Regulations of sodium channels by Wnt signalling in cardiomyocytes

By

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Master of Science in Cellular and Molecular Medicine

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Abstract

Background: The canonical Wnt/β-catenin pathway is activated in a variety of heart diseases, such as myocardial infarction and cardiac hypertrophy, that are associated with altered ion channel expressions and increased risk of cardiac arrhythmias. Previous work from our lab has demonstrated that the Wnt/β-catenin signalling (Wnt signalling) inhibits sodium (Na^+) current in rat cardiomyocytes. In this project, we aim to investigate the mechanisms that underlie the inhibition of Na^+ current by Wnt signalling in both rat and human cardiomyocytes.

Results: In both neonatal rat ventricular myocytes (NRVMs) and human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs), activation of the Wnt/β-catenin signalling led to reduced level of Na^+ channel gene transcript (*Scn5a*), channel protein ($Na_v1.5$) and channel current density. This suggests that reduced *Scn5a* expression is likely the primary mechanism for reduced Na^+ current. In addition, we found that activation of the Wnt/β-catenin signalling in both NRVMs and iPSC-CMs upregulated *Tbx3* transcript and protein levels, which is a transcription factor that is known to suppress *Scn5a* transcription. In NRVMs, siRNA-mediated *Tbx3* knockdown attenuated (by ~30%) Wnt-induced reductions in *Scn5a* and $Na_v1.5$ levels.

Conclusions: Our findings are consistent with the conclusion that Wnt/β-catenin signalling inhibits Na^+ current in both rat and human cardiomyocytes by reducing *Scn5a* levels, with *Tbx3* as one of the mediators.

Keywords: Wnt signalling, Cardiomyocyte, Cardiac sodium channel, *Scn5a, Tbx3*

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I would also like to thank my family for always being supportive for me. The financial support from my parents and emotional comfort from my cousin really helped me when I tried to fit in the environment at the beginning of my study.

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Table of Contents

List of abbreviations

AVN – Atrioventricular node

CCS – Cardiac conduction system

CHIR – CHIR-99021

Fz – Frizzled receptor

GSK-3β – Glycogen synthase kinase 3 beta

HEPES – Hydroxyethyl piperazineethanesulfonic acid

HF – Heart failure

hiPSC-CMs – Human induced pluripotent stem cell-derived cardiomyocytes

LRP 5/6 – Low-density lipoprotein receptor-related protein 5/6

ms – Milliseconds

$Na_v1.5$ – Cardiac sodium channel

NFAT– nuclear factor of activated T cells

NRVMs – Neonatal rat ventricular myocytes

RT-qPCR – Real-time quantitative polymerase chain reaction

SAN – Sinoatrial node

SCN5A – Gene encoding $Na_v1.5$

Tbx3 – T-box factor 3

TCF – T-cell factor

Wnt – Wingless/Int-1 gene family

1. Introduction

1. Wnt signalling pathway

The Wnt signalling pathways are highly conserved and are functioning from worms to animals,[1, 2] and play important roles in development, physiology and pathophysiology. Wnt ligands are secreted, soluble proteins. After secretion, they bind to their respective receptors located on the plasma membrane of target cells and activate the intracellular signalling cascades. Nineteen different Wnt genes and ten different Wnt receptor (Frizzled) genes are expressed in mammals.[3] Wnt signalling pathways are divided into the canonical, β-catenin-dependent pathway and the noncanonical, β-catenin-independent pathway. This project focuses on the canonical Wnt pathway. The non-canonical Wnt pathways include the planar cell polarity pathway and Wnt/Ca^{2+} pathway.

2. Canonical Wnt pathway

In the absence of Wnt ligand proteins, the β-catenin in the cytosol is quickly phosphorylated by the GSK-3β complex, leading to the degradation of β-catenin. When canonical Wnt ligands (such as Wnt3a) are present, their binding to the receptor (Frizzled) and co-receptor (low-density lipoprotein Receptor-related protein 5/6, LRP5/6) leads to the translocation of Axin, a key component of the GSK-3β complex, from the cytosol to the plasma member, which inhibits GSK-3β complex (Figure 1). The subsequent accumulation of β-catenin in the cytosol allows its translocation to the nucleus where, together with other factors such as TCF, it regulates the transcription of Wnt target genes. The Wnt/β-catenin pathway is critical for embryonic heart

development, but it has a low activity in healthy adult heart. However, recently studies have demonstrated that the Wnt/β-catenin pathway is activated in many heart disease in both patients and animal models, such as cardiac hypertrophy, myocardial infarction and heart failure.[4-6] _ENREF_17 For example, mouse hearts after myocardial infarction (MI) are found to have elevated levels of both Wnt proteins (such as Wnt2 and Wnt4) and Wnt receptors.[7] In myocardial tissues of patients with ischemic heart disease, β-catenin was found to be frequently localized in the nuclei (indicating Wnt pathway activation).[6]

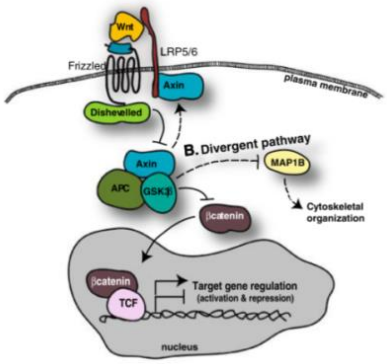

Figure 1. Canonical Wnt/β-catenin signalling pathway

In the Wnt/β-catenin pathway, the binding of a Wnt ligand protein (such as Wnt3a) to its receptor and co-receptor on membrane causes the recruitment of Axin, a key component of the β-catenin degradation complex, to the plasma membrane, which inhibits the phosphorylation and degradation of β-catenin. Following the accumulation of β-catenin in cytosol, the translocation of β-catenin into nucleus regulates target gene expressions. (Reproduced with permission from: Kimberly A mulligan, Benjamin N. R. Cheyette. Wnt Signalling in Vertebrate Neural Development and Function. *Journal of Neuroimmune Pharmacology*, 2012)[8]

3. Sodium current in ventricular myocytes

Ion channels are proteins that allow the flux of ions across the cell membrane generating ionic currents critical for the rhythmic heart contractions.[9] In ventricular myocytes, Na^+ enters cells through voltage-gated sodium (Na^+) channels causing a large Na^+ current that triggers an action potential.[9] The cardiac Na^+ channel protein has two subunits: α subunit that forms the pore and conducts Na^+ ions, and an auxiliary β subunit that interacts with the α subunit and regulates its gating.[10] Loss-of-function mutations in *Scn5a* gene (encoding the $Na_v1.5$ α subunit) lead to reductions in both peak Na^+ current and action potential upstroke in cardiomyocytes,[11] and underlie the lethal ventricular arrhythmias in about 20% of the Brugada syndrome patients. In addition, reduction of Na^+ current is found in myocardial infarction and heart failure and plays a role in arrythmias.[12] Previous data from our lab have shown that Wnt/β-catenin signalling, which is activated in myocardial infarction and heart failure, inhibits Na^+ currents in neonatal rat ventricular myocytes (Fig. 2).[13]

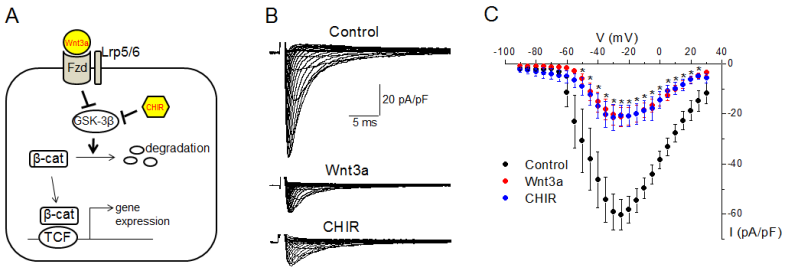

Figure 2. Activation of canonical Wnt/β-catenin pathway by treatment with either Wnt3a or CHIR (panel A), decreases sodium (Na^+) current density (panels B and C) in neonatal rat ventricular myocytes (NRVMs). NRVMs were prepared and treated with Wnt3a or CHIR for 48 h. Na^+ current was recorded with the whole-cell patch-clamp technique. Abbreviations: β-cat, β-catenin; TCF, T cell factor; GSK-3β, glycogen synthase kinase 3β. (reproduced with permission from: Liang W *et al.*, Wnt signalling suppresses voltage-dependent Na channel expression in postnatal rat

cardiomyocytes. *Journal of Physiology,* 2015).[13]

4. T-box 3 (Tbx3)

Tbx3 is a transcription factor of the T-box family. The human *TBX3* gene has 7 exons and is located in chromosome 12. The Tbx3 protein contains a DNA-binding domain (i.e., T-box)[14], two repression domains and a putative activation domain (Fig. 3A). *Tbx3* is expressed in the cardiac conduction system (Fig. 3B), including the sinoatrial node (SAN), atrioventricular node (AVN), and the His-bundle and proximal branches. However, *Tbx3* is not expressed in healthy atrial or ventricular myocytes. In SAN and AVN, *Tbx3* suppresses the expression of atrial/ventricular-specific genes such as *Scn5a* and *Gja1* (encoding Cx43), and *Tbx3* knockout mice showed malformations of SAN and AVN with ectopic expression of atrial/ventricular-specific genes.[15, 16]

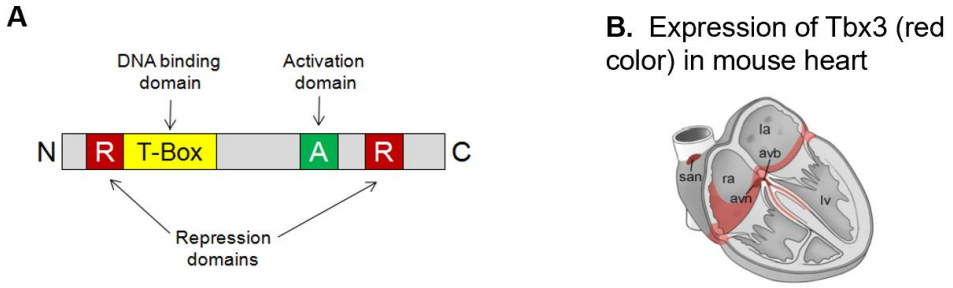

Figure 3. A. Schematic of Tbx3 protein, which contains a DNA binding domain (T-Box, yellow), two repression domains (red), and a putative activation domain (green). Figure was generated based on information from *http://atlasgeneticsoncology.org*

B. Red color indicates expression pattern of *Tbx3* in mouse heart at the late fetal stage. (Reproduced with permission from Boogaard *et al.* Genetic variation in T-box binding element functionally affects *SCN5A/SCN10A* enhancer. *Journal of Clinical Investigation*, 2012)

2. Objective and Hypothesis

1. **Objective:** The objective of this project is to investigate the mechanisms of Wnt-mediated inhibition of Na^+ current in cardiomyocytes.

2. **Hypothesis:** Based on our observations that canonical Wnt signalling upregulates *Tbx3*, a known suppressor of *Scn5a*, in cardiomyocytes, we will test the hypothesis that Wnt signalling inhibits Na^+ currents by reducing *Scn5a*, with *Tbx3* as a mediator.

The studies from this project have been included in the following two manuscripts: The following sections are reproduced from these manuscripts with permission.

1. Lu A, Kamkar M, Chu C, Wang J, Gaudet K, Chen Y, Lin L, Liu W, Marbán E, Liang W. Direct and Indirect Suppression of Scn5a Gene Expression Mediates Cardiac Na^+ Channel Inhibition by Wnt Signalling. *Canadian Journal of Cardiology*, 2020;36(4):564-576

2. Lu, Aizhu and Chu, Cencen and Xia, Ying and Wang, Jerry and Beanlands, Rob SB and Liu, Peter and Davis, Darryl R. and Liang, Wenbin. *Inhibition of β-catenin Upregulates Voltage-gated Na^+ Current in Brugada Syndrome Cardiomyocytes* (March 30, 2021). Available at SSRN: https://ssrn.com/abstract=3815857 or http://dx.doi.org/10.2139/ssrn.3815857

3. Methods

1. Culture of neonatal rat ventricular myocytes (NRVMs)

Primary culture of neonatal rat ventricular myocytes (NRVMs) were prepared as we described,[13] beginning with trypsin (Amersham Biosciences, Piscataway, NJ) and collagenase (type II, Worthington Biochemical, Freehold, NJ) digestion of ventricles from 2-day-old rats (Sprague-Dawley, Harlan, Charles River, Montreal). Isolated NRVMs were resuspended in M-199 medium (ThermoFisher) supplemented with 10% fetal bovine serum (FBS), 19.4 mM glucose, 2 mML-glutamine, 2 unit/mL penicillin, 0.8 μg/mL vitamin B12, 10 mM HEPES, and 1× MEM nonessential amino acids. Cardiac fibroblasts were removed by two 60-min preplating steps. Cells were plated at 200,000 cells/cm^2 in 6- or 12-well plates (BD Biosciences) precoated with 0.1% gelatin (StemCell Technologies). FBS in medium was reduced to 2% at day 2 (2 days after plating). Wnt/β-catenin pathway was activated by adding to the culture medium of recombinant Wnt3a protein (1, 10, 100, or 300 ng/ml, R&D systems) or CHIR-99021 (3-10 μM, Selleck Chemicals) for 48 h.

2. Human induced pluripotent stem cells-derived cardiomyocytes (iPSC-CMs)

Healthy human iPSCs were generated by Dr. J.C Wu's lab with Sendai virus from peripheral blood mononuclear cells donated by a healthy volunteer (female, 41-year-old). Differentiation of iPSCs into cardiomyocytes was performed using a 2-D monolayer protocol in a chemically defined medium[17]. Briefly, iPSCs (passage 15-40) at 80-90% confluency were treated with 6 μM CHIR-99021 (Selleck Chemicals) from day 0 to 2 in CDM3 medium (RPMI 1640

supplemented with 213 µg/mL L-asorbic acid-2-physphate and 500 µg/mL recombinant human albumin). Cells were maintained from day 2 to 4 in CDM3 medium containing 2 µM Wnt-C59 (Selleck Chemicals). At day 4, cells were cultured in control CDM3 medium (without other added factors) and spontaneous beating cells were observed under a microscope at day 7-10. Cells were glucose-starved from day 10 to 14 to purify cardiomyocytes. To minimize well-to-well variations during the differentiation, cells at day 14-20 were lifted with TrypLE (Life Technologies), pooled, and replated into new plates at a density of 250,000 cells/cm2 in CDM3 medium. Cells at day 30-40 of differentiation were used in this study.

3. Real-Time Quantitative PCR

Total RNA was isolated from NRVMs and iPSC-CMs with an RNeasy mini kit (Qiagen) and cDNA was synthesized with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time quantitative PCR was performed with Taqman Gene Expression Assays (Life Technologies) on a 7900HT Fast Real-Time PCR System (Applied Biosystems) or LightCycler96 (Roche). Transcript level of target genes was normalized to that of *Hprt1* mRNA in the same sample, and analyzed with the $2^{-\Delta\Delta C(t)}$ method.

***Table**. Taqman primer/probes used for real-time quantitative RT-PCR.*

(Reproduced with permission from Lu A, *et al.,* Direct and Indirect Suppression of Scn5a Gene Expression Mediates Cardiac Na^+ Channel Inhibition by Wnt Signalling. *Canadian Journal of Cardiology*, 2020)

Gene Symbol	Exons Spanned by Probe	Amplicon Size (bp)	ThermoFisher Assay ID
Axin2	3-4	67	Rn00577441_m1
Hprt1	8-9	64	Rn01527840_m1
Nkx2.5	1-2	67	Rn00586428_m1
Tbx2	2-3	150	Rn01460173_m1
Tbx3	1-2	108	Rn00710902_m1
Tbx5	8-9	66	Rn01481891_m1
Tbx18	7-8	79	Rn01445129_m1
Scn5a	22-23	54	Rn00565502_m1
Shox2	2-3	97	Rn00564672_m1

4. siRNA for *Tbx3* knockdown

Knockdown of *Tbx3* in NRVMs was performed using the Silencer® Select siRNA technique (ThermoFisher) which employs the latest improvements in siRNA design and off-target effect prediction algorithms. NRVMs were cultured in 6-well plates and transfected with either a siRNA that targets exon 2 in rat *Tbx3* mRNA (0.075 nmol/well, ThermoFisher ID: s165547), or a control non-silencing siRNA (0.075 nmol/well, ThermoFisher ID: AM4621), using Lipofectamine RNAiMAX (9 uL/well, ThermoFisher).

Forty eight hours later, NRVMs were treated with 300 ng/ml Wnt3a protein for 48 hours. NRVMs were then collected for qRT-PCR and western blot assays as described below.

5. Western Blotting

NRVMs and iPSC-CMs were homogenized in RIPA buffer containing a protease/phosphatase inhibitor cocktail (Thermo Scientific). Protein concentration was determined by BCA assay[18] and cell lysates (20 μg protein per lane) were run on a 4-12% SDS-polyacrylamide gel and transferred onto a PVDF membrane. The transferred membrane was incubated with a primary antibody overnight at 4°C, followed by a 2-h incubation with a peroxidase-conjugated secondary antibody (1:2000). Primary antibodies used were: rabbit anti-Nav1.5 (1:1000, a gift from Dr Hugues Abriel), and rabbit anti-Tbx3 (ThermoFisher, 1:500). Immunoreactivity was detected by chemiluminescence (ECL Western blotting analysis system, Amersham Biosciences). Equal protein loading of the gels was assessed by re-probing the membrane with rabbit anti-calnexin (Abcam, 1:2000). Band densities in western blot experiments were quantified using the “gel analysis tool” of the ImageJ program (https://imagej.nih.gov).

6. Electrophysiology

Electrophysiology experiments were carried out using standard whole-cell patch-clamp technique[19] with a MultiClamp 700B amplifier (Axon Instruments) at a sampling rate of 20 kHz and low-pass Bessel-filtered at 5 kHz. NRVMs and iPSC-CMs were placed in a perfusion chamber on the stage of an inverted microscope, perfused at room

temperature with bath solutions (see below). Microelectrodes had tip resistances of 2-5 MΩ when filled with internal pipette solutions. Ionic currents were recorded in voltage-clamp mode with series resistance compensated by 70-80%. Voltage-gated sodium currents were recorded with NRVMs bathed in a solution containing (mM): NaCl 20, TEA-Cl 50, CsCl 67, MgCl2 1, CaCl2 1, CdCl2 0.1, glucose 10, and HEPES 10, pH=7.4 with CsOH. Pipette solution consisted of (in mM) NaCl 5, CsF 125, EGTA 10, HEPES 10 and Mg-ATP 5, pH=7.2 with CsOH (E_{Na}= +35.3 mV). Cells were held at -120 mV and I_{Na} was elicited by a family of voltage steps to potentials ranging from -90 to +30 mV with 5 mV increments.

7. Statistical Analysis

Data are expressed as mean ± SEM with $p<0.05$ considered significant. Sample number indicates the number of biological replicates. Differences between two means were evaluated by two-tailed Student's t-test. Differences among multiple means were assessed by one-way analysis of variance (ANOVA). When significance was detected by ANOVA, differences among individual means were evaluated post hoc by Bonferroni's test.

8. Results

1. Wnt/β-catenin signalling reduces *Scn5a* transcript and $Na_v1.5$ protein in NRVMs

To activate Wnt/β-catenin signalling in cardiomyocytes, primary cultures of neonatal rat ventricular myocytes (NRVMs) were exposed to Wnt3a protein or CHIR (CHIR-99021, a cell-permeant small-molecule inhibitor of GSK-3β) for 48 hours. Both Wnt3a and CHIR reduced $Na_v1.5$ protein (Fig. 4A), which is the pore-forming α subunit of the voltage-gated Na^+ channel in cardiomyocytes. mRNA of *Scn5a*, the gene encoding $Na_v1.5$, was also reduced by Wnt3a (0.17±0.03 vs. control 1.03±0.12, n=6, p<0.01, Fig. 4B). These observations suggest that Wnt-induced reductions in Na^+ current, as demonstrated previously by us (Figure 2)[13] is partly, if not completely, secondary to reduced *Scn5a* expression.

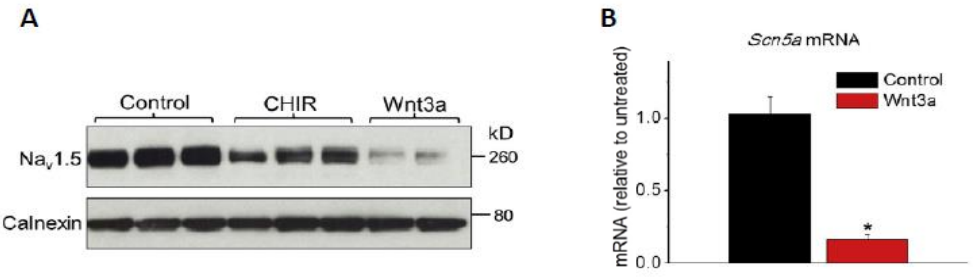

Figure 4. A. Western blot showing reduced $Na_v1.5$ after the treatment of CHIR (3 μM) or Wnt3a (300 ng/mL) for 48 hours in neonatal rat ventricular myocytes (NRVMs). Calnexin was used as a loading control. **B.** Quantitative reverse transcription polymerase chain reaction (qRT-PCR) showing reduced *Scn5a* mRNA in NRVMs after the treatment of Wnt3a, n = 6 samples from 2 independent NRVM isolations, *p< 0.01 vs control. *(Reproduced with permission from* Lu A, *et al.,* Direct and Indirect Suppression of Scn5a Gene Expression Mediates Cardiac Na^+ Channel Inhibition by Wnt Signalling. *Canadian Journal of Cardiology*, 2020)

2. Inhibition of cardiac Na^+ channel by Wnt/β-catenin signalling in NRVMs is associated with upregulation of *Tbx3*

To investigate how Wnt/β-catenin signalling reduces *Scn5a*, we examined if transcription factors known to regulate *Scn5a* are affected by Wnt/β-catenin. Specifically, we examined transcription factors that inhibit *Scn5a* expression (*Tbx2*, *Tbx3*, *Tbx18* and *Shox2*)[16, 20, 21] _ENREF_20 and factors that promote *Scn5a* expression (*Tbx5* and *Nkx2.5*).[22, 23] _ENREF_23. qRT-PCR showed that *Tbx3* transcript was selectively increased by Wnt3a (5.7±0.75 vs. control 0.97±0.14, n=4, p<0.01, Fig. 5A). With respect to biological gradient, Wnt3a provided a dose-dependent increase in *Tbx3* transcript and protein (Fig. 5B, 5C) which mirrored transcript and protein decreases in *Scn5a*/$Na_v1.5$. These data suggest that inhibition of cardiac Na^+ channel by Wnt/β-catenin signalling is associated with upregulation of *Tbx3.*

Figure 5. A. qRT-PCR showing selective upregulation of *Tbx3* mRNA among *Scn5a* regulators in Wnt3a-treated NRVMs, n = 6 samples (for *Tbx2, Tbx5,* and *Nkx2.5*) or 4 samples (for *Tbx3, Tbx18,* and *Shox2*) from 2 NRVM isolations, ⁎P < 0.01 vs control. **B.** qRT-PCR showing dose-dependent, Wnt3a-induced upregulation of *Tbx3* and downregulation of *Scn5a* in NRVMs, ⁎p< 0.05 vs 0 ng/mL Wnt3a, n = 3 samples for each concentration of Wnt3a. **C.** Representative western blot images showing dose-dependent, Wnt3a-induced upregulation of Tbx3 protein and downregulation of $Na_v1.5$ in NRVMs. The detection of Tbx3 in control NRVMs (0 ng/mL Wnt3a) in this experiment

could be due to contamination of atrioventricular node cells (which express Tbx3) in this batch of NRVMs.

(Reproduced with permission from Lu A, *et al.,* Direct and Indirect Suppression of Scn5a Gene Expression Mediates Cardiac Na^+ Channel Inhibition by Wnt Signalling. *Canadian Journal of Cardiology*, 2020)

3. siRNA-mediated *Tbx3* knockdown attenuates Wnt3a-induced reductions in *Scn5a* transcript and $Na_v1.5$ protein in NRVMs

To evaluate the role of *Tbx3* in Wnt-induced *Scn5a* and $Na_v1.5$ reductions, NRVMs were transfected with siRNA targeting *Tbx3* (Tbx3-siRNA) prior to Wnt3a treatment. Tbx3-siRNA abrogated the ability of Wnt3a to stimulate production of *Tbx3* transcript and protein ($p<0.01$, Fig. 6A and 6C). In agreement with a role for *Tbx3*, Wnt3a-induced reductions in *Scn5a* mRNA (Fig. 6B, n=5) and $Na_v1.5$ protein (Fig. 6D, n=4) were notably attenuated ($p<0.05$) by *Tbx3* knockdown. Taken together, our data indicate that Tbx3 upregulation by Wnt3a significantly contributes to $Na_v1.5$ inhibition in cardiomyocytes; however this accounts for only 30% of the overall $Na_v1.5$ reductions within cardiomyocytes suggesting that other mechanisms are also involved.

Figure 6. A. qRT-PCR showing blocking of *Tbx3* increases by siRNA-Tbx3 in Wnt3a-treated NRVMs. n=5 samples from 2 NRVM isolations, *p<0.01. **B.** qRT-PCR showing attenuation of Wnt3a-induced *Scn5a* mRNA reductions by siRNA-Tbx3. n=5 samples from 2 NRVM isolations, *p<0.01. **C.** Representative western blot of NRVMs pretreated with either control-siRNA (left 4 lanes) or Tbx3-siRNA (right 4 lanes); Tbx3-siRNA completely blocked Wnt3a-induced *Tbx3* increases and attenuated $Na_v1.5$ reductions. **D**. Summary of western blot showing attenuated effects of Wnt3a on $Na_v1.5$ in cells pretreated with Tbx3-siRNA. n=4 samples from 2 NRVM isolations, *p<0.05. *(Reproduced with permission from* Lu A, *et al.,* Direct and Indirect Suppression of Scn5a Gene Expression Mediates Cardiac Na^+ Channel Inhibition by Wnt Signalling. *Canadian Journal of Cardiology*, 2020)

4. Wnt/β-catenin signalling reduces both *SCN5A* mRNA and $Na_v1.5$ protein and increases *Tbx3* in iPSC-CMs

Activation of Wnt/β-catenin signalling was induced in healthy iPSC-CMs by treatment with CHIR-99021 (CHIR) for 48 h. CHIR is a cell-permeant small-molecule inhibitor of GSK-3β

and is a commonly used Wnt/β-catenin pathway activator. Consistent with our observations in NRVMs, CHIR led to a 71% reduction in *SCN5A* mRNA (0.29±0.03, n=8, vs. control 1.0±0.05, n=9) in iPSC-CMs (Fig. 7A). Similarly, western blot analyses showed reduced $Na_v1.5$ protein in iPSC-CMs after CHIR treatment (Fig. 7B and 7C). CHIR treatment of iPSC-CMs also led to increases in *TBX3* mRNA (Fig. 8A) and Tbx3 protein (Fig. 8B) as we observed in NRVMs, suggesting that the upregulation of *Tbx3* by Wnt/β-catenin signalling is conserved in both rat and human cardiomyocytes.

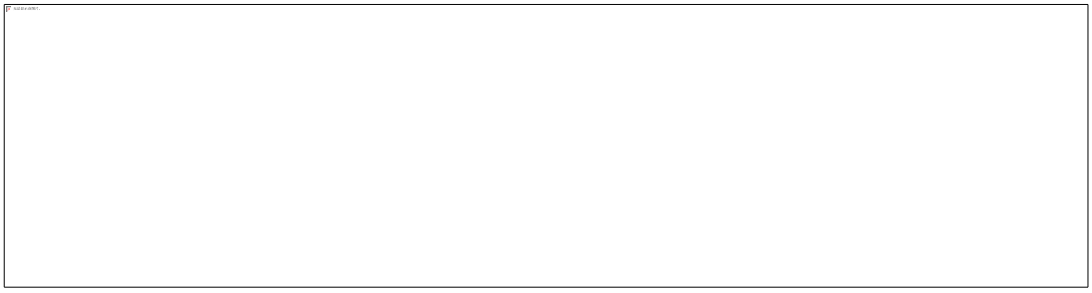

Figure 7. A. qRT-PCR showing reduced *SCN5A* mRNA in iPSC-CMs (Healthy Line 2) after treatment with CHIR (5 μM, n=8 samples from 2 cell differentiations) for 48 h. Control group was treated with equal amount of DMSO (n=9 samples from 2 cell differentiations). Data were analyzed by two-tailed *t*-test. **B**. Representative western blot showing reduced $Na_v1.5$ in iPSC-CMs after treatment with CHIR or DMSO (control) for 48 h. **C**. Quantification of $Na_v1.5$ band densities in panel B (normalized to calnexin), showing reduced $Na_v1.5$ in CHIR group (n=5 samples from 2 cell differentiations) as compared to control, DMSO-treated group (n=6 samples from 2 cell differentiations). Data were analyzed by two-tailed *t*-test.

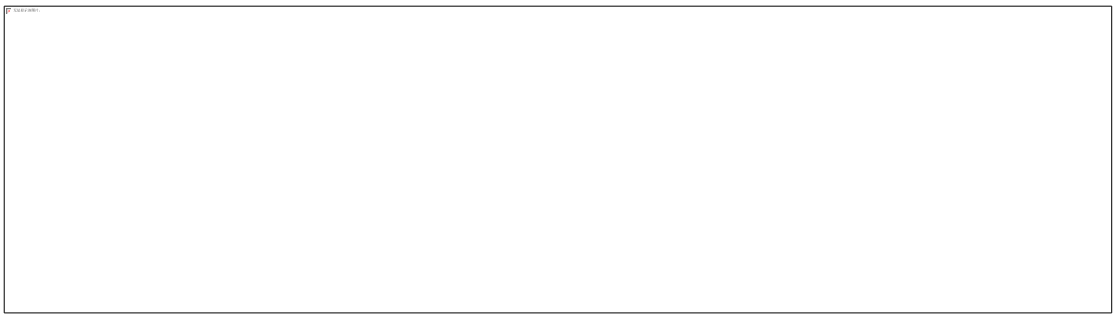

Figure 8. A. qRT-PCR showing CHIR-induced upregulation of *Tbx3* mRNA, an inhibitor of *Scn5a*/$Na_v1.5$, in iPSC-CMs (n=8 samples from 3 bathes of cell differentiation) after CHIR treatment for 48 h. Control groups were treated with equal volume of DMSO. *p<0.05 by two-tailed *t*-test. **B**. Western blot showing increased Tbx3 protein in iPSC-CMs after treatment with CHIR for 48 h.

5. Wnt/β-catenin signalling reduces Na^+ current in iPSC-CMs

To investigate if reduced $Na_v1.5$ protein in CHIR-treated iPSC-CMs is associated with changes in voltage-gated Na^+ current (I_{Na}), whole-cell patch-clamp recording was conducted in healthy iPSC-CMs. I_{Na} was recorded with a bath solution containing 20 mM Na^+ (E_{Na}= +35 mV) and 0.1 mM Cd^{2+} (to inhibit L-type Ca^{2+} current, $I_{Ca,L}$) as we previously used for I_{Na} recording of rat cardiomyocyte.[13] CHIR treatment led to a 69% reduction in peak I_{Na} density (-10.5±2.8 pA/pF at -20 mV, n=9 cells vs. control -33.6±4.1 pA/pF, n=6 cells Fig. 2Ac).

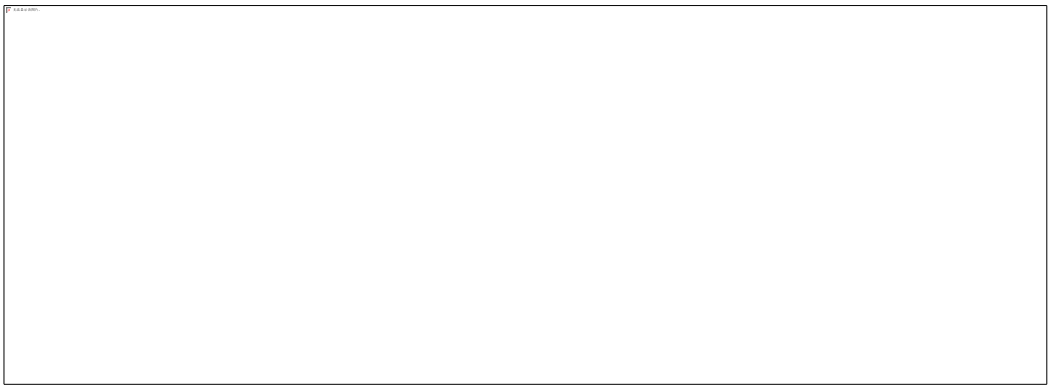

Figure 9. A. Voltage protocol used to elicit I_{Na}. Cells were held at -120 mV and I_{Na} was elicited by 50-ms voltage steps ranging from -90 to +30 mV with 5-mV increments with a cycle length of 600-ms. **B**. Representative I_{Na} recorded from healthy iPSC-CMs cultured in control medium (containing DMSO, top panel) or in medium containing 5 μM CHIR (bottom panel) for 48h. Dashed lines indicate zero current. **C**. Current-voltage relationship of I_{Na} in DMSO-treated (control, n=6 cells from two bathes of cells, black dots) and CHIR-treated (n=9 cells from two batches of cells, blue dots) iPSC-CMs. *p<0.05 vs. control by two-tailed *t*-test.

6. Discussion

The large I_{Na} amplitude in healthy cardiomyocytes is responsible for the rapid depolarization of the cell (action potential upstroke), causing the fast conduction of action potentials within the myocardium which is critical for the rhythmic beating of a healthy heart. In various heart diseases, such as myocardial infarction and heart failure, the myocardium undergoes "electrical remodeling" in response to the injury. As part of the electrical remodeling, I_{Na} is reduced in cardiomyocytes which slows down the action potential conduction in the ventricular myocardium providing the substrate for lethal arrhythmias.[12, 24, 25] However, the mechanisms for I_{Na} reduction in heart diseases are not fully clear.

The Wnt/β-catenin pathway has a low activity in healthy myocardium, but recently studies showed that this pathway has elevated activity in heart diseases, such as myocardial infarction and heart failure,[4-7] which are associated with I_{Na} reductions._ENREF_17 We and others have recently demonstrated that Wnt/β-catenin signalling inhibits I_{Na} in rat and mouse cardiomyocytes,[13, 26] suggesting that Wnt signalling may be a contributing factor to the I_{Na} reductions in heart diseases. In the present study, we further demonstrated that Wnt/β-catenin signalling also similarly inhibits I_{Na} in human iPSC-derived cardiomyocytes, suggesting that this is a conserved mechanism and may be potentially relevant to human heart diseases. iPSC-derived cardiomyocytes provided a convenient system to study human heart biology and diseases in cell culture. However, two major limitations of iPSCs-derived cardiomyocytes need to be acknowledged.[27, 28]_ENREF_48 First, after cardiac differentiation of iPSCs, the culture contains atrial, ventricular and nodal cardiomyocytes. However, studies showed that the inhibition of *SCN5A* and I_{Na} by Wnt signalling is conserved in human, rats[13] and mice.[26, 29] In

addition, studies in neonatal rat ventricular myocytes (NRVMs) and mouse atrial cells,[13, 26, 29] are consistent with findings in human iPSC-derived cardiomyocytes of the present study, suggesting that the I_{Na} inhibition by Wnt signalling is conserved in atrial and ventricular myocytes. Secondly, iPSC-derived cardiomyocytes have been found to have an immature phenotype.[28, 30] Therefore, our findings cannot be directly translated to adult human cardiomyocytes. Future studies using mature iPSC-CMs, such as engineered heart tissues_ENREF_57,[31] are warranted to test if our findings can be faithfully recapitulated in a more adult-like context. Nevertheless, we have previously demonstrated that $Na_v1.5$ protein is reduced by Wnt/β-catenin signalling in both neonatal and adult rat ventricular cardiomyocytes,[13, 32]_ENREF_26 suggesting that this effect is not specific for a certain developmental stage of the cardiomyocytes.

The present study suggests that that reduced *Scn5a* transcript is likely the primary reason for reduced I_{Na} and $Na_v1.5$ in both human and rat cardiomyocytes after Wnt signalling activation. Other studies from our lab demonstrated that the reduced *Scn5a* transcript is caused, at least partly, by the direct suppression of *Scn5a* transcription by Wnt/β-catenin signalling via the binding of TCF4 (the effector of Wnt pathway) to the *Scn5a* promoter region.[32] Furthermore, the present study found that Wnt/β-catenin signalling increased Tbx3 mRNA and protein in both human and rat cardiomyocytes, suggesting that this is a conserved phenomenon. Tbx3 is a transcriptional transcription factor that is expressed in the sinoatrial node (SAN) and atrioventricular node (AVN) in the adult heart where it suppresses the expression of atrial/ventricular-specific genes.[33, 34] Previous studies have shown that Tbx3 suppresses *Scn5a* expression.[21, 34] Our Tbx3 knockdown studies demonstrated that the Wnt-

induced Tbx3 upregulation also contributes to *Scn5a*/$Na_v1.5$ reductions in neonatal rat ventricular myocytes. Future studies are needed to investigate if other mechanisms, such as post-translational modifications of $Na_v1.5$ protein, are also involved in Wnt-induced I_{Na} reduction.

7. Future Directions

This project demonstrated the inhibition of Na^+ current and $Na_v1.5$ by Wnt signalling in cultured rat and human cardiomyocytes. It will be important to investigate the involvement of Wnt inhibition of Na^+ current in heart diseases and its potential as a therapeutic target. Recent data from other projects in our lab have suggested that the inhibition of Wnt/β-catenin pathway, via shRNA-mediated β-catenin knockdown, was able to rescue the reduce Na^+ current in iPSC-derived cardiomyocytes of patients with Brugada Syndrome (an inherited heart disease with reduced Na^+ current). Another project from our lab demonstrated that Wnt pathway genes are upregulated in the mouse ventricular tissues after myocardial infarction (MI), and the downregulation of *Scn5a* transcript after MI was attenuated in mouse with cardiac knockout of β-catenin. Future studies are warranted to investigate if small molecule inhibitors of the Wnt/β-catenin pathway are able to rescue I_{Na} in heart disease.

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