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Hypertrophic cardiomyopathy-linked mutation in Troponin T causes myofibrillar disarray and pro-arrhythmic action potential changes in human iPSC cardiomyocytes

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Abstract

Background—Mutations in cardiac troponin T (TnT) are linked to increased risk of ventricular arrhythmia and sudden death despite causing little to no cardiac hypertrophy. Studies in mice suggest that the hypertrophic cardiomyopathy (HCM)-associated TnT-I79N mutation increases myofilament Ca sensitivity and is arrhythmogenic, but whether findings from mice translate to human cardiomyocyte electrophysiology is not known.

Objectives—To study the effects of the TnT-I79N mutation in human cardiomyocytes

Methods—Using CRISPR/Cas9, the TnT-I79N mutation was introduced into human induced pluripotent stem cells (hiPSCs). We then used the matrigel mattress method to generate single rod-shaped cardiomyocytes (CMs) and studied contractility, Ca handling and electrophysiology.

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Results—Compared to isogenic control hiPSC-CMs, TnT-I79N hiPSC-CMs exhibited sarcomere disorganization, increased systolic function and impaired relaxation. The Ca-dependence of contractility was leftward shifted in mutation containing cardiomyocytes, demonstrating increased myofilament Ca sensitivity. In voltage-clamped hiPSC-CMs, TnT-I79N reduced intracellular Ca transients by enhancing cytosolic Ca buffering. These changes in Ca handling resulted in beat-to-beat instability and triangulation of the cardiac action potential, which are predictors of arrhythmia risk. The myofilament Ca sensitizer EMD57033 produced similar action potential triangulation in control hiPSC-CMs.

Conclusions—The TnT-I79N hiPSC-CM model not only reproduces key cellular features of TnT-linked HCM such as myofilament disarray, hypercontractility and diastolic dysfunction, but also suggests that this TnT mutation causes pro-arrhythmic changes of the human ventricular action potential.

Graphical abstract

Summary of the generation and characterization of the hiPSC model, which describes how the HCM-linked TnT-I79N mutation can increase arrhythmia risk in humans.

Keywords

human induced pluripotent stem cells; hypertrophic cardiomyopathy; troponin T; ventricular AP; arrhythmia; calcium

1. Introduction

Familial hypertrophic cardiomyopathy (HCM) is commonly caused by mutations in genes encoding sarcomeric proteins and is the most common cause of sudden cardiac death in the young [1–3]. Prominent clinical features of HCM include unexplained left ventricular hypertrophy and cardiomyocyte disarray [4]. Among causal HCM genes, mutations in cardiac troponin T (TnT) have been linked to a high incidence of sudden cardiac death at a relatively young ages (<45), often with little to no cardiac hypertrophy on autopsy [5, 6]. The mechanisms underlying the particularly high incidence of cardiac arrhythmia burden in the setting of TnT mutations remains enigmatic, although a high degree of myocyte disarray has been suggested [7]. TnT is a regulatory protein that is required for the formation of the troponin complex and Ca-dependent regulation of actomyosin [8]. In vitro studies demonstrate that HCM-linked TnT mutations almost universally increase myofilament Ca sensitivity [9]. Studies in mice suggest that the myofilament Ca sensitization caused by malignant TnT mutations such as TnT-I79N generates susceptibility to ventricular arrhythmias [10], but myocyte disarray, a prominent finding in humans, is absent in murine

hearts [11]. Based on murine studies, several underlying arrhythmia mechanisms have been proposed [12], but none of these hypotheses have been tested in human cardiomyocytes (CM), which have very different electrophysiological properties compared to murine CM. For example, the duration of the human action potential (AP) is 10 times longer than that of the murine AP. Furthermore, unlike the mouse AP, the human AP has a high phase 2 plateau at positive membrane potentials and rapid phase 3 repolarization, mostly due to differences in repolarizing K^+ currents [13].

The technologic breakthrough of human induced pluripotent stem cells (hiPSCs) has allowed the modeling of genetic diseases in human CMs, including HCM [14]. hiPSC-CMs robustly express myofilament proteins, and have intracellular Ca handling and cytosolic Ca buffering parameters comparable to those of adult primary ventricular CMs [15]. Recent improvements in culture methods enables the generation of single rod-shape CM that can be used for contractile measurements and electrophysiological studies [16]. Here, we used CRISPR/Cas9 approaches to generate hiPSCs that are heterozygous for TnT-I79N, a mutation associated with increased risk of ventricular arrhythmias and sudden death in humans. We then compared the contractility, Ca handling and electrophysiology to isogenic control hiPSC-CMs that lack the I79N mutation. We find that TnT-I79N hiPSC-CMs not only reproduce key features of TnT-linked HCM such as myofibrillar disarray, but also exhibit increased cytosolic Ca binding that results in Ca-dependent triangulation of the AP. Our results support that pro-arrhythmic AP changes are caused by an HCM-linked TnT mutation in human CMs.

2. Materials and Methods

Data averaged from multiple days were used for analysis. All protocols were approved by Vanderbilt University Medical Center Institutional Review Board. All experiments were done at room temperature, except the 2D hiPSC monolayer impedance assay (37 degree C). Expanded methods are provided in the data supplement.

2.1 In vitro cardiac induction

HiPSC-derived cardiomyocytes (hiPSC-CMs) were generated using the small molecules CHIR 99021 (Selleck Chemicals) and IWR-1 (Sigma) [2, 3]. At day 30 post-cardiac induction, hiPSC-CMs were dissociated and stored in liquid nitrogen. For single-cell studies, hiPSC-CMs were thawed and plated at low density on flexible matrigel mattress in culture medium for 5 days prior to experimentation [2]. This so called “matrigel mattress” method generates single rod-shaped hiPSC-CMs with aligned myofilaments and sarcomere length similar to that of acutely-isolated adult rabbit CMs [16].

2.2 Expression of mutant TnT-I79N protein

The fraction of mutant TnT-I79N protein expressed in hiPSC-CMs was quantified using nano-liquid chromatography mass spectrometry (nLC-MS). Briefly, 1×10^6 of the cryopreserved day 30 hiPSC-CMs were lysed with 1X SDS sample buffer (New England BioLabs Inc., #B7703S). 5–10 μ L protein samples were loaded onto a 15% SDS-PAGE gel. Digestion was performed with a ProteoExtract All-in-One Trypsin Digestion Kit (Catalog

#650212-1KIT, EMD Millipore, Billerica, MA) to get the dried tryptic peptides. The fraction of mutant TnT-I79N protein expressed in hiPSC-CM was quantified using nLC-MS.

2.3 Immunocytochemistry

Immunostaining of CMs was carried out as previously described [16]. Briefly, CMs were fixed in 2% paraformaldehyde for 5 min at room temperature, then permeabilized with 0.2% Triton X-100 (Sigma) for 10 min. Samples were blocked in PBS solution with 1% BSA and incubated for 1 hour at room temperature. Primary antibody α -actinin (Sigma) and cardiac troponin T (Invitrogen) were added in PBS solution with 1% BSA (1:100) and incubated overnight at 4°C. Samples were washed in PBS. Secondary antibodies specific to the primary IgG isotype were diluted (1:1000) in the same solution as the primary antibodies and incubated in dark at room temperature for 1 hour. Samples were washed in PBS and mounted. Sarcomeric structures were examined with Olympus 1X81 microscope coupled to Slidebook software. For each image, the extent of α -actinin disorganization were scored in blinded fashion by two independent observers on a scale from 0 to 2: 0, no α -actinin disorganization; 1, mild α -actinin disorganization (affecting <50% of sarcomere); 2, severe α -actinin disorganization (affecting $\geq$ 50% of sarcomere). The sarcomere length for each cell was measured as an average distance of α -actinin staining. For morphometric analysis, images were imported into Image J and analyzed using standard plugins.

2.4 Cell shortening and Ca transient measurements

HiPSC-CMs were loaded with Fura-2 acetoxymethyl ester, Fura-2 AM (Molecular Probes Inc., Eugene, OR) as described [15, 16]. Ca transient and cell shortening recorded during 0.2 Hz field stimulation in Tyrode's solution containing 2 mmol/L $[Ca]_o$ for 20s. Then stimulation was switched off followed by application of 10 mmol/L caffeine to record caffeine transient. To examine $[Ca]_o$ -dependence of contractility, cell shortening recorded in Tyrode's solution with different $[Ca]_o$ (0.2, 0.5, 1, 2 and 3 mmol/L) under 0.2 Hz field stimulation.

2.5 Cytosolic Ca buffering measurement

Cytosolic Ca-buffering was measured in voltage-clamped cells using the "Trafford" method [15, 17]. Briefly, the experiments were performed in voltage-clamped hiPSC-CMs at room temperature. HiPSC-CMs were loaded with 25 μ mol/L Fluo-4 salt through pipette solution to measure cytosolic $[Ca]_{free}$. 10 mmol/L caffeine was rapidly applied to release Ca from sarcoplasmic reticulum (SR). The Ca released from the SR is extruded via Na/Ca exchanger (NCX), generating inward NCX current. Integration of NCX current yielded the total amount of Ca released from the SR. Cytosolic buffering curves were generated by plotting $[Ca]_{total}$ as a function of $[Ca]_{free}$. Maximal cytosolic buffering capacity (B_{max}) and the apparent dissociation constant (K_d) were estimated for each myocyte using a modified Michaelis-Menten equation: $[Ca]_{total} = C + (B_{max} * ([Ca]_{free} - x_{off}) / (K_d + ([Ca]_{free} - x_{off})))$. Only curves that were fit well were used for final analysis.

2.6 Action potential measurement

To avoid the potential effects of exogenous Ca buffering, APs were recorded using pipette solutions without Ca chelating agents such as EGTA unless otherwise indicated. Cells were superfused with Tyrode solution. The glass pipette had access resistance of 4–6 MΩ after filling with the internal pipette solution containing (in mmol/L) 110 KCl, 5 NaCl, 5 Mg-ATP, 10 HEPES, 5 phosphocreatine, pH adjusted to 7.2 with KOH. APs were recorded using Axopatch 200B, Digidata 1550A and pClamp 10.5 software (Axon Instruments, Foster City, CA, USA) for data amplification and acquisition. A 2-ms current pulse at 20% above threshold was provided to evoke APs at a cycle length of 2 s (0.5 Hz). Electrophysiological data were analyzed using Clampfit 10.5 and were prepared by Prism. EMD57033 and blebbistatin were kept in DMSO as 3 mmol/L stock solution, and the final concentration of DMSO in experiment solutions was adjusted to 0.1%. CMs were pretreated with 3 μmol/L EMD57033 or blebbistatin for 1 min and then APs measured using pipette solution without EGTA. To measure the APs under excess cytosolic Ca buffering, 14 mmol/L EGTA was added into pipette solution. The AP triangulation index was defined as the APD(90–30)/APD90 ratio. The beat-to-beat instability index of APs was obtained from 14 consecutive APs per cell by calculating the ratio of interquartile range to median APD50 [18].

3. Results

3.1 Generation of TnT-I79N hiPSC-CMs

To study the effect of the I79N mutation in human cardiomyocytes, we used the CRISPR/Cas9 technique to introduce a heterozygous I79N mutation into the *TNNI2* gene of a hiPSC line derived from a healthy donor. This approach is advantageous when access to patient samples is limited while simultaneously providing a control hiPSC line that is isogenic to the I79N line. The control hiPSC line was generated from a healthy male donor with normal karyotype (Online Figure 1). The hiPSC line expressed the pluripotency markers Oct4, SSEA4, SSEA3, and Tra-1–60, and can differentiate into cells of all three germ layers (Online Figure 2). Sequencing identified a hiPSC colony with a heterozygous I79N mutation (Figure 1A). The I79N hiPSC line did not have genetic changes at the predicted off-target sites by Sanger sequencing of PCR amplicons. We next generated myocytes using a standard small molecule method [19] and quantified the expression of I79N mutant protein in hiPSC-CMs using mass spectrometry. Of the total TnT protein, 43% was mutant TnT-I79N (Online Figure 3). These results indicate that the mutant TnT-I79N protein was expressed at similar levels as wild-type TnT and therefore likely incorporated into the myofilaments of cardiomyocytes.

3.2 TnT-I79N causes sarcomere disorganization without cellular hypertrophy

Severe myocyte disarray with little to no hypertrophy is a typical finding in patients with HCM-linked TnT mutations [5, 6]. Hence, we examined cell size and sarcomere structure of I79N hiPSC-CMs. Sarcomeres were labeled with antibodies against TnT and α-actinin, and average sarcomere length and the degree of sarcomere organization scored in blinded fashion (Figure 1B). Compared to the organized sarcomere structure of control hiPSC-CMs, I79N hiPSC-CMs exhibited a significantly higher degree of sarcomere disorganization (Figure 1C) and decreased average sarcomere length (Figure 1D). There were no significant

differences in cell length, cell width, and cell volume between control and mutation containing CMs (Online Figure 4). These results indicate that the I79N mutation causes significant sarcomere disorganization in the absence of cellular hypertrophy.

3.3 TnT-I79N increases systolic contractility and impairs relaxation

We measured the contractility of single cells using video edge detection [16]. Average diastolic cell length was not significantly different between the two groups (I79N 66.25 ± 4.82 vs. control 73.05 ± 3.50 μm , $n=16$ per group, $p=0.25$). In response to the pacing stimulus, the percentage of cell shortening was significantly increased in I79N CMs (Figure 2B). Time to peak shortening was not altered (I79N 0.76 ± 0.05 vs. control 0.80 ± 0.07 s, $N=16$, $p=0.67$). In contrast, time to 90% relaxation was significantly prolonged in I79N (Figure 2C). We next evaluated the $[\text{Ca}]_o$ -dependence of cell shortening. At low $[\text{Ca}]_o$ (0.2 mmol/L), cell shortening was comparable between I79N and control CMs. At higher $[\text{Ca}]_o$ of 0.5, 1, 2, and 3 mmol/L, cell shortening was significantly increased in I79N (Figure 2D). Cell relaxation was also impaired at higher $[\text{Ca}]_o$ (Figure 2E).

Consistent with the single cell measurements, field-stimulated 2D monolayers of I79N hiPSC-CM also exhibited hypercontractility, impaired relaxation and a leftward shift of the contractile response to Ca (Online Figure 5). Taken together, these results indicate that TnT-I79N sensitized myofilaments to Ca with increased contractility and impaired relaxation, especially at higher $[\text{Ca}]_o$. These findings are consistent with previous reports that the I79N mutation increases myofilament Ca sensitivity in mouse skinned cardiac muscle fibers [10].

3.4 TnT-I79N increases cytosolic Ca buffering

Troponin C (TnC) accounts for a substantial proportion of Ca binding in the cytosol and contributes close to 50% of fast cytosolic buffering during the cardiac cycle [20]. Since TnT interacts with TnC, mutations in TnT may alter Ca binding to troponin C and hence cytosolic Ca buffering. Our previous studies demonstrated that hiPSC-CMs exhibit cytosolic Ca buffering properties comparable to acutely-isolated adult rabbit CMs [15]. Cytosolic Ca buffering was measured in voltage clamped hiPSC-CMs (Figure 3) using the Trafford protocol to assess rapid cytosolic Ca binding in response to changes in free cytosolic Ca ($[\text{Ca}]_{\text{free}}$) that occurs during a cardiomyocyte contraction [15, 17]. Ca was released from the SR by applying caffeine and the change in total amount of cytosolic Ca ($[\text{Ca}]_{\text{total}}$) calculated by integration of the NCX current (Figure 3A&B). Cytosolic buffering curves were generated by plotting $[\text{Ca}]_{\text{total}}$ as a function of $[\text{Ca}]_{\text{free}}$ (Figure 3C) and fit using the Michaelis-Menten equation to estimate K_d and B_{max} for each cell. Maximal binding capacity B_{max} , an estimate of total cytoplasmic Ca binding sites, was not significantly different between the two groups (Figure 3D). On the other hand, the apparent dissociation constant, K_d , was significantly decreased (Figure 3E), indicating a higher affinity for Ca in I79N CMs. Importantly, the SR Ca content – estimated by the NCX integral – was not significantly different between the groups (Figure 3F). Taken together, these data indicate that the Ca-sensitizing I79N mutation alters cytosolic Ca buffering properties by increasing cytosolic Ca binding affinity.

3.5 TnT-I79N mutation reduces action potential-triggered Ca transients

We next investigated the effect of I79N-induced Ca buffering changes on cytosolic Ca handling in intact hiPSC-CMs. Cells were loaded with the Ca indicator Fura-2AM and APs were triggered by field stimulation at 0.2 Hz. The I79N mutation significantly reduced Ca transient amplitude (Figure 4A–B). Diastolic Ca was comparable between two groups (F_{ratio} I79N 1.20 ± 0.03 vs. control 1.20 ± 0.02). Time to peak (I79N 0.59 ± 0.07 vs. control 0.52 ± 0.05 s) and 90% decay time (I79N 2.28 ± 0.21 vs. control 2.32 ± 0.16 s) were also not different between groups. We next applied 10 mmol/L caffeine to trigger SR Ca release. As in voltage-clamped cells, the peak of caffeine-induced Ca transient was significantly smaller in I79N CMs than control (Figure 4C), even though the total amount of Ca released from the SR (measured by NCX integral, Figure 3D), was not different. Taken together, these results indicate that the increased cytosolic buffering caused by the TnT-I79N mutation significantly reduced the AP triggered Ca transient in intact hiPSC-CMs.

3.6 Myofilament Ca sensitization by TnT-I79N alters the shape of the human AP

We next tested the hypothesis that altered cytosolic Ca buffering in I79N CMs alters AP morphology. APs were recorded in current clamp mode without any exogenous Ca buffers added to the intracellular solutions. There was no difference in resting membrane potential, upstroke velocity and peak amplitude between the two groups (Online Table 2). The I79N mutation significantly accelerated AP repolarization and shortened early repolarization (APD30, APD50 and APD70; Figure 5D). Interestingly, late repolarization (APD90) was not different between the two groups (Figure 5D). As a result, APs from I79N CMs were significantly more triangulated than APs recorded from control CMs. Moreover, I79N APs exhibited significantly increased beat-to-beat instability of APD90 compared to control CMs (Figure 5B–C). To test whether acutely increasing myofilament Ca sensitivity reproduces the observed effects of I79N on AP in control CMs, we treated control cells with the Ca sensitizer EMD57033. Analogous to the effect of the I79N mutation, 3 $\mu\text{mol/L}$ EMD57033 also shortened early repolarization in control CMs, caused AP triangulation, and produced beat-to-beat instability (Figure 5B–C, E).

If myofilament Ca sensitization is responsible for the AP triangulation, normalizing myofilament Ca sensitivity should rescue AP triangulation in I79N CMs. Blebbistatin is a myosin ATPase inhibitor with minimal effects on membrane ion channel function that reduces myofilament Ca sensitivity in skinned TnT-I79N fibers to that of wild-type fibers [10]. Pretreatment with 3 $\mu\text{mol/L}$ blebbistatin completely abolished the differences in AP repolarization (Figure 6A–B). Further, one would expect a similar rescue of AP triangulation in the presence of excess intracellular buffering given the proposed mechanism of I79N resulting in enhanced cytosolic Ca buffering. The addition of 14 mmol/L EGTA to the pipette solution completely reversed AP triangulation (Figure 6C–D).

What is the mechanism responsible for the AP triangulation? In ventricular CMs, Ca extrusion via the Na-Ca exchanger (NCX) generates an inward current that contributes to phase 3 of the AP. The smaller Ca transients of I79N CMs (Figure 4) likely generates less NCX currents and may explain the shortening of early repolarization observed in TnT-I79N CMs (Figure 5). To test this hypothesis, extracellular Na^+ was rapidly replaced with Li^+ ,

which allows influx of Li^+ through Na channels and hence a normal AP upstroke, but blocks Ca extrusion via NCX. Li^+ eliminated the differences in APD30, APD50 and APD70 in I79N and control CMs (Figure 7A-B). In the presence of Li^+ , APs were shortened in both groups (albeit to a greater extent in control CM), suggesting that the NCX inward currents contribute significantly to AP repolarization in hiPSC-CMs. Taken together; these results indicate that the I79N mutation causes AP triangulation via increased cytosolic Ca buffering which lowers systolic Ca transients, and consequently, reduces NCX currents.

4. Discussion

Here we report the first hiPSC model of TnT-linked HCM to study the cellular mechanisms leading to increased susceptibility of sudden death. TnT mutations account for approximately 15% of genotype-positive HCM cases. This subset of HCM cases are characterized by relatively mild or subclinical hypertrophy with a high incidence of sudden death [21]. In contrast to the five previously published hiPSC-HCM models [1, 22–26], we used gene-editing via CRISPR/Cas9-induced double-strand breaks to generate mutant hiPSC-CMs. This approach has the major advantage of providing isogenic control hiPSC-CMs for comparison rather than having to use population control hiPSC-CMs to control for influences of genetic background and epigenetic memory. We found that mutant TnT-I79N protein is robustly expressed (43% of total TnT protein) and caused myofibrillar disarray without myocyte hypertrophy (Figure 1). This result is consistent with clinical findings of extensive myocyte disarray in absence of marked hypertrophy in patients carrying TnT mutations [6]. We also examined how TnT-I79N alters human cardiomyocyte contractility, Ca handling, and electrophysiology. We found that I79N caused enhanced contractility and impaired relaxation because this mutation sensitized myofilaments to Ca (Figure 2). The resulting increase in cytosolic Ca buffering altered myocyte Ca handling (Figures 3–4) and shortened early repolarization via a Ca-dependent, NCX-mediated mechanism (Figures 5–7) resulting in AP triangulation. AP triangulation is an established predictor of increased arrhythmia risk [18]. Taken together, our results not only provide a human cellular model that produced the cellular equivalents of the major clinical features of TnT-linked HCM, but also suggests a new mechanism of arrhythmogenesis. The pathogenic TnT mutation caused pro-arrhythmic AP changes in human CMs.

4.1 Pathogenesis of TnT-I79N-linked HCM

Two general hypotheses exist on the pathogenesis of TnT mutations in HCM: (1) mutant TnT protein incorporates into myofilament to exert a dominant-negative effect on the cardiac structure and/or function; (2) mutant protein acts as a null allele leading to haploinsufficiency [27]. We found that I79N and WT TnT protein were expressed at similar levels in hiPSC-CMs. Although we did not have antibodies to distinguish mutant from WT TnT to directly demonstrate incorporation of I79N in the sarcomere, our protein expression data coupled with the functional data of myofilament Ca sensitization indicate that I79N is functionally incorporated into the thin filament which resulted in myofibrillar disarray (Figure 1). Since this was not observed in murine TnT-I79N models [11], this result will enable future studies of the underlying mechanisms responsible for myofibrillar disarray caused by I79N.

4.2 TnT-I79N increased contractility and impaired relaxation

Increased systolic function and impaired diastolic function are hallmarks of human HCM and are consistently phenocopied in HCM mouse models [2, 3, 28, 29]. Our new TnT-I79N hiPSC model robustly exhibits this phenotype with enhanced contractility and slow relaxation observed in both single CMs (Figure 2) and 2D monolayers (Online Figure 6). Importantly, myocardial contractility was increased despite the sarcomere disorganization and shortened sarcomere length (Figure 1), suggesting that the hypercontractility is a direct consequence of the I79N mutation. Our data supports a mechanism of increased myofilament Ca sensitivity in the presence of TnT-I79N, consistent with previously reported results in skinned mouse fibers [10]. The Ca sensitizing effect of TnT-I79N was concluded based upon a leftward shift in the contractility relationship between extracellular [Ca] and cell shortening (Figure 2). The same mechanism is likely also responsible for the impaired relaxation observed in the TnT-I79N hiPSC-CMs. Our results further support the utility of hiPSC-CMs as an experimental model for mechanistic studies of HCM and drug screening.

4.3 The TnT-I79N mutation increased myofilament Ca sensitivity and cytosolic Ca buffering

Myofilament activation and force development is largely dependent on intracellular [Ca] [12]. The degree of myofilament Ca sensitivity is dynamically regulated by the Ca binding affinity of TnC, thin filament regulation of actin-myosin interaction and actin-myosin cross-bridge cycling [30]. Because TnC binds the majority of Ca in cytosol, the increased Ca binding affinity of TnC would be predicted to increase myofilament Ca sensitivity [31]. Previous studies demonstrated that HCM-linked TnT mutants almost universally increase myofilament Ca sensitivity of force development in skinned fiber, often associated with slow Ca dissociation from TnC on the thin filament and increase the cross-bridge detachment rate [8, 10, 32]. Consistent with these skinned fiber studies, we observed that the I79N mutation reduced the apparent dissociation constant (= increasing cytosolic binding affinity, K_d) without changing the total number of Ca binding sites in the cytosol (=maximal binding capacity, B_{max}). Since Ca binding to the troponin complex accounts for the majority of fast cytosolic Ca buffering during the cardiac cycle [20], the most likely explanation is that myofilament Ca sensitization by TnT-I79N increases cytosolic Ca buffering properties through increasing myofilament Ca binding.

Based on the increased Ca binding to TnT-I79N containing myofilaments (Figure 3), one would predict reduced peak intracellular [Ca] during systole, which was confirmed by our experimental results (Figure 4). During relaxation, the extra Ca bound to Ca sensitized myofilament could be expected produce a slower decay of Ca. This was observed in mouse CMs expressing human TnT-I79N [2]. However, here we failed to observe prolonged Ca transients in human CMs expressing TnT-I79N (Figure 4). A possible explanation is that humans have a much slower heart rate compared to mice, and the slow off rate of Ca caused by the TnT-I79N mutation is not rate limiting for Ca removal from the cytosol in a human CM.

4.4 Myofilament Ca sensitization causes pro-arrhythmic AP triangulation via suppression of NCX activity

Based on work in mice, we have previously speculated that myofilament Ca sensitization by TnT-I79N increases myofilament Ca binding and reduces $[Ca]_i$, resulting in shortening of the AP plateau phase [3]. Here, we report this phenomenon also occurs in human CMs (Figure 5). The AP remodeling of TnT-I79N hiPSC-CMs was prevented by myofilament Ca de-sensitization with blebbistatin and reproduced in control CMs treated with the Ca-sensitizer EMD57033 (Figures 5 – 6), providing strong support that changes in myofilament Ca sensitivity are responsible for the AP remodeling in human CMs.

How can increased myofilament Ca sensitivity lead to the AP remodeling? The most likely explanation is increased myofilament buffering reduces free $[Ca]$ during systole and therefore, NCX activity. NCX is primarily active during the plateau phase generating an inward current by exchanging a single Ca ion for three sodium ions. The reduced Ca diminishes the activity of the NCX, resulting in less inward current during phase 2 and 3 of the AP. Reduced NCX activity will shorten the plateau phase of AP, but not necessarily affect the terminal AP duration [33, 34]. This is what we observed in TnT-I79N CMs: APD30, APD50 and APD70 were reduced, but APD90 was not significantly different. Consistent with our hypothesis that Ca-dependent NCX is responsible, the AP differences were eliminated by the replacement of Na^+ with Li^+ ; which cannot be transported by the NCX and hence block NCX contributions to phase 2 and 3 of the AP (Figure 7). In addition, intracellular Ca buffering with EGTA abolished the AP differences in TnT-I79N CMs, further confirming the role of intracellular Ca as the primary driver of the AP remodeling in TnT-I79N CMs. Together, these results suggested that $[Ca]_i$ -dependent NCX current modulation of the AP plateau phase is conserved among human, mouse, and rat CMs [3, 35].

The AP remodeling of TnT-I79N CMs resulted in a more triangular shape (triangulation). Triangulation of the AP is considered a strong predictor of arrhythmia risk in a number of studies [18, 36, 37]. Consistent with our results from hiPSC-CMs, AP triangulation after exposure to myofilament Ca sensitizers has also been observed in feline and rabbit hearts [10], which are species that have an AP morphology similar to humans. Triangulation is considered pro-arrhythmic for a number of reasons: First, it can result in Na or Ca channel reactivation to generate early afterdepolarizations [38, 39]. Second, during the faster early repolarization more Na channels will start to recover from inactivation, but the recovery is incomplete and results in slow conduction and risk for reentrant arrhythmias [40]. Finally, the slow repolarization promotes dispersion of APD at the tissue level, an established risk factor for ventricular arrhythmia in humans [41].

5. Conclusion

Here, we established the first hiPSC-CM model of HCM due to a TnT mutation and compared it to isogenic control cells. We found that TnT-I79N hiPSC-CMs not only reproduce key cellular features consistent with clinical HCM such as myofilament disarray and diastolic dysfunction, but also exhibit altered cytosolic Ca buffering that results in Ca-dependent triangulation of AP. Our results also suggest a possible cellular mechanism of

increased risk of sudden death in patients with TnT mutations is the pro-arrhythmic AP changes in human CMs described in this manuscript. As many genotype positive but phenotype patients have no signs of dysfunction on imaging studies negative (ie., no cardiac hypertrophy), CRISPR/Cas9 generated hiPSC-CM models, such as ours, could present an opportunity for identifying patients at risk for sudden death or developing therapies to delay the progression of diastolic dysfunction in patients with known TnT mutations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

hiPSCs	Human-induced pluripotent stem cells
CM	Cardiomyocyte
HCM	Hypertrophic cardiomyopathy
Ca	Calcium
SR	Sarcoplasmic reticulum
NCX	Na/Ca exchanger
AP	Action potential
APD30, 50, 70, 90	AP duration at 30, 50, 70 and 90% repolarization

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Highlights

- TnT-I79N hiPSC-CMs reproduce key cellular features of clinical HCM.
- TnT-I79N mutation reduces Ca transients by increasing cytosolic Ca buffering.
- TnT-I79N mutation causes pro-arrhythmic action potential changes.

Figure 1. TnT-I79N hiPSC-CMs exhibited sarcomere disorganization and sarcomere shortening (A) Sanger sequencing confirmed the presence of the c.236 T>A (p. I79N) in one allele of the *TNNT2* gene. (B) Representative images of different severity of α-actinin disorganization in the control and TnT-I79N CMs. TnT-I79N CMs exhibited a significantly higher level of α-actinin disorganization (C) and a shorter sarcomere length (D). TnT-I79N, n=62; control, n=57.

Figure 2. TnT-I79N hiPSC-CM exhibit enhanced contractility and impaired relaxation
 (A) Representative traces of cell shortening in single CMs. At $[Ca]_o$ of 2 mmol/l, TnT-I79N CMs showed an increased shortening (B) and a prolonged time from peak shortening to 90% baseline (C). At higher $[Ca]_o$ of 0.5, 1, 2 and 3 mmol/l, cell shortening was significantly increased in TnT-I79N CMs (D). At higher $[Ca]_o$ of 1, 2 and 3 mmol/l, the time from peak shortening to 90% baseline was slower in TnT-I79N CMs than control (E). * $P < 0.05$, ** $P < 0.01$ compared to control; TnT-I79N, $n = 15$; control, $n = 22$.

Figure 3. TnT-I79N increases cytosolic Ca buffering

(A) Representative voltage-clamp traces after rapidly applied caffeine-induced SR Ca release. (B) Representative integration of the NCX current yielded the total amount of Ca released from the SR. (C) Representative cytoplasmic buffering curves generated using the Trafford method by plotting cytosolic $[Ca]_{free}$ against change in total cytoplasmic Ca concentration ($[Ca]_{total}$). (D) and (E) Average B_{max} and K_d in hiPSC-CMs. (F) The change of total cytoplasmic Ca levels after caffeine application, a measure of SR Ca content. TnT-I79N, $n=12$; control, $n=16$.

Figure 4. TnT-I79N hiPSC-CM exhibit reduced Ca transients

(A) Representative traces of intracellular Ca transient during field-stimulation (top), and Ca-transients triggered by rapid caffeine application (bottom) in Fura-2-loaded control and TnT-I79N CMs. Compared to control, TnT-I79N mutation reduced both field-stimulated Ca transients (B) and caffeine-induced Ca transients (C) in single CMs. TnT-I79N, n=15; control, n=22.

Figure 5. Calcium sensitization in hiPSC-CM results in action potential (AP) triangulation and beat-to-beat instability

(A) Representative recordings of APs in TnT-I79N and control CMs. Without EGTA dialysis, APs of TnT-I79N CM and control CMs pretreated with myofilament Ca sensitizer EMD57033 exhibited increased AP triangulation (B) and beat-to-beat instability (C). Early repolarization (APD30, APD50 and APD70) was shorter in TnT-I79N CMs than isogenic control, but there was no significant difference in APD90 (D), which was reproduced in control CMs pretreated with EMD57033 (E); TnT-I79N, n= 18; control, n=20; control +EMD57033, n=12.

Figure 6. AP triangulation in TnT-I79N CMs was prevented by myofilament Ca desensitization with blebbistatin or excess cytosolic Ca buffering

(A) Representative AP recordings after treatment with 3 μ mol/L blebbistatin. (B) Average data. Blebbistatin treatment abolished AP triangulation in TnT-I79N CMs, but had no effect in isogenic control CM. n=5 per group.

(C) Representative AP recordings with excess cytosolic buffering. (D) Intracellular dialysis with 14 mmol/L EGTA abolished the AP difference in TnT-I79N and control CMs. n=5 per group.

Figure 7. Block of NCX reverses AP triangulation in TnT-I79N hiPSC-CMs
 Acute block of Ca efflux via NCX by replacing extracellular Na^+ with Li^+ abolished the differences of AP morphology between TnT-I79N and control CMs. (A) Representative recorded APs, (B) average data; TnT-I79N, n=5; control, n=6.