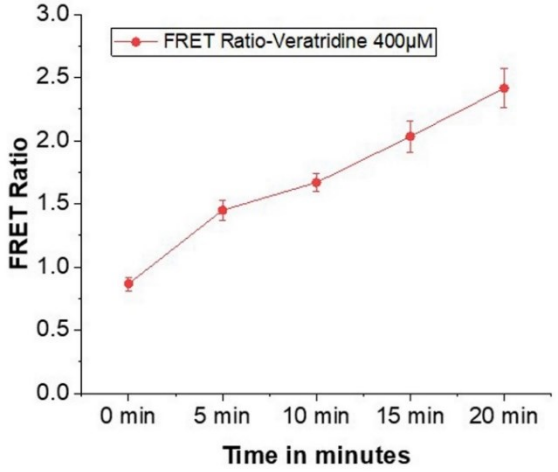

Figure 3 Veratridine-induced depolarization in *S. cerevisiae* cells that express full-length human NaV1.7.

To check the effect of tetracaine (NaV1.7 inhibitor) on cell membrane potential, yeast cells overexpressing hNaV1.7 were incubated with increasing concentrations of tetracaine. The compound exhibited concentration-dependent inhibition in the FRET ratio, with the order of potency (IC_{50} = 0.46 μM) being approximately the same as previously reported [11,13] (**Figure 4**). This FRET assay using an *S. cerevisiae*-based platform will not only speed up the screening of NaV1.7 inhibitors or analgesics, but is also cost-effective. Thus, it will ultimately help pharmacological industries in high-throughput screening of NaV1.7 inhibitors.

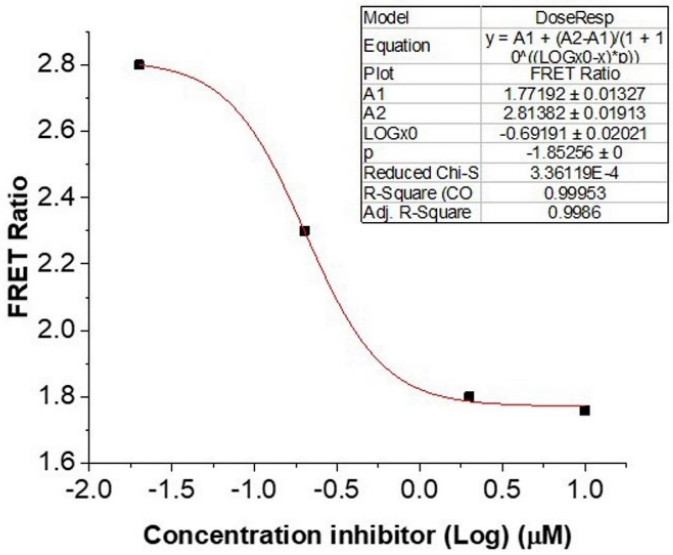

Figure 4 NaV1.7 inhibition curves for tetracaine at 4 different concentrations (0.02, 0.2, 1 and 10 μM) in the FRET assay. X-axis represents concentrations of tetracaine on the log scale.

Next, yeast cells expressing hNaV1.7 and control host cells were incubated with a fixed concentration of inhibitor (0.46 μM) for 10 min at room temperature, followed by the addition of veratridine at time points 5, 10, 15 and 20 min to measure the maximal depolarizing response. A significant change in the fluorescent counts of the acceptor dye was observed in hNaV1.7 overexpressing cells, unlike in the host control yeast cells (**Figures 5(A)** - **5(C)**). The emission spectra of the acceptor dye remained constant for control yeast cells after adding the activator at all time periods, suggesting no movement of the acceptor dye due to the absence of hNaV1.7 channels (**Figure 5(A)**). This finding further suggested that any change in the FRET ratio in the control cells was due to a change in the emission spectra of the donor dye.

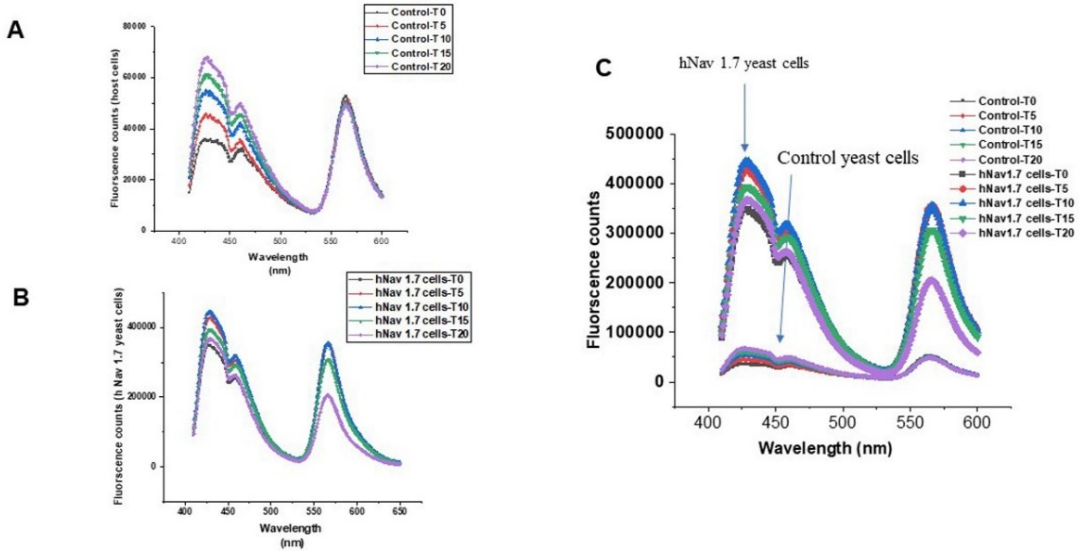

Figure 5 Change in fluorescent counts of the acceptor dye in hNaV1.7-expressing yeast cells compared to host control yeast cells after incubation with 0.46 μM concentration of tetracaine for 10 min. After the baseline emissions were recorded, veratridine (400 M) was added, and the ratio of the fluorescence intensity signals emitted at 435 and 570 nm was measured at intervals of 5 min, where T0 was 0 min, T5 was 5 min, T10 was 10 min, T15 was 15 min, and T20 was 20 min. A) Fluorescent counts of the control cells. B) Fluorescent count of hNaV1.7 cells. C) Overlaid fluorescent count for control and hNaV1.7 cells.

It should be noted that to study channel and inhibitor interactions, structural information is required to uncover the specificity and binding affinity of inhibitors to hNaV1.7 channels. However, for the cryo-EM study of full-length NaV1.7 in a complex with toxins, we needed large-scale transfected mammalian cell growth (~16 L) to obtain sufficient protein for structure determination [22]. Therefore, a higher-yield expression and purification procedure to obtain functional channel domains would be beneficial for characterizing toxin-channel interactions. In this study, we used the *S. cerevisiae*-based D-crypt™ platform, which will facilitate the large-scale production of purified proteins to 500 L [12,23]. Therefore, we expect that this D-crypt™ platform will help to scale up the production of channel proteins, which will ultimately help in the structural and functional characterization of the binding interactions between inhibitors and the NaV1.7 channel.

Furthermore, previous studies have shown that assays developed to screen NaV1.7 inhibitors are biased toward nonselective pore blockers and fail to detect the most potent selective VSD4 blockers, including PF-05089771 (PF - 771) and GX-936 [24]. Therefore, cloning of the mutant form of NaV1.7 is necessary to differentiate selective NaV1.7 blockers from nonselective NaV1.7 blockers. The novel D-crypt™ platform can also be used to clone the mutant form of NaV1.7, to discover selective NaV1.7 blockers. The emergence of selective molecular inhibitors will enable a better understanding of the etiology of dose-limiting side effects that limit the development of earlier sodium channel blockers, and enable researchers to link antinociceptive responses in efficacy studies to subtype selectivity.

Conclusions

Based on the results of the study, several conclusions were obtained, namely;

1) The D-crypt platform is a suitable platform for expressing full-length functionally active hNaV1.7 channel.

2) The platform reportedly helps in the large-scale production of difficult-to-express proteins up to 500 L, which will help in identifying more specific NaV1.7 inhibitors by characterizing channel-inhibitor interactions.

NaV1.7 is often challenging to clone, express, purify, and manufacture because of several issues such as inherently low solubility, random mutations in the coding sequences of the channels, structural instability, host toxicity, and low yield. This protein is considered a difficult-to-express protein (DTE - Ps), like other membrane proteins. Therefore, cloning the full-length hNaV1.7 channel remains a challenge and

a careful cloning procedure is required to eliminate the issues described above. The proprietary expression vectors of the D-crypt™ platform contain modified upstream elements to maintain the correct reading frame, allowing the reassembly of several fragments of hNaV1.7 in a single transformation, thereby making the entire cloning process straightforward. The activity of the channel was checked by FRET assay, suggesting the successful cloning of the active hNaV1.7 channel protein in the D-crypt™ platform. We expect that this platform can also be used for cloning the full-length mutant form of hNaV1.7, which is required for the discovery of selective blockers of hNaV1.7. Additionally, D-Crypt is optimized to reduce the time, cost, and risk associated with producing high-quality recombinant proteins for drug development programs.

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