Identification of an \( I_{\text{Na}} \)-dependent and \( I_{\text{to}} \)-mediated proarrhythmic mechanism in cardiomyocytes derived from pluripotent stem cells of a Brugada syndrome patient

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Brugada syndrome (BrS) is an inherited cardiac arrhythmia commonly associated with SCN5A mutations, yet its ionic mechanisms remain unclear due to a lack of cellular models. Here, we used human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) from a BrS patient (BrS1) to evaluate the roles of \( Na^+ \) currents (\( I_{\text{Na}} \)) and transient outward \( K^+ \) currents (\( I_{\text{to}} \)) in BrS induced action potential (AP) changes. To understand the role of these current changes in repolarization we employed dynamic clamp to “electronically express” \( I_{\text{K1}} \) and restore normal resting membrane potentials and allow normal recovery of the inactivating currents, \( I_{\text{Na}} \), \( I_{\text{Ca}} \) and \( I_{\text{to}} \). HiPSC-CMs were generated from BrS1 with a compound SCN5A mutation (\( p.A226V \) & \( p.R1629X \)) and a healthy sibling control (CON1). Genome edited hiPSC-CMs (BrS2) with a milder \( p.T1620M \) mutation and a commercial control (CON2) were also studied. CON1, CON2 and BrS2, had unaltered peak \( I_{\text{Na}} \) amplitudes, and normal APs whereas BrS1, with over 75% loss of \( I_{\text{Na}} \), displayed a loss-of-\( I_{\text{Na}} \) basal AP morphology (at 1.0 Hz) manifested by a reduced maximum upstroke velocity (by ~80%, \( p < 0.001 \)) and AP amplitude (\( p < 0.001 \)), and an increased phase-1 repolarization pro-arrhythmic AP morphology (at 0.1 Hz) in ~25% of cells characterized by marked APD shortening (~65% shortening, \( p < 0.001 \)). Moreover, \( I_{\text{to}} \) densities of BrS1 and CON1 were comparable and increased from 1.0 Hz to 0.1 Hz by ~100%. These data indicate that a repolarization deficit could be a mechanism underlying BrS.

The Brugada syndrome (BrS) is a rare cardiac rhythm disorder associated with an increased risk of malignant ventricular arrhythmias\(^1,2\). The signature ‘coved-type’ ST-segment elevation in the right precordial leads (V1–V3) of the electrocardiogram (ECG) may occur spontaneously or be induced by a provocative drug test with sodium channel blocking drugs\(^1,2\). Fetal arrhythmic events in BrS often occur during sleep/rest, a condition associated with slow heart rates\(^1\).

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SCN5A encodes the pore-forming subunit (Nav1.5) of the cardiac sodium channels. SCN5A variants account for ~80% of BrS, with known genetic mutations, and there are over 300 genetic variants in SCN5A associated with BrS. Several studies have established loss of sodium channel function associated with these SCN5A variants. However, the low penetrance and variable expressivity of SCN5A reported for BrS raise questions about the quantitative role of the Na\(^+\) current in the ionic mechanism of BrS.

A repolarization disorder hypothesis of BrS has been made based on studies using perfused ventricular wedges and isolated cardiac myocytes of animals. Yet the role of the decreased Nav1.5 current (I\(_{\text{Na}}\)) remains to be defined. The genetically engineered haploinsufficient (SCN5A\(^+/−\)) and SCN5A\(^{1798\text{insD}/+}\) mouse models, and the SCN5A\(^{E558X/+}\) pig model, have shown mainly conduction defects without the loss-of-dome like increased phase-1 repolarization change in the action potential (AP). Moreover, from the two latest reports in which BrS patient-specific human induced pluripotent stem cells (hiPSC) derived cardiomyocytes (hiPSC-CMs) were adopted, no loss-of-dome-like AP change was observed.

In the current study, we selected hiPSC-CMs generated from a BrS patient with a severe reduction in I\(_{\text{Na}}\). Next, we converted the hiPSC-CMs to more mature, native ventricular myocyte-like cells by in silico injection of a synthetic inward rectifier K\(^+\) current (I\(_{\text{K1}}\)) and challenged the cells with slow pacing frequencies. We then identified an I\(_{\text{Na}}\) deficiency-dependent loss-of-I\(_{\text{Na}}\) basal AP pattern of BrS at normal heart rates and an increased phase-1 repolarization proarrhythmic AP pattern at a low heart rate. We further obtained evidence that associated the heart rate-induced AP changes with transient outward K\(^+\) current (I\(_{\text{to}}\)). We therefore conclude that a loss-of-I\(_{\text{Na}}\) and an elevated I\(_{\text{to}}\) could together make the ventricular BrS cardiomyocytes undergo proarrhythmic changes.

Results

Impact of SCN5A mutations of BrS1 on SCN5A/Na\(_{1.5}\) expression and I\(_{\text{Na}}\). A BrS patient with p.A226V and p.R1629X mutations of SCN5A was selected in this study (Fig. 1). First, Tsa201 cells heterologously expressing WT-, A226V- and R1629X-SCN5A were prepared. Immunofluorescence (Fig. 2Aa) and Western blotting (Fig. 2Ab) assays showed that, compared with WT control, A226V-SCN5A cells expressed comparable levels of full-length Na\(_{1.5}\) whereas R1629X-SCN5A cells showed a much lower level and smaller size of a truncated Na\(_{1.5}\). Cells co-expressing A226V-SCN5A and R1629X-SCN5A showed both the full length and truncated Na\(_{1.5}\). Next, I\(_{\text{Na}}\) was recorded in tsa201 cells heterologously expressing WT-, A226V- and R1629X-SCN5A. The average I\(_{\text{Na}}\) density (pA/pF) in A226V-SCN5A cells was ~50% of WT-SCN5A (p < 0.01) and it was almost undetectable in R1629X-SCN5A cells (Table S1, Fig. 2B). The steady state (SS)-inactivation (Fig. 2C) and -activation (Fig. 2D) of I\(_{\text{Na}}\) in A226V-SCN5A cells were largely unaffected (Table S1).
Next, a ~50% reduction of the SCN5A mRNA was noted in hiPSC-CMs generated from the BrS patient (BrS1) compared with the sibling control (CON1) indicating a possible nonsense-mediated decay (Fig. 3A). The I\textsubscript{Na} density measured in BrS1 was ~25% of CON1 (Fig. 3B,C). Relative to the CON1, BrS1 showed marginal/moderate changes in the rate of SS-inactivation and activation, and a more significant change in the rate of recovery from inactivation (Table S1, Fig. 3D–F).

Identification of a loss-of-I\textsubscript{Na} basal AP pattern with BrS1. HiPSC-CMs are known for their lack of I\textsubscript{K1}, which is responsible for their depolarized resting membrane potential (RMP) of ~−60 mV compared with the ~−90 mV of quiescent adult ventricular and atrial myocytes\textsuperscript{15,16}. The more positive resting membrane potential in hiPSC-CMs importantly results in inactivation of the fast I\textsubscript{Na}, leaving very limited I\textsubscript{Na} available during the phase-0 depolarization and thereby potentially masking the impact of I\textsubscript{Na} deficiency on the AP. The depolarized state of hiPSC-CMs could also lead to I\textsubscript{Na} inactivation and the absence of phase-1 repolarization in hiPSC-CMs\textsuperscript{16}.

Indeed, APs recorded from spontaneously contracting CON1 and BrS1, or BrS1 paced at 1.5, 1.0, 0.5, 0.2 Hz, were comparable without displaying a phase-1 repolarization morphology (Table S2, Figure S3).

To overcome this limitation, a synthetic, cardiomyocyte membrane potential-dependent in silico I\textsubscript{K1} was injected into hiPSC-CMs using the dynamic clamp technique\textsuperscript{15} to produce a physiologically polarized resting membrane potential (~−84 mV). Thereafter, ventricular-like hiPSC-CMs (I\textsubscript{K1}\textsuperscript{positive}) from CON1 (Fig. 4A) paced at a 1.0 Hz displayed a phase-1 repolarization morphology that resembles normal human ventricular myocytes.

In contrast, an AP rounded pattern, clearly distinguishable from the phase-1 repolarization pattern of CON1, was observed in ventricular-like BrS1 (Fig. 4B). Such APs were characterized by over 75% reduction in the maximum upstroke velocity (dV/dt\textsubscript{Max}) and reduced AP amplitude (APA) and overshoot (Table S3, Fig. 4C). This AP pattern, referred as a “loss-of-I\textsubscript{Na}” AP morphology, reflects a marked I\textsubscript{Na} deficiency in BrS1 during depolarization.

Identification of a heart rate-induced increased phase-1 repolarization proarrhythmic AP change in BrS1. In BrS patients, sleep and rest are the most common triggers of ST-segment elevation, ventricular arrhythmia, and sudden death\textsuperscript{1,3}. To test the effect of heart rate on the pathophysiology of BrS, APs were recorded from CON1 and BrS1 paced at 1.0 Hz, 0.5 Hz and 0.1 Hz. Responding to reduced pacing frequencies from 1.0 Hz to 0.1 Hz, CON1 showed slight changes in APD (±27% change in APD90 and the maximal shortening per cell was <33%) (Table S4, Fig. 5A, Table S5). In contrast, diverse changes in APD were observed with BrS1 paced at 0.1 Hz. The majority (75%) of BrS1 (the non-early repolarization subgroup) demonstrated a marked prolongation of APD90 or moderate shortening (<28% per cell) like CON1 (Fig. 5B1). However, a fraction (25%) of

Figure 2. Expression of SCN5A/Nav1.5 and I\textsubscript{Na} density documented in tsa201 heterologous expression system. tsa201 cells transfected with equal amount of WT-SCN5A, A226V-SCN5A, R1629X-SCN5A and A226V-SCN5A + R1629X-SCN5A (50% each) were assayed for SCN5A/Nav1.5 expression and I\textsubscript{Na} density. (Aa) Representative immunofluorescence staining images of Na\textsubscript{1.5} (purple) in tsa201 cells. Arrows indicate the Na\textsubscript{1.5} staining. Levels of Na\textsubscript{1.5} were semi-quantified and plotted in a bar-graph (n = 3). (Ab) Representative Western blotting image of Na\textsubscript{1.5} in tsa201 transfected cells (n = 3). (B) Peak I\textsubscript{Na} density measured in transfected tsa201 cells. (C) and (D) Steady state (SS)-inactivation and activation curves of I\textsubscript{Na} measured in tsa201 expressing WT- and A226V-SCN5A. *p < 0.05; vs. WT-SCN5A.
Figure 3. SCN5A and Nav1.5 expressions and \(I_{Na}\) measured in hiPSC-CMs. (A) Level of SCN5A mRNA in BrS1 (hiPSC-CMs from a BrS patient with compound SCN5A mutations) and CON1 (the sib of BrS1) determined by qPCR. \(n=3\). (B) Representative traces of sodium currents in the control (CON1) and patient (BrS1). (C) Peak \(I_{Na}\) density measured in BrS1 and CON1. (D–F) Steady state (SS)-inactivation, activation and recovery from inactivation curves of \(I_{Na}\). Values given are mean ± SEM. † \(p<0.01\); vs. CON1.

Figure 4. A basal action potential pattern of BrS identified in BrS1. (A and B) Representative AP waveforms recorded from a CON1 and a BrS1 paced at 1.0 Hz. The solid and dashed arrows indicate APA measured at phase-0 and phase-2, respectively. (C) The main AP parameters of CON1 and BrS1 are plotted and compared in bar-graphs. Values given are mean ± SEM. †\(p<0.00001\); vs. CON1 (by unpaired \(t\)-test).
BrS1 (the early repolarization subgroup) displayed a drastic shortening of APDs (APD90 reduced by 66% in average and ≥ 50% per cell) accompanied by decreased dV/dtMax and APA, and a more depolarized resting membrane potential (Table S4, Fig. 5B2, Table S5). The marked reduction in APD, particularly the phase-2 APD indicated by an over 50% reduction in the ratio of APD20/APD90, represented an increased phase-1 repolarization AP pattern highly resembles the proarrhythmic loss-of-dome AP change recorded from canine right ventricle epicardium model under the repolarization disorder hypothesis.

On the other hand, CON2 and BrS2 (at both 24 °C and 34 °C) paced at 1.0 and 0.1 Hz displayed APDs within the normal range, similar to CON1. In agreement with that observed in tsa201 cells,17 we observed an increased dV/dtMax and APA/overshoot in hiPSC-CMs at 34 °C compared with that 24 °C, indicating an increased Ina at

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**Figure 5.** Effects of low heart rates on APs of BrS1 and CON1 hiPSC-CMs. (A, B1 and B2) (Top): representative superimposed AP waveforms recorded from CON1 and two BrS1 subgroups (non-early repolarization and early repolarization), sequentially paced at 1.0 Hz, 0.5 Hz and 0.1 Hz. (A, B1 and B2) (Bottom): superimposed AP waveforms continually recorded from CON1 and two BrS1 subgroups sequentially paced at 1.0 Hz and 0.1 Hz. (C) Main AP parameters plotted and compared among groups in bar-graphs. Values given are mean ± SEM. Statistics were performed with two-way repeated measures ANOVA followed by the Bonferroni post hoc testing. *p < 0.05; †p < 0.01; ‡p < 0.001. Black symbols: Intra-(sub) group comparisons. 0.1 Hz/0.5 Hz vs. 1.0 Hz. Pink symbols: vs. BrS1 (non-ER subgroup) at the same frequency. Green symbols: vs. CON1 subgroup at 1.0, 0.5 and 0.1 Hz.
34 °C (Fig. 6). Normal levels of \( I_{Na} \) were observed with BrS2 and \( dV/dt_{Max} \) at 34 °C and 24 °C were all ≥ 190, which is greater than the 110~185 level of controls (CON1 and CON2) and significantly larger than the 41.9 ± 5.3 levels observed in BrS1.

Evaluating the role of \( I_{to} \) in mediating the heart rate-dependent AP changes. In cardiac myocytes, \( Ca^{2+} \)-independent \( I_{to} \) consists of a fast recovery component (\( I_{to,f} \)) mediated mainly by Kv4.3 subunits and a slow recovery component (\( I_{to,s} \)), mediated by Kv1.4 subunits. \( I_{to} \) contributes to the phase-1 repolarization of the AP. Slow heart rates favor the recovery of \( I_{to} \)8,19, whereas increased \( I_{to} \) levels could accentuate the phase-1 notch of the AP20–22.

To explore the potential involvement of \( I_{to} \) in heart rate-induced AP changes, the level of \( I_{to} \) in CON1 and BrS1 paced at 1.0 Hz and 0.1 Hz were measured and compared (Fig. 7). \( I_{to} \) densities of both CON1 and BrS1 paced at 1.0 Hz were comparable (~5 pA/pF at 40 mV), which is ~50% of that in isolated human sub-epicardial ventricular myocytes (10.6 ± 1.08 pA/pF at 40 mV)18. A ~100% increase (p < 0.00001) of \( I_{to} \) densities was recorded in both CON1 and BrS1 when the heart rate was reduced to 0.1 Hz. Moreover, variations in the levels of \( I_{to} \) among different hiPSC-CMs, particularly of those paced at 0.1 Hz, were identified in a fraction of cells (19~23%) which showed much higher \( I_{to} \) densities. Our data suggest potential heterogenic expression of \( I_{to} \) among hiPSC-CMs and \( I_{to,s} \) could be the dominant component of \( I_{to} \).

To further validate the role of \( I_{to} \) in heart rate-induced AP changes, BrS1 that displayed an increased phase-1 repolarization AP change at 0.1 Hz were treated with 4-Aminopyridine (4-AP), which is known to block both \( I_{to,f} \) and \( I_{to,s} \)23,24 at higher concentrations (0.5 mM to 10 mM could effectively block 55−85% of \( I_{to} \))22. As 4-AP at 1.0 mM and above could also block the rapid and slow components of the delayed rectifier \( K^{+} \) currents (\( I_{Ks} \) and \( I_{Kr} \)) by over 25% and 39%, respectively25, we applied 0.05 mM and 0.5 mM 4-AP to BrS1 and noted that 0.05 mM 4-AP caused moderate APD prolongations, while 0.5 mM 4-AP completely reversed the increased phase-1 repolarization (Table S6, Fig. 8A). The significant increase in the ratio of APD20/APD90 suggested that 4-AP treatment recovered the loss of phase-2 repolarization.

Blocking \( I_{to} \) induced increased phase-1 repolarization-like change in BrS1. Ajmaline and flecaïnide are commonly employed in the clinical setting as challenges to attempt to unmask the characteristic BrS ECG phenotype and its associated arrhythmic events7. We observed that BrS1 paced at 1.0 Hz responded to flecaïnide (10 μM) with a significant shortening in APDs (with an average of 43.6% reduction in APD90).
accompanied by reductions in dV/dtMax from 31.4 ± 7.7 to 12.6 ± 2.7 V/s (p < 0.01) and APA/overshoot, confirming an INa inhibition (Table S7 and Fig. 8B).

Computer simulation of the effects of INa and ICa,L on APDs. Adopting the O’Hara-Rudy model (2013) of human epicardial ventricular myocytes, computer simulation was performed to examine the effects of INa and ICa,L on APDs. While APD shortening was not seen in the presence of lower INa (INa reduced to 15%), it was observed with INa reduced to 15% (no effect with 20%) and ICa,L increased to 5 folds (Figure S5A). On the other hand, the shortened APD was not observed with reduced ICa,L in the presence of reduced INa (Figure S5B).

Discussion
In cardiomyocytes derived from a BrS patient (BrS1) with a severe SCN5A haploinsufficiency, and an over 75% reduction in INa, we identified a highly distinguishable loss-of-INa AP pattern at normal pacing frequency (1.0 Hz) and an increased phase-1 repolarization AP pattern characterized by marked reduction in APDs and more depolarized resting membrane potential at a slow pacing frequency (0.1 Hz). Observation of these changes in repolarization required use of dynamic clamp to “electronically” express ICa,L and allow recovery from inactivation of the major inactivating currents INa, ICa, and Ica,L. In contrast, cardiomyocytes without a marked INa reduction (BrS2) and two normal controls (CON1 and CON2) failed to show heart rate-induced AP abnormalities. Importantly, similar low levels INa were observed in four separate hiPSC lines from the same patient (Table S1), and one line was selected for our experiments. We further correlated the reduced heart rate (from 1.0 Hz to 0.1 Hz) with a ~100% increase in ICa,L in the presence of reduced INa (Figure S5B).

Thus, the results from the current study suggest repolarization abnormalities. The repolarization hypothesis of BrS implies that the loss-of-dome change is limited to epicardial myocytes and corresponds to a transmural dispersion of repolarization causing an ST-segment elevation in the ECG. The fact that cardiac myocytes from

Figure 7. Effects of heart rates on INa in hiPSC-CMs. (A-left and B-left) Representative traces of INa currents recorded from CON1 (n = 26) and BrS (n = 22) paced at 1.0 Hz and 0.1 Hz. (A-middle and B-middle) I-V relationships of INa show the current densities. (A-right and B-right) Graphic demonstrations and comparisons of INa (recorded at +50 mV) in each cell are presented. Values given are mean ± SEM. §p < 0.0001; ‡p < 0.0001; vs. 1.0 Hz (by paired t-test and one-way ANOVA test).
a BrS patient predisposed with a severe $I_{Na}$ deficiency (such as BrS1) could develop arrhythmic changes at slow heart rates, and also associated with an elevated $I_{to}$ supports the repolarization disorder hypothesis that a low $I_{Na}$ in conjunction with a high $I_{to}$ could result in an increased phase-1 repolarization $^{7,8}$.

Interestingly, the heart rate-sensitive and $I_{to}$-mediated basal and pro-arrhythmogenic AP phenotypes identified in BrS hiPSC-CMs might mirror a switch between the daytime/active/asymptomatic and sleep/bradycardia/disease onset states seen in many BrS patients. Even with a severe $I_{Na}$ deficiency, APs of BrS1 remain largely ‘normal’ at normal heart rates which keep patients in ‘event-free’ clinical status. A precipitating factor is needed, such as sodium channel blockade $^{7}$, to trigger or unmask arrhythmic events. $I_{to}$, coordinated by $I_{Na}$ and $I_{Ca,L}$, plays a key role in shaping the AP of ventricular epicardial myocytes. $I_{to,s}$ and $I_{to,f}$ coexist in ventricular myocytes and are distinguishable by the either tens or thousands of milliseconds taken for recovery from inactivation $^{23,24}$. The levels of $Kv1.4$ and $Kv4.3$ genes in human right ventricular myocytes have been confirmed, and the level of $Kv1.4$ is $\sim 37.5\%$ that of $Kv4.3$ and the $Kv4.3$-dependent $KChIP2$ is $>7$-fold that of $Kv4.3$ $^{27}$. $I_{to,s}$ plays a role in ventricular myocytes and slow heart rates could facilitate its recovery from inactivation. An accentuated phase-1 notch in canine epicardial ventricular myocytes paced at 0.125 Hz $^{19}$ and 0.5 Hz $^{20}$, and in ferret ventricle myocytes paced at 0.2 Hz $^{23}$, was noted. Moreover, canine ventricular myocytes paced at 0.125 Hz were more vulnerable to an ischemia-induced loss-of-dome AP change $^{28}$. Furthermore, it has been shown that hiPSC-CMs responded to 1.0 Hz, 0.5 Hz and 0.1 Hz with 20%, 40% and 100% recovery of $I_{Na}$, respectively; and hiPSC-CMs express a relatively high level of $Kv1.4$ ($\sim 80\%$ of $Kv4.3$) and a much lower level of $KChIP2$ compared $Kv4.3$ $^{36}$. Our data from hiPSC-CMs are consistent with that of Cordeiro et al. $^{28}$, and indicate that $I_{to,s}$ could be a main component of $I_{to}$ in hiPSC-CMs and play a dominant role at 0.1 Hz when there is more time to recover from inactivation. Our data

**Figure 8.** Effects of 4-AP and flecainide on APs. (A) Representative superimposed AP waveforms of a BrS1 cell paced at 0.1 Hz and subsequently exposed to 0.05 mM and 0.5 mM 4-AP. Main AP parameters (showing only the results of 0.5 mM 4-AP) are presented in bar-graphs. (B) Representative superimposed AP waveforms of a V-like BrS1 hiPSC-CM paced at 1.0 Hz followed by flecainide (10 µM) treatment. Main AP parameters are presented in bar-graphs. Values given are mean ± SEM. * $p < 0.05$; † $p < 0.01$; ‡ $p < 0.001$; †† $p < 0.0001$ (by paired $t$-test); vs. 0.1 Hz (for 4-AP) or 0.1 Hz (for flecainide).
Further suggest that, like that among different subtypes of human ventricular myocytes such as those from the right ventricle pericardium and endocardium, \( I_{\text{Na}} \) could be heterogeneously expressed among hiPSC-CMs and a small fraction of cells that express a distinguishable higher level of \( I_{\text{Na}} \) could behave like the right ventricle epicardium myocytes prone to the 'loss-of-dome' AP change (Fig. 7). Treatment with 4-AP also reversed the increased phase-1 repolarization which we attribute to block of \( I_{\text{Na}} \) channels (rather than potentiation of \( I_{\text{Ca,L}} \) which was reported in heterologous experiments\(^{39} \)).

Our experimental findings from hiPSC-CMs are supported by results from a computer simulation study using the O’Hara-Rudy non-diseased human ventricular epicardial myocyte model (2011)\(^{26} \). When \( I_{\text{Na}} \) was reduced to 10–30% of the control level, a subsequent 1.7–2.4-fold \( I_{\text{Na}} \) increments was associated with 'loss-of-dome' AP change\(^{39} \). Ca\(^{2+} \) handling was not investigated in our study, but has been reported to have a role in arrhythmogenesis in an hiPSC-CM model of Brugada\(^{13} \).

Although BrS patient-derived hiPSC-CMs represent the most advanced model that reflects the basal cellular phenotype associated with \( SCN5A \) defects and \( I_{\text{Na}} \) deficiency, further dissecting the mechanism behind the BrS is met with limitations. Beside a weaker than normal inward rectifier current (\( I_{\text{K1}} \)) even when \( I_{\text{Na}} \) is increased using dynamic clamp, these cells have lower expression of slowly reactivating \( I_{\text{Na}} \). Thus, hiPSC-CMs do not fully recapitulate the epicardial action potential phenotype, and the spike and dome configuration of the AP is not obvious.

Despite our novel and compelling findings, our study has limitations. First, our data may not fully explain the mechanisms behind many BrS cases in which the \( SCN5A \) defects are associated with only a milder/moderate \( I_{\text{Na}} \) deficiency. Indeed, our data from BrS2 demonstrated that the heterologous T1620M variant\(^{13} \) shows a minimal, if any, impact on \( I_{\text{Na}} \) in hiPSC-CMs recorded at both 24°C and 34°C, reflected by the normal dV/dt\(_{\text{Max}} \) and APA/dV/dt\(_{\text{Max}} \) overshoot. A recent report further supports this notion by showing normal APs from the hiPSC-CMs of BrS patients without any genetic mutations\(^{22} \). The hiPSC-CMs (\( I_{\text{Na}}, I_{\text{K1}} \)–dominant, demonstrated in this study and a previous report\(^{28} \)) compared with that in human epicardial ventricular myocytes (\( I_{\text{Na}}, I_{\text{K1}} \)–dominant\(^{12,24} \)) may compromise the value of this model. This may also explain the lack of consistency between the cellular phenotypes and the clinical findings of the patients. Second, we acknowledge that we did not correct these mutations with gene editing in the hiPSC-CMs to rescue the disease phenotype. However, we believe that using a healthy sibling and a standard cell line are appropriate controls in this study, as done previously by our lab and others\(^{31–35} \). Finally, although hiPSC-CMs were studied a longer than 30 days after differentiation, prolonged culture times can have effects on \( I_{\text{Na}} \) properties\(^{30} \). These effects should be taken into account when interpreting the data and further studies are needed to access the potential impact on hiPSC-CMs as an electrophysiological model.

Conclusions
Using hiPSC-derived cardiomyocytes from a BrS patient with \( SCN5A \) mutations, we demonstrate that a severe \( I_{\text{Na}} \) deficiency could lead to a remodeled baseline APs vulnerable to heart rate-induced, \( I_{\text{Na}} \)-sensitive proarrhythmic \textit{increased phase-1 repolarization} changes. Our data support a coordinated role of \( I_{\text{Na}} \) and \( I_{\text{K1}} \) in the mechanism of BrS.

Materials and Methods
For a full description of the materials and methods, see Supplemental Material.

\textbf{hiPSC-CMs from BrS patients and controls.} To obtain a BrS hiPSC-CM model with marked \( I_{\text{Na}} \) deficiency, a BrS patient (II-2) with compound heterozygous mutation in \( SCN5A \) (a missense mutation: c.677 C>T, p.A226V, and a nonsense mutation: c.4885 C>T, p.R1629X)\(^{14} \), and a sibling control, were included in this study (Figure S1). All experimental protocols were approved by the institutional review board (Institutional Review Board of SingHealth, Singapore) and all methods were performed following the relevant guidelines and regulations. Data and clinical biological samples were taken following written informed consent. hiPSCs were generated from the dermal fibroblasts of the BrS patient and sibling control (Fig. 1). All hiPSC lines were expanded as adherent cultures in feeder-free conditions on Matrigel-coated dishes in the presence of chemically defined medium (E8 Essential Medium, Life Technologies). Differentiation of hiPSC to cardiomyocytes (CMs) was performed following a previously reported protocol based on small molecules-mediated canonical Wnt pathway modulation\(^{37} \). Four hiPSC clones, which showed good self-renewal potential and cardiac differentiation efficiency, were selected. Eventually, single CON1 and BrS1 clone, evidenced by yielding >55% cardiac troponin T (cTnT) positive hiPSC-CMs quantified by fluorescence-activated cell sorting (FACS) analysis (Figure S6), were adopted. hiPSC-CMs were further stained with anti-\(\alpha\)-actinin, anti-\(\beta\)-MHC and anti-cardiac titin antibodies (Fig. 1D). The BrS1 and CON1 hiPSC-CMs were dissociated into single cells 35–45 days after cardiac differentiation and used for electrophysiology assays.

We also used cardiomyocytes (CON2), a commonly used control hiPSC line (iCell\(^{8} \) Cardiomyocytes, from a female Caucasian with the age unknown), and a genome edited BrS hiPSC-CM line (BrS2) which carries the p. T1620M. SCN5A mutation known to have a milder impact on \( I_{\text{Na}} \) in heterologous expression systems\(^{8,17} \). Both lines were commercially obtained from Cell Dynamic International (Madison, WI, USA).

\textbf{Whole-cell patch-clamp assays.} An Axopatch 200B Amplifier controlled by pClamp10 software together with a Digidata 1440 acquisition system (Molecular Devices, Sunnyvale, USA) was used to record ionic currents and APs.

\( I_{\text{Na}} \) in tsa201 cells and hiPSC-CMs\(^{32} \), \( I_{\text{Na}} \)\(^{12,24,28} \) and the ultra-rapid delayed rectifier K\(^{+} \) current (\( I_{\text{K1}} \))\(^{38,39} \) in hiPSC-CMs were measured using standard voltage-clamp protocols.

Dynamic clamp was performed using a Cybercyte System from Cytocybernetics (Buffalo, USA)\(^{15} \). With an injection of a synthetic \( I_{\text{K1}} \) current, the ventricular-like hiPSC-CMs (\( I_{\text{K1}} \)\(^{\text{positive}} \)) were defined as having AP
amplitude (APA) over 100 mV, APD at 90% repolarization (APD90) over 200 ms and lack of \( I_{K_{Na}} \), which is highly expressed in the atrial-like human embryonic stem cell-derived cardiomyocytes and hiPSC-CMs (Figure S2).

**Statistical analyses.** Numerical data are presented as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM). Comparisons were made with unpaired and paired (two-tailed) Student’s t-test, one-way repeated measures ANOVA followed by the Tukey’s post hoc testing, and two-way repeated measures ANOVA followed by the Bonferroni post hoc testing using GraphPad Prism 5.0 (GraphPad Software, La Jolla, USA). A p-value of <0.05 was considered statistically significant.

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Conceived and designed the experiments: H.W., R.L., S.A.C. and R.L.R. Performed the experiments: D.M., Z.L., L.J.L., Y.Z., G.L., O.I., J.W., Y.Y.C. and B.K.P. Analyzed the data: H.W., D.M., Z.L., Y.Z. and R.L.R. Contributed reagents/materials/analysis tools: R.L., W.S.T., C.K.C., B.Y.T., D.C., K.L.H., P.L., R.Y.Y.Y., R.L.R., A.D.K. and G.C.L.B. Contributed to the writing of the manuscript: H.W., S.A.C., J.W., C.R.B., A.O.V. and R.L.R.

Additional Information
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Competing Interests: Drs Bett and Rasmusson are co-founders and owners of Cytopcybernetics Inc. which builds and sells the Cybercyte dynamic clamp system used in this study. Cytopcybernetics produced Figure S4 on Flecanide block of the action potential. Other authors have no competing financial and non-financial interests as defined by Nature Research, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

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