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Modeling Timothy syndrome with iPS cells

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Abstract

Genetic mutations in ion channel genes that are associated with cardiac arrhythmias have been identified over the past several decades. However, little is known about the pathophysiological processes. An important limitation has been the difficulty of using human cardiomyocytes to study arrhythmias and identify drugs. To circumvent this issue, we have developed a method using human induced pluripotent stem cells to generate cardiomyocytes from individuals with Timothy syndrome (TS), a genetic disorder characterized by QT prolongation, ventricular tachycardia and autism. The TS ventricular-like cardiomyocytes exhibit deficits in contraction, electrical signaling and calcium handling, as revealed by live cell imaging and electrophysiological studies. We tested candidate drugs in TS cardiomyocytes and found that roscovitine could successfully rescue these cellular phenotypes. The use of a human cellular model of cardiac arrhythmias provides a useful new platform not only to study disease mechanisms but also to develop new therapies to treat cardiac arrhythmias.

Keywords

Timothy syndrome; calcium channelopathy; QT prolongation; calcium signaling; drug testing

1. Introduction

Long QT Syndrome (LQTS) is a heart disorder that can lead to sudden cardiac death and is therefore a major concern for patients and clinicians. Genetic mutations leading to LQTS are present in about 1 of 7,000 people and result from mutations in 13 genes(1). In addition to having a genetic basis, QT prolongation can occur as a side effect by many approved drugs and is a common cause of drug failure in clinical trials, often resulting in major setbacks to drug discovery efforts. Although the genes that cause LQTS have been identified, the mechanisms that underlie the disease in humans are incompletely understood. Most of our knowledge on cardiac arrhythmias to date comes from rodent models. Rodent models are far from ideal models for human arrhythmogenic disorders, particularly considering the electrophysiological differences leading to heart rates ten times faster in mice than humans (2, 3, 4, 5). Because a perfect model system for cardiac arrhythmias does not exist, developing methods to generate pluripotent stem cells from human patients and to differentiate these cells into cardiomyocytes could provide new opportunities to investigate the molecular and cellular basis of LQTS, and to ultimately identify new therapeutics.

Timothy syndrome (TS) is a multisystem disorder caused by gain-of-function mutations in the L-type calcium channel *CACNA1C* gene(2). Common cardiac phenotypes in patients include a rate-corrected QT interval between 480 ms and 700 ms and ventricular tachycardia, often resulting in sudden death (2). While lethal arrhythmias can sometimes be prevented with automated defibrillators, there is essentially no effective treatment for the disease. In heterologous systems such as frog oocytes, non-excitable cell lines and rodent myocytes, excess calcium flux from the extracellular region has been shown to occur through mutant channels, due to ineffective channel inactivation(2, 3). Mouse models of TS

do not fully recapitulate the human disorder and, in particular, lack the postnatal lethality that occurs in patients with TS(4, 5). This is likely because mouse cardiomyocytes have different electrical properties from their human counterparts. In order to investigate human-specific disease mechanisms of TS, we therefore generated human cardiomyocytes from TS patients using induced pluripotent stem cell (iPSC) technology, examined cellular phenotypes in contraction, electrical signal and calcium handling, and performed drug tests to find lead compounds for TS.

2. Calcium handling in the heart

Voltage-gated calcium channels are major protein complexes expressed in the plasma membrane of excitable cells such as cardiomyocytes, skeletal myocytes and neurons. These channels are composed of an α_1 subunit that contains the pore and voltage sensor and β , γ and $\alpha_2\delta$ subunits that regulate channel gating and trafficking. Voltage-gated calcium channels are divided into L, N, P/Q and T type channels based on their α_1 subunits, which confer specific physiological properties and susceptibility to blockers and toxins. Cardiac cells express T-type and L-type calcium channels (LTCCs), which regulate calcium signaling and contractility. In the heart, α_{1c} (*CACNA1C*, $\text{Ca}_v1.2$, Fig. 1) and β_{2b} are the dominant subunits that form the LTC at the plasma membrane(6). The LTCC is essential for generating the cardiac action potential and for triggering muscle contraction in a process called excitation-contraction (E-C) coupling (Fig. 2)(7). Calcium influx through LTCCs in the plasma membrane induces calcium release through ryanodine receptors (RyRs) in the sarcoplasmic reticulum (SR). The Ca^{2+} released from the SR binds to troponin C in myofilaments and induces a series of allosteric changes in the myosin filaments leading to muscle fiber contraction. The subsequent removal of Ca^{2+} is primarily mediated by the activity of the SR Ca^{2+} ATPase (SERCA) that pumps Ca^{2+} into the SR stores, with a minor contribution of the sodium calcium exchangers NCX and plasma membrane calcium ATPase. Disruption of *CACNA1C* gene that encodes $\text{Ca}_v1.2$ in mice leads to an almost complete loss of heart beat and embryonic lethality, indicating that $\text{Ca}_v1.2$ plays an essential role in heart development and function(8). Therefore, genetic mutations in the *CACNA1C* gene associated with Timothy syndrome, including gain-of-function mutations, could affect $\text{Ca}_v1.2$ channel properties, resulting in altered E-C coupling in the heart and causing severe arrhythmias.

3. Timothy syndrome

Timothy syndrome is a calcium channelopathy caused by a mutation in the $\text{Ca}_v1.2$ channel. The first case of TS was described in 1992 and involved LQTS (type 8), congenital heart defects, and syndactyly (webbing of fingers and toes)(9). As therapy began to extend the lives of affected children, it became clear that TS is a multisystem disorder that includes lethal arrhythmia, congenital heart defects, syndactyly, autism, and intermittent hypoglycemia(2). The disorder results from a recurrent, *de novo* missense mutation in exon 8a of *CACNA1C*, the gene that encodes the $\text{Ca}_v1.2$ protein (2). Exon 8a is an alternatively spliced exon of the *CACNA1C* channel and is found in about 20% of the channels in the adult brain and heart. This gain-of-function mutation results in a Gly406Arg substitution that alters voltage and calcium-dependent channel inactivation, leading to excess calcium influx in response to depolarization. In addition to this mutation, three other mutations in the *CACNA1C* channel have been associated with TS. TS type 2 was reported in two individuals with *CACNA1C* mutations in exon 8, which is mutually exclusive with exon 8a, and is found in the majority of channels in the adult heart. One of these patients had a Gly406Arg mutation that caused an extremely long QT interval (QTc 620–730 ms), and the other had a Gly402Ser mutation that coupled with somatic cell mosaicism, causing a milder phenotype. Recently, a case of TS type 3 was reported in an individual with an Ala1473Gly

mutation in exon 38 of *CACNA1C*(10, 11). All of the TS mutations have been found to occur in structurally similar regions of the Ca_v1.2 protein. The α 1 subunit of voltage-gated calcium channels contains four pseudo repeats of six transmembrane domains that contain parts of the pore and voltage sensor and are connected by intracellular loops that bind accessory subunits and regulatory proteins. The TS mutations occur at the C-terminal end of the sixth transmembrane domain, in pseudo repeat 1 for TS1 and TS2 and pseudorepeat 4 for TS3 (Fig. 1).

Approximately 30 patients have been reported with TS1, two with TS2 and only one with TS3 (2, 10, 11). In general, TS1 patients have a milder phenotype than TS2 and TS3 patients, most likely because the TS1 mutation occurs in a minor splice isoform of the Ca_v1.2 channel (10). All TS patients have long QT intervals, 70% have T-wave alternation, 84% have ventricular tachycardia and 81% have atrioventricular (AV) block (2:1). A variety of congenital heart defects are also observed in TS patients: 59% have patent ductus arteriosus, 29% have patent foramen ovale, 18% have ventricular septal defect, 6% have tetralogy of Fallot, and 50% have hypertrophic cardiomyopathy (2). These congenital heart defects suggest that Ca_v1.2 channels play important roles in cardiovascular development as well as their role in the adult heart.

In addition to cardiac deficits, TS patients display a variety of other phenotypes. 100% TS1 patients have cutaneous syndactyly of the second and third and fourth and fifth fingers, have small teeth and are bald at birth (2). Ca_v1.2, therefore, is believed to play a role in the development of hair follicles and in the apoptotic death of the progenitor cells that form the digits. Further, 80% of TS patients are reported to have an autism spectrum disorder, 20% have seizures, and 25% have intellectual disability (2), indicating that Ca_v1.2 is also important in the development and function of the brain.

4. Studies of TS in mouse and cell models

Most of our knowledge of TS comes from studies of channels containing the TS mutation expressed in frog oocytes, HEK cells or CHO cells (2). Thiel *et al.* and Barrett and Tsien demonstrated that TS mutations altered calcium-dependent inactivation as well as voltage-dependent inactivation in rat ventricular myocytes and HEK cells overexpressing TS mutants, respectively(3, 14). Prolonged action potentials and delayed afterdepolarizations were observed in rat ventricular cardiomyocytes overexpressing TS mutant channels, while voltage-dependent inactivation changed slightly in the myocytes(3). Cheng *et al.* generated transgenic mice with TS mutant expression controlled under a cardiac promoter, and found that they display similar phenotypes as human patients, including QT prolongation, torsade de pointes and hypertrophy. In contrast, Bader and colleagues have reported that heterozygous mice of a knock-in mouse model for the Gly406Arg mutation (type 2) develop autistic behavior without any cardiac abnormalities(4, 5). Neither of these TS mouse models were observed to display postnatal lethality, a key phenotype of human patients with TS (patient average life, ~2.5 years old). Based on the *in vivo* studies performed to date, cardiac phenotypes differ between mice and humans. Therefore, to understand human-specific mechanisms of QT prolongation and severe arrhythmias, developing human models of TS is imperative.

5. Cellular phenotypes of TS-iPSC derived cardiomyocytes

a) Generation of cardiomyocytes from TS patients

To generate iPSC lines from TS patients, we harvested skin fibroblasts from two TS patients using four retroviruses containing *SOX2*, *OCT3/4*, *KLF4* and *C-MYC*(15). At the time the patients were recruited, the retroviral method using these four factors was considered one of

the most reliable methods(18, 19), although currently, there are several integration-free methods available using modified mRNA and episomal vectors(16, 17). To alleviate concerns about unpredicted effects of retroviral genes on the genome of our generated iPSC lines, we mapped the integration sites of the retroviruses using nested PCR. We found that all the lines had independent retroviral insertion sites, and the sites did not occur in the coding region of any gene in the iPSC lines used for *in vitro* cardiac differentiation. Based on cell morphology, proliferation potential, viral silencing and the results from karyotyping, immunocytochemistry, teratoma formation and gene expression profiling using RT-PCR and microarray, we successfully differentiated four control and five patient iPSC lines, as well as a human embryonic stem cell (ESC) H9 line, into neurons or cardiomyocytes. The resulting cells were studied in a variety of assays to examine cellular phenotypes of TS. Currently, we use integration-free methods with episomal vectors (20) instead of retroviral methods. Comprehensive genomic analyses of iPSCs are also required to identify chromosomal aberrations and point mutations that might exist in the donor fibroblasts, might occur in the reprogramming process or might be acquired during long-term cell culturing. As conventional karyotyping is not sufficient to detect *de novo* mutations, we use single-nucleotide polymorphism (SNP) array karyotyping for newly generated iPSC lines with episomal vectors. This assay allows us to perform a more comprehensive analysis of the iPSC lines to select clones of iPSCs derived from controls and patients.

One challenge in the field has been the ability to select clones of iPSCs for *in vitro* differentiation, as the examination of phenotypes is dependent on investigators' experiences in the manual passage of the cells and is based on the morphology of the cells. Although common selection markers for the identification of properly reprogrammed cell clones are not completely known, the expression of *Esrrb1*, *Utf1*, *Lin28* and *Dppa2* could potentially be used to predict cell reprogramming into iPSCs, instead of previously used genes like *Oct4* (21).

To generate human cardiomyocytes from iPSCs, we exploited a method using embryoid bodies (EB) with 5–20% fetal bovine serum (FBS) to prepare cell aggregates. Although the efficiency of this technique is relatively low compared to methods using EB or monolayer cultures with defined growth factor cocktails(22, 23), we successfully observed three major types of myocytes in control and TS cells at day 40–60 after induction of differentiation: embryonic nodal-, atrial- and ventricular-like cardiomyocytes. In contrast, when we used the protocol established by Yang and colleagues(22), >90% of the generated cardiomyocytes demonstrated ventricular-like action potentials, making it very difficult to find nodal- and atrial-like populations(24) (M. Yazawa, unpublished). Therefore, the approach using EBs with FBS enabled us to examine cellular phenotypes in all types of TS cardiomyocytes, making it an ideal method to investigate the cellular basis of severe arrhythmias in TS. RT-PCR analysis revealed that the spontaneously contracting EBs at day 37 expressed cardiac markers and both exon 8 and 8a of *CACNA1C* but not *NANOG*, indicating that these contain cardiomyocytes but not iPSCs.

b) Irregular and slow contraction

To examine the contractile properties of cardiomyocytes, we collected time-lapse images of spontaneously contracting EBs and analyzed their movement using image analysis software. Because EBs contain heterogeneous cell populations and the shape of EBs can affect spontaneous contraction, we collected movies of >100 EBs derived from five TS iPSC lines and compared them to EBs derived from five control lines. Control EBs contracted at approximately 60 bpm at day 37, similar to the resting heart rate in humans, whereas TS EBs contracted at only ~30 bpm. Notably, the contraction of the TS EBs was significantly more irregular than the contraction of control ones, regardless of whether they were grown at room temperature or at 37 °C (M. Yazawa, unpublished). These results indicate that

contracting EBs from multiple TS-derived iPSC lines have disease-specific defects such as bradycardia and arrhythmias.

c) Sarcomere formation and channel property

To further characterize defects in TS cardiomyocytes, we dissociated contracting EBs into single cells and stained them using antibodies to the sarcomere markers α -actinin and cardiac Troponin I. The immunocytochemistry indicated that there was no difference in sarcomere maturation between TS and control cardiomyocytes. We confirmed this result by examining the ultrastructure of organelles such as the SR and mitochondria in cardiomyocytes using electron microscopy. We additionally sought to determine whether the TS mutation altered voltage-dependent inactivation of LTCC in these cells. We performed whole-cell patch clamp experiments using Ba^{2+} as a charge carrier to measure L-type currents and found that TS cardiomyocytes displayed a significantly reduced voltage-dependent inactivation; in contrast, a change in the current-voltage relationship or the peak amplitude of the Ba^{2+} current was not observed. Interestingly, all three types of TS cardiomyocytes demonstrated an altered voltage-dependent inactivation, indicating that all of the cardiomyocytes derived from the patients expressed TS mutant channels. The TS channel properties that we observed are consistent with those reported in previous studies using heterologous cells overexpressing TS channel mutants(2).

d) Prolonged action potentials and delayed afterdepolarization

Depolarization by sodium channels opens LTCCs in cardiomyocytes, and Ca^{2+} influx through LTCCs contributes to the plateau phase of the cardiac action potential (AP). We examined whether the shape or duration of the AP was altered by the TS $\text{Ca}_v1.2$ mutation using current-clamp recording to record spontaneous APs in control and TS cardiomyocytes. Human cardiac cell populations generated from iPSCs contain a mixed group of nodal-like, ventricular-like and atrial-like myocytes(25), therefore it was necessary to use single-conditioned cardiomyocytes for electrophysiological recordings. We harvested the mRNA from each clamped cell to conduct single-cell RT-PCR using a primer set to detect cardiac ventricular myosin light chain 2v. Current clamp recording revealed that the ventricular-like myocytes derived from TS patients had APs that were three times as long as those of control cells, indicating that our model of TS reflects cardiac phenotypes of patients showing QT prolongation. The atrial- and nodal-like cells, however, showed a normal shape and duration of the spontaneous action potentials although, interestingly, all three types of cardiomyocytes from TS patients had increased L-type currents in voltage-clamp measurements. In addition, a large number of depolarizing events that failed to trigger a full AP were observed in TS ventricular-like cardiomyocytes. These irregular APs were similar to the delayed afterdepolarizations (DADs) that arise following ectopic Ca^{2+} release through RyRs and excess NCX activation and are associated with cardiac arrhythmias(6). The fact that these phenotypes are broadly consistent with cardiac phenotypes in TS patients supports that human iPSC-derived cardiomyocytes could provide a useful platform to study human disease mechanisms of TS, and perhaps other genetic arrhythmias as well.

e) Abnormal calcium handling

$\text{Ca}_v1.2$ channels play an essential role in the heart by converting electrical signals to chemical signals to activate Ca^{2+} -induced Ca^{2+} release (CICR) from the SR, leading to muscle contraction. Although $\text{Ca}_v1.2$ channels have been studied for decades, the effect of altering $\text{Ca}_v1.2$ inactivation on CICR still remains unknown. To address how the TS mutation affects Ca^{2+} signaling in CMs, cytoplasmic calcium imaging was conducted using fast line-scan imaging of the human cardiomyocytes loaded with the Ca^{2+} indicator Fluo-4 AM (~2.0 ms/line). The Ca^{2+} transients were found to be highly irregular in spontaneously contracting TS ventricular-like cardiomyocytes. In addition, the TS mutation led to

significantly larger and more prolonged Ca^{2+} transients, suggesting that channel inactivation is important for maintaining the timing and the amplitude of calcium release from the SR. In contrast, calcium handling in atrial- and nodal-like cardiomyocytes derived from TS patients appeared to be unaffected by the inactivation of TS $\text{Ca}_v1.2$ channels.

f) Remaining questions concerning cardiac phenotypes in TS

It was surprising that the spontaneous action potentials and calcium transients were normal in TS atrial- and nodal-like cardiomyocytes in spite of the altered voltage-dependent inactivation of the TS $\text{Ca}_v1.2$ channels, a sharp contrast from what was observed in the ventricular-like cardiomyocytes. One possible explanation could be that compensation by other ion channels might occur. Alternatively, $\text{Ca}_v1.2$ could have different roles in regulating electrical signal and calcium handling in these cell populations, which might explain why most TS patients exhibit ventricular deficits but no major abnormalities in the atrium. To further elucidate this issue, additional electrophysiological recordings of potassium and sodium currents as well as biochemical assays for all three types of TS cardiomyocytes will be required. It will also be imperative to be able to reliably distinguish the different types of cardiac cells in culture, as this would allow for more robust tests and for the development of better models of cardiac arrhythmias. To do this, reporter systems could be constructed for each cardiac cell type using promoters of cardiac ventricular myosin light chain 2v and other myofilaments and transcription factors.

Another key remaining question is whether ventricular arrhythmia occurs in TS human hearts due to a cell autonomous or a whole organ defect. In our studies, we used single-conditioned, spontaneously contracting cardiomyocytes to examine cellular phenotypes in electrical activities and calcium handling, in order to be certain which cell type exhibited abnormal cardiac function. Although small cell clusters composed of several cardiomyocytes (3–10 cells) could be examined, the size and cell population of these clusters are largely heterogeneous after dissociation. In order to conduct electrophysiological recordings and live cell imaging of these clusters, fluorescent cell-type reporters and uniform cell culture systems will be necessary. A bioengineering approach to generate defined human cell clusters with cardiac reporters could enable us to address how TS mutations lead to ventricular tachycardia *in vivo*. This would be highly advantageous over mouse models of TS, as mice have different electrophysiological machinery for maintaining their high heart rate (> 600 bpm) from humans (~60–70 bpm) and the TS mice don't show lethal arrhythmias.

Most spontaneously beating cardiomyocytes derived from human iPSCs have a resting membrane potential of about –50 mV at day 60–90 after differentiation (M. Yazawa, unpublished work). Currently, one of the key limitations of using human pluripotent stem cells in the cardiovascular field is the difficulty of generating human mature cardiomyocytes, for instance producing human adult ventricular myocytes with a proper resting potential of about –90 mV and formation of the sarcomere with a T-tubule (Fig. 2). We found that culturing a long-term *in vitro* system for over 8 months changed the gene expression of a variety of ion channels in beating EBs and brought the resting potential deeper (~ –70 mV)(26), however an 8-month culture for cardiac differentiation is not practical due to cost and the potential for additional chromosomal aberrations and point mutations. Jung *et al.* generated human cardiomyocytes from a patient with a mutation in RyR2 that is associated with catecholaminergic polymorphic ventricular tachycardia (CPVT) using a similar protocol for cardiac differentiation, and used non-beating cardiomyocytes to examine calcium sparks and electrical activity in CPVT cardiomyocytes(27). Interestingly, they reported resting potentials of –80 mV in the myocytes at day ~90, and SR calcium buffering molecule calsequestrin proteins were detected in these cells to a similar degree as in human adult heart tissues. These findings

suggest that non-beating cells that can respond to electrical stimuli could be useful to further characterize cellular phenotypes in TS and other arrhythmias and also to explore the maturation process of human differentiating cardiac cells; in these studies, β -agonists and mechanical stretching on bioengineering devices could be used to accelerate cell maturation.

As patients with TS exhibit severe ventricular arrhythmias leading to sudden death, the human cardiomyocytes derived from these patients have defects in contraction, electrical activity and calcium transients. Irregular APs that are similar to DADs were observed in TS cardiomyocytes. DADs are induced by a partial depolarization following ectopic Ca^{2+} releases and sodium/calcium exchanges associated with cardiac arrhythmias. To examine dynamic calcium handling in synchronization with a variety of electrical activities, simultaneous recordings of action potential and cytoplasmic calcium signaling would be informative. Computational modeling could also be used to dissect the contribution of each channel associated with irregular action potentials(28).

Because calcium signaling regulates the expression and function of molecules essential for heart physiology, the altered calcium handling in TS cardiomyocytes might affect the expression and function of potassium and sodium channels and calcium handling molecules such as RyRs, SERCA and NCX. Further characterization of the molecular phenotypes in human ventricular-like cardiomyocytes derived from TS would provide insight into the pathological process in TS hearts and may allow for the identification of unique markers for severe ventricular arrhythmias.

6. Cellular phenotypes of TS TS-iPSC derived neuronal cells

One of the advantages of using disease-specific iPSCs is the ability to examine cellular phenotypes in multiple cell types. In addition to the effects on heart physiology, some TS patients show autism, ASDs and delays in language development(2), suggesting that studying neuronal precursor cells and neurons derived from TS patients could be valuable. We have developed methods to generate cortical neuronal precursor cells and neurons from iPSCs and found that TS-derived neuronal cells have defects in calcium signaling, activity-dependent gene expression, and differentiation(29). More specifically, these cells showed an increased expression of upper cortical layer markers and a decrease in callosal projection markers compared to control cells. In addition, the TS mutation caused ectopic expression of tyrosin hydroxylase in neurons leading to an increase in the production of neurons that synthesize norepinephrine and dopamine. These findings provide strong evidence that $\text{Ca}_v1.2$ regulates the differentiation of cortical neurons in humans and offers new insights into the causes of autism in TS patients. To further investigate the pathophysiological process that leads to autistic behaviors, the injection of neurons into brain slices or into brains of live animals could be a useful method to explore *in vivo* defects in cell survival, proliferation, differentiation, synapse formation, and electrical activity. It would also be interesting to compare the human molecular and cellular phenotypes to those in rodent models of TS; this would provide insight into the mechanisms of neuropsychiatric involvement in TS patients as well as the validity of different models of the disease.

7. Therapeutic approaches to TS

Several drugs, including the LTCC blocker verapamil, beta-blockers and the sodium channel blocker ranolazine, have been suggested as potential therapies for TS based on laboratory studies. In the clinic, however, these blockers had a limited effect on TS, and most TS patients died before puberty due to arrhythmias(2, 6). Itzhaki and colleagues reported that another LTCC blocker, Nifedipine, abolished all arrhythmic events in a human iPSC model of LQTS type 2, but unfortunately the long-term application of Nifedipine prevented beating of either control or patient EBs(30).

To prevent life-threatening arrhythmias such as ventricular fibrillation and torsade de pointes in TS patients, implantable defibrillators (ICDs) are useful, but the usage of ICDs and/or blockers is limited in TS infants. Basic researchers using rodent models of TS have reported that the ablation of the anchoring molecule AKAP150 and inhibition of the calmodulin-dependent protein kinase II (CaMKII) can restore normal functions in $\text{Ca}_v1.2$ channels(3, 4). Yarotsky *et al.* found that Roscovitine (Ros), a cyclin-dependent kinase inhibitor, increased voltage-dependent inactivation of $\text{Ca}_v1.2$ channels and restored normal kinetics in TS $\text{Ca}_v1.2$ channels in HEK 293 cells expressing $\text{Ca}_v1.2$ channel constructs(31, 32). Based on this finding, we investigated whether Ros could restore normal calcium handling in TS cardiomyocytes. We examined the effect of different concentrations of Ros on the timing and amplitude of spontaneous Ca^{2+} transients in TS cardiomyocytes. While treatment with 10 μM Ros did not have an immediate effect on TS cardiomyocytes, 33.3 μM Ros significantly reduced both the irregular timing and amplitude of Ca^{2+} transients. After washing out Ros, the irregular Ca^{2+} transients were again observed in TS cardiomyocytes. These results demonstrate that Ros indeed has a beneficial effect on the phenotypes in TS cardiomyocytes.

To determine whether Ros rescues the electrophysiological phenotypes of TS cardiomyocytes, whole-cell patch voltage clamping was performed to measure Ba^{2+} currents in the presence and absence of Ros (33.3 μM). Ros significantly reduced Ba^{2+} currents and increased the channel inactivation in TS cardiomyocytes (15), and had only a mild effect on control cells. To confirm these beneficial effects of Ros on the electrical defects in TS cardiomyocytes, spontaneous action potentials were recorded using current clamp mode. We found that Ros also reduced the duration of APs in TS cardiomyocytes and decreased the frequency of abnormal depolarizing events (15). These results demonstrate that cardiomyocytes generated from patient iPSCs with TS can be used to screen potential drugs, and also support that Ros might be a leading compound for treating patients with TS and other cardiac arrhythmias.

We additionally explored whether Ros has beneficial effects on cell phenotypes in TS-derived neuronal cells. Treatment of human TS-derived neurons with roscovitine (10–15 μM) caused a 68% reduction in the proportion of tyrosin hydroxylase-positive neurons without affecting the fraction of cells expressing the neuronal marker MAP2 (29). This result indicates that the increase in tyrosin hydroxylase expression likely results from altered calcium signaling due to affected inactivation of TS $\text{Ca}_v1.2$ channels and, further, that restoring channel inactivation in mature neurons can decrease the abnormal expression of tyrosin hydroxylase in patient neuronal cells.

In sum, these results reveal that Ros is a putative future therapy for TS patients. However, Ros was originally identified as a cdk inhibitor for cancer treatments and may have unpredicted side effects in humans. For future pharmaceutical application for the treatment of TS, screening Ros derivatives and analogs will be required. Cyclosporine A could be an alternative approach to rescue cardiac phenotypes in Timothy syndrome patients, because it is a well-known immunosuppressive drug targeting calcineurin and increases voltage-dependent inactivation of $\text{Ca}_v1.2$ through calcineurin pathways(33). To verify drug candidates before clinical trials, cellular models of Timothy syndrome using patient-specific iPSCs could be a useful platform. To develop automated high-throughput systems for chemical screens, biochemical assays to detect molecular markers for ventricular arrhythmias would be ideal. Before this is possible, further characterization of the molecular defects in TS iPSC-derived cardiomyocytes will be necessary. While an electrophysiological approach is available using an automated patch clamp rig, calcium handling and contraction in human cardiomyocytes on automated fluorescent microscopes could easily be used to develop medium-throughput screening systems to find new drugs for TS and other

arrhythmias. Conducting screens using a chemical library requires a large-scale iPSC culture (> tens million cells) and cardiac differentiation. The most efficient way to develop this model at such a large scale is to expand cardiovascular progenitor cells (CPCs) and induce directed differentiation into cardiomyocytes, however how to identify and maintain human CPCs *in vitro* still remains unknown. Although many challenges remain, we are hopeful that these interventions will eventually lead to better approaches to develop human models of cardiac arrhythmias and discover new therapeutics.

8. Conclusion

By generating human cardiac cells containing the genetic information of patients with cardiac disorders, we have been able to investigate the cellular and molecular mechanisms of cardiac diseases in the actual cells that are affected. Compared to iPSC models of other LQTSs (30, 34), ventricular-like cardiomyocytes derived from TS iPSCs show a prolonged action potential and irregular APs that are similar to DADs. These results suggest that the duration of action potentials in iPSC models of LQTS are consistent with the QT interval in patients, as TS patients demonstrated a longer QT interval than other LQTS patients (2, 15, 34). Therefore, modeling cardiac arrhythmia using iPSC-derived cardiomyocytes could be a useful tool to further elucidate disease mechanisms and test the effect of drugs on cardiac action potentials. Thus far, we have studied the role of Cav1.2 channels in heart development and function and how ventricular fibrillation occurs in patients with TS. Our approaches could help characterize phenotypes in this human cardiac disorder and identify potential drug candidates. A major limitation to finding new drugs for therapeutic applications has been that most initial drug screens have used rodent models, which often fail to reveal toxicity and side effects that might occur in human cells and are not ideal for identifying therapies that are effective in human cells. By developing a screening assay based on human iPSC-derived cardiomyocytes, we hope to develop a platform that would be widely useful for future pharmaceutical approaches toward the treatment of not only TS but also other cardiac arrhythmias.

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Figure 1. Timothy syndrome mutations in L-type calcium channel α_{1C} subunit

The α_{1C} forms the ion channel pore to permeate calcium ions and is composed of four homologous domains (DI- IV). The mutations that cause TS (*de novo* gain-of-function mutations) are localized in the cytoplasmic end of transmembrane segment 6 of DI and DIV.

Figure 2. Cellular phenotypes in excitation-contraction coupling in cardiomyocytes derived from Timothy syndrome patients

Depolarization opens L-type calcium channels (LTCC), inducing calcium influx. Calcium influx activates the ryanodine receptor calcium release channels (RyR) in the sarcoplasmic reticulum (SR) and triggers the release of Ca^{2+} from the SR. The calcium transient activates the myofilaments that generate contraction. Relaxation is promoted when Ca ions are removed from the cytosol by Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase (SERCA) in SR membranes and the NCX and other calcium ATPase in the plasma membrane (PM). Human cardiomyocytes derived from Timothy syndrome patients showed a variety of cellular phenotypes in the excitation-contraction coupling such as excess calcium influx, irregular and prolonged action potentials, abnormal calcium transients and irregular contraction.