SNTA1-deficient human cardiomyocytes are associated with increased structural components, calcium handling disorder, and shorter field potential duration

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Abstract

Background

α -1-syntrophin (SNTA1), a protein encoded by *SNTA1*, is highly expressed in human cardiomyocytes. Mutations in *SNTA1* are associated with arrhythmia and cardiomyopathy. Previous research on *SNTA1* has been based on nonhuman cardiomyocytes. Our study was designed to identify phenotype of *SNTA1*-deficient using human cardiomyocytes.

Methods

SNTA1 was knocked out in H9 cell line using CRISPR-Cas9 system. H9SNTA1KO cells were then induced to differentiate into cardiomyocytes using small molecule inhibitors. The phenotypic discrepancies associated with *SNTA1*-deficient cardiomyocytes were investigated.

Results

SNTA1 was truncated in PH1 domain by a stop codon (TGA) using CRISPR-Cas9 system. *SNTA1*-deficient did not affect the pluripotency of H9SNTA1KO, and they retain their *in vitro* ability to differentiate into cardiomyocytes. However, H9SNTA1KO derived cardiomyocytes exhibited increased structural components, weak calcium transient intensity, low levels of calcium in sarcoplasmic reticulum, lower cardiac contractility, and shorter field potential duration. Early treatment of *SNTA1*-deficient cardiomyocytes with ranolazine improved the calcium transient intensity and cardiac contractility.

Conclusions

SNTA1-deficient cardiomyocytes can be used to research the etiology, pathogenesis, and potential therapies for myocardial diseases. The *SNTA1*-deficient cardiomyocyte model suggests that the maintenance of cardiac calcium homeostasis is a key target in the treatment of myocardial-related diseases.

Introduction

SNTA1 becomes an important signaling scaffold protein between the extracellular matrix and the intracellular cytoskeleton by connecting with dystrophin-associated protein complex (DAPC) [1]. The point mutations of *SNTA1* can cause long QT syndrome [2–3], Brugada syndrome [4], sudden infant death syndrome [5], atrial fibrillation [6], etc. The SNTA1 binds the motif of PDZ domain of Nav1.5. Nav1.5 is an important type of cardiac voltage-gated sodium channels. SNTA1 plays a critical auxiliary role in the correct subcellular localization, expression, and function of Nav1.5. SNTA1 is involved in the regulation of membrane volume on Kir2.1 and Kir2.2 channels [7]. In Snta1 knockout mice, left ventricular posterior wall

thickening and abnormal myocardial performance index (MPI) has been reported, which indicates the presence of myocardial hypertrophy in the knockout mice [8].

At present, the results of research on *SNTA1* are from nonhuman cells, such as CHO cells, H9C2 cells, *Xenopus* oocytes, etc. A large number of patient cardiomyocytes can be derived from patients' iPSCs. Patients' cardiomyocytes were studied for abnormal phenotypes, however, there are a lot of differences between patients and normal donors in terms of the genetic background. Different genetic background hampered the further research in patients' iPSCs. With the widespread applications of gene editing technologies in eukaryotic cells [9–11], genetically modified human embryonic stem cells (hESCs) establishment help to resolve the impediment of the different genetic background [12–13]. In order to investigate the phenotype of *SNTA1*-deficient cardiomyocytes, *SNTA1*-deficient cardiomyocytes induction from the CRISPR-Cas9-modified hESCs were researched, and then compared with the phenotype of wild type (WT) cardiomyocytes under the same genetic background. This research provides an example of using human cell to study the phenotype of cardiomyocytes caused by the *SNTA1* knockout, meanwhile, providing a case that the exploration of the mechanism of pathogenesis of gene knockout using human cardiomyocytes.

Methods

Embryonic stem cell culture and the design for *SNTA1* knockout

The H9 embryonic stem cell was purchased from WiCell Research Institute Inc. The H9 cells were cultured in E8 medium, and when the cells reached 80% confluence, they were digested by 0.5 mM EDTA. Using the CRISPR-Cas9 system, Zhang Lab web tools were used to design single guide RNA (sgRNA) targeting *SNTA1*. The sgRNA targeting site was used to select public exons close to start codon: we selected exon 2 design sgRNA (5'-attggcaggacag-3') and confirmed deletion by western blots.

Embryoid body (EB) formation test and monolayer differentiation

With 0.5 mM EDTA, 6×10^6 cells were suspended in PSCeasy Medium (Cellapy, China) with 2 μ M thiazovivin (TZV) and plated in a 6-wells plate without Matrigel for 4 days, then the EBs were cultured in RPMI medium 1640 with 20% knockout serum replacement (KSR) in the Matrigel-coated plate. The embryonic stem cells were induced to differentiate into cardiomyocytes using CardioEasy Kit (Cellapy, China) in the Matrigel-coated plate for monolayer differentiation.

RNA-sequencing (RNA-seq) analysis and qRT-PCR

After RNA was extracted from *SNTA1*-deficient cardiomyocytes (KO) and WT, RNA-seq was analyzed by BGI Tech. Solutions Co., Ltd. (Liuhe, China). Total cellular RNA was extracted with TRIzol (Invitrogen, USA) and treated with DNase I (Beyotime, China) for approximately 30min at 37°C to eliminate DNA contamination. RNA was reverse transcribed using the Prime-Script TM reverse transcription system (TaKaRa, Japan). Relative gene expression levels were examined by qRT-PCR using the iCycler iQ5 (Bio-

Rad, USA) with TB Green™ Premix Ex Taq™ II (Takara, Japan). The relative quantification was calculated according to the $\Delta\Delta$ CT method.

Flow cytometry

The cells were digested with 0.5 mM EDTA to prepare single-cell suspensions, and then incubated with the antibody for 30 min in PBS at room temperature (RT). The samples were detected by Flow cytometer (Beckman, EPICS XL) and the results were analyzed using the Flow Jo VX software.

Immunofluorescent staining

Cells were fixed in 4% paraformaldehyde for 30 min, washed three times in PBS, permeabilized with 0.3% Triton X-100 for 10 min at RT, and blocked in 3% BSA for 30 min at RT. Then the cells were incubated with the primary antibody for 24 h at 4°C, washed three times in PBS, and then the cells were incubated with the secondary antibody and DAPI (100 nM) for 1 h at RT. The cells were subsequently washed again three times in PBS, and imaged with the confocal microscopy (Leica, TCS5 SP5).

Calcium transient assay and caffeine-evoked calcium release test

The cardiomyocytes were plated on 35 mm confocal dish loaded with 4 μ M Fluo-4 AM (Yeasen, China) and incubated at 37°C for 20 min in PBS (Servicebio, China) containing 0.04% Pluronic F-127 (Yeasen, China). PBS was changed to the Cardiomyocytes Maintenance Medium (Cellapy, China). Loaded samples were transferred under a TCS-SP5-RS confocal microscope (Leica, Germany). Laser emission at 488 nm was used for stimulation and emitted fluorescence at 530 nm was acquired. Samples were then stimulated with freshly prepared solution of caffeine (20 mM), and emitted fluorescence acquired to record transient alteration in cytosolic calcium levels.

Contractility measurement and microelectrode array test

The contractility of cardiac myocytes was measured according to the protocols reported previously [14-15]. The electrical activity of cardiac myocytes was detected using Maestro Multiwell Microelectrode Array (MEA). The experimental was carried out according to the protocol of micro electrode array.

Statistical methods

The data of measurement and count were all presented as mean $\pm$ standard deviation. The difference between two groups was analyzed by one-tail or two-tail student's *t* test, and the rate was compared by Fisher's Exact test, three groups of data and more were analyzed using single-factor or two-factor analysis of variance, followed by Tukey multiple comparison test. The confidence interval was 95%, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001, representing four levels of statistical significance.

Results

Establishment of homozygous *SNTA1*-deficient hESCs (H9SNTA1KO)

We selected the second exon of *SNTA1*, corresponding to the pleckstrin homology 1 (PH1) domain, as the target site of sgRNA (Fig.1A). The result of DNA sequencing indicated that an adenine nucleotide was inserted before the protospacer adjacent motif (PAM) region after gene editing (Fig.1B). A stop codon (TGA) appeared at the amino acid position 149 in the SNTA1 and terminated the SNTA1 protein prematurely in the PH1 domain. The rate of *SNTA1* knockout were evaluated using the Synthego analysis sequencing result (Fig.1C). Immunofluorescence staining for pluripotency was performed on H9SNTA1KO. Both SSEA4 and NANOG were positive with H9SNTA1KO. (Fig.1D). The expression of pluripotent markers *SOX2*, *DPPA4*, *OCT-4*, and *NANOG* in H9SNTA1KO were similar to WT using qRT-PCR analysis (supplementary data Fig.1A). The karyotype analysis of H9SNTA1KO was normal (46, XX) (supplementary data Fig.1B). Also, western blots confirmed H9SNTA1KO to be deficient in *SNTA1* (supplementary data Fig.1C).

H9SNTA1KO differentiated into cardiomyocytes

Schematic of the H9SNTA1KO which were induced into cardiomyocytes using small molecule inhibitors (Fig.2A). The image of H9SNTA1KO induced using small molecule inhibitors from stage 1 through 3 (Fig.2B1, B2, B3, respectively). We noticed the beating of KO on the 10th day of cardiac differentiation (Fig.2B4). KO were enriched with metabolic selection using glucose-free medium containing lactate. The KO layer was formed after cell density adjustment (Fig.2C). In order to identify the kind of differentiated cells, we stained the cells with TNNT2 and α -actinin cardiomyocytes markers. The result showed the beating cells, which differentiated from H9SNTA1KO, were positive for TNNT2 and α -actinin (Fig.2D). We examined the expression of the TNNT2 in WT and KO before metabolic selection using flow cytometry assay. The results indicated that H9SNTA1KO were capable of differentiating into cardiomyocytes (Fig.2E). MYL2, a specific marker of ventricular muscle, was detected in WT and KO after metabolic selection using flow cytometry assay. The results showed that the H9SNTA1KO had a normally-differentiated cardiomyocyte subtype (Fig.2F).

Structural components increased in KO

In order to study the phenotype of KO, The RNA of KO was analyzed by RNA-seq after being cultured on the 30th day. The data were analyzed using KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment to discover the gene activation of hypertrophy-related pathway in KO (supplementary data Fig.2A, B, C, respectively). The main structural components of KO were detected by qRT-PCR, including TNNT2, MYH6, MYH7, MYL2-MYL7 etc. The results suggested that the structural components involved in contraction of KO were elevated at the transcription level (Fig.3A). For instance, the results indicated that the expression levels of TNNT2, MYL2, and α -actinin in KO were higher than those in WT cultured on the 45th day (Fig.3B, C, D, respectively). The expression of MYL2 was detected using western blotting technique. The results showed the expression was higher in KO (supplementary data Fig.2D). The p-Ca²⁺/calmodulin-dependent protein kinase II (p-CaMKII) level, which is usually with abnormal calcium handling, was detected by western blots. The results confirmed that p-CaMKII levels increased in KO (supplementary data Fig.2E).

The KO had normal diameter and shorter field potential duration (FPD)

In general, the diameter of a cell is affected by structural components increasing. Comparing the diameters of KO to those of WT used flow cytometry forward scatter (FSC). The results showed the diameters of KO were not different from those of the WT (Fig.4A, B, C, respectively) on the 15th, 35th, and 45th day. The mutations of *SNTA1* were usually involved in the variation of QT interval. *SNTA1* was thought to be the long QT syndrome (LQT) type 12 gene. A variation of QT interval was simulated by the FPD using MEA. The FPD of KO were measured with the MEA (Fig.4D, E, respectively). The results confirmed that the FPD values of the KO were shorter than those of WT.

The KO exhibited calcium transient abnormality

The abnormalities in structural components of cardiomyocytes are usually caused by the abnormality of calcium handling. We carried out calcium transient test in WT and KO (Fig.5A). The space-averaged calcium transients displaying the parameters measured for analysis of calcium handling (Fig.5B). The results showed that the peak value of calcium release was remarkably decreased in KO (Fig.5C). The time to peak and the decay time of calcium transient were shorter in KO (Fig.5D, E, respectively). In addition, the contraction duration of KO was shorter (Fig.5F). Furthermore, the beating rate of KO was increased (Fig.5G). All of these results confirmed that intracellular calcium handling of KO was abnormal.

Abnormal caffeine-evoked Ca²⁺ release in KO

There was a disorder of intracellular calcium handling in KO with the peak of calcium release decreased. The peak of intracellular calcium release is directly related to the sarcoplasmic reticulum (SR) calcium storage in cardiomyocytes. Therefore, we conducted caffeine-evoked Ca²⁺ release test on the KO (Fig.6A). The results indicated that the peak of caffeine-evoked calcium release was decreased in KO (Fig.6B) with the time of peak and Decay time from 50% peak being shorter (Fig.6C, D, respectively). The calcium ion level in SR was decreased in the KO compared with the WT. The release of calcium ion from SR is related to the contractility of cardiomyocytes. There was a decrease SR calcium level in the KO, the contractility of KO was detected. The contractility of cardiac myocytes was measured using quantification of muscle contraction method (Fig.6E). The results showed that the amplitude of contraction was decreased in KO compared with WT (Fig.6F, G, respectively).

Impairment of calcium handling in KO

We found there was an abnormal calcium handling in KO. We detected the expression of some genes involved in the calcium by qRT-PCR. The result indicated that *SERCA2a*, *BIN1*, and *CASQ2* were abnormal at transcriptional levels (Fig.7A). The *SERCA2a*, *BIN1*, and *CASQ2* levels were further detected using western blots. The results showed that expression level of *SERCA2a* and *CASQ2* decreased, while *BIN1* increased (Fig.7B, C, D, respectively). Overall, there was impairment in calcium handling in KO.

Early application of ranolazine improving the calcium handling of KO

The application of ranolazine significantly promoted the intracellular calcium handling, prevented intracellular calcium overload, and increased calcium loading in SR during diastole. Ranolazine (10 μ M) was added on the 20th day cultured WT and KO for 24 h, respectively. Then, the calcium transient test was performed on the KO (Fig.8A). The result showed the time to peak in KO treated with ranolazine was shorter compared with no-treatment (Fig.8B). The peak of calcium transient test increased in KO treated with ranolazine compared with no-treatment (Fig.8C). The contraction duration became shorter in KO treated with ranolazine compared with no-treatment (Fig.8D). The beating rate was elevated in KO treated with ranolazine compared with no-treatment (Fig.8E). The results of calcium transient test of WT treated with ranolazine were in the supplementary data Fig.3A-E. The results showed that early application of ranolazine elevated the peak of calcium release in KO. We speculated the increasing calcium transient would ameliorate the contraction force. Hence, we detected the contraction force of KO treated with ranolazine. The result showed the contractility force was increased in KO treated with ranolazine (Fig.8F, G respectively). All the results demonstrated that ranolazine improved calcium release from SR in KO at early application.

Discussion

SNTA1 is highly expressed in human heart. As a scaffold protein, SNTA1 binds to the C-terminal of sodium voltage-gated channel alpha subunit 5 (SCN5A) and played a vital regulatory role in SCN5A [5]. Recently, it has been reported that SNTA1 interacted with the N-terminal domain of SCN5A [7]. The MPI was elevated in *Snta1* knockout mice; the results indicated that *Snta1* knockout mice had left ventricular dysfunction and hypertrophy [8]. Our cell model highlighted the indispensable role of SNTA1 in the cardiomyocyte of calcium handling (Fig. 9). In Human Gene Mutation Database (HGMD), diseases caused by *SNTA1* point mutation, including LQT, Brugada, sudden infant death syndrome, cardiomyopathy, atrial fibrillation, etc., were recorded. For elucidating the functions of SNTA1, CHO, 293T, and mice were used as research model. The major limitation is the transition from animal to human studies due to the species differences. The results of animal experiments do not completely replace human experiments. In general, it is difficult to obtain a large number of human cardiomyocytes. The hESCs differentiated into cardiomyocytes provide an opportunity to investigate cardiac development and disease as well as providing a platform to perform drug and toxicity tests.

Currently, there are three methods for myocardial differentiation, including EB differentiation method, heart organoids method, and small molecule inhibitors differentiation method. Every method has own characteristics. Traditional spontaneous 3D method by EB differentiation of hESCs into cardiomyocytes is inefficient. It is not easy to obtain a single-type cardiomyocyte. Heart organoids technique simulates the whole organ and is not conducive to the study of the ventricular muscle alone [16]. A single type of human ventricular-like muscle can be obtained using small molecule inhibitors, and the ventricular-like muscle can be purified using metabolic purification method *in vitro* [17]. The H9SNTA1KO was established using the CRISPR-Cas9 system, and then induced the H9SNTA1KO to differentiate into

cardiomyocytes using small molecule inhibitors. The result confirmed that *SNTA1*-deficient did not affect the differentiation of cardiomyocytes and subtypes of cardiomyocyte using flow cytometry assay.

Furthermore, Transcriptomic discrepancies were investigated between KO and WT using RNA-seq, after cardiomyocytes were purified using the metabolic method [18]. Analysis of RNA-seq data revealed that KO had activation of genes associated with hypertrophy. qRT-PCR and immunofluorescence quantitative analysis on genes associated with the structure of cardiomyocytes were performed. Thus, the activation of genes associated with cardiac hypertrophy was confirmed. The results suggested that the expression of structural genes was enhanced on the 45th day of cardiac differentiation. The KO exhibited hypertrophy phenotype like the *Snta1*-knockout animal's cardiac muscle. We also found that TNNT2, MYH7, MYH6, and MYH7/MYH6 increased in transcriptional level on the 45th day of cardiac differentiation. In the HGMD, point mutations in *SNTA1* were associated with arrhythmia. *SNTA1* encodes the α 1-syntrophin that is responsible for LQT12 in an autosomal-dominant manner (*SNTA1* is a disputed-evidence or limited-evidence gene in genotype–phenotype correlations [19]). In common, the disease phenotypes of KO were more serious than the other point mutation. The disease phenotype was most serious in the knockout cell or knockout animal, but the *Snta1*-knockout mice did not exhibit any arrhythmia in Kim's paper [8]. When human cardiomyocytes were used, the arrhythmia might be exhibited. We compared the FPD of KO to the WT using MEA. The parameters of FPD in MEA simulates to the QT intervals of electrocardiograph. With the results indicating that FPD of KO was shorter than that of WT. *SNTA1* point mutations caused complex genetic arrhythmias, including LQT, Brugada, and sudden infant death. *SNTA1* interacted with the N- and C-terminal of SCN5A [2], and also adjusted the density of human Kir2.1, Kir2.2 in cardiomyocyte membrane [7]. *SNTA1* is also involved in the dystrophin/utrophin protein complex and help the target protein to the right location in cardiomyocyte [20]. In our research, the mutation we created terminated *SNTA1* at the 149 amino acid position, resulting in the termination of *SNTA1* at the PDZ domain. The short QT syndrome caused by *SNTA1* deficiency may be associated with channel dysfunction.

The diameter of KO was compared with the WT on the 15th, 35th, and 45th day of cardiac differentiation. The result that the diameter of KO was not different from the WT. *SNTA1* binds to the dystrophin glycoprotein complex through its PH2 and SU domains. *SNTA1* also binds to the cytoskeleton protein, such as actin, microfilament, and microtubule [21]. The organization of cytoskeleton in KO may be disrupted by *SNTA1* deficiency, but the diameter of KO did not be influenced. However, we only harvested ventricular-like muscle cells with no morphological maturity. We speculated that the immature morphological features of the cardiomyocytes influenced the result of diameter measurement.

The function of cardiomyocytes is precisely dependent on the calcium handling. Calcium is a critical intracellular signaling molecules, which mediates various biological processes, including excitation-contraction coupling (EC), enzyme activity, gene transcription, and cell death [22–23]. Calcium is necessary for heart contraction. Particular attention was paid to the transport and storage of calcium in cardiomyocytes since an abnormal calcium handling plays a key role in the pathogenesis of cardiomyopathy and arrhythmia [24–25]. The EC of cardiomyocytes requires the coordinated transport of

calcium in and out cell. In the physical process of myocardial contraction, Nav1.5, which is a sodium channel on the membrane of ventricular myocytes, is activated by an electrical stimulation from the pacemaker, and then Nav1.5 channel opens and the extracellular sodium flows into the cells forming an ascending branch of action potential. The CACNA1C in T-tubules induced the extracellular calcium influx into cell (calcium sparks) by depolarization of membranes, and calcium influx activated the RyR2 on the SR, with the calcium in SR released through the RyR2 into the cytoplasm. Elevated free calcium in the cytoplasm causes myofilament contraction. In the process of calcium-induced-calcium-release (CICR), there are three proteins to help maintain the function of the T-tubule [26]. The first protein is junctophilin 2 (JPH2), which is the major structural protein in cardiomyocytes between the T-tubules and the SR. It drags the T-tubules closer to SR to form the junctional membrane complex, facilitating CICR [27–29]. The CICR is a critical procedure of EC in ventricular myocytes. The second protein is caveolin 3 (CAV3), a member of caveolin protein family that contribute to the formation of caveolae and provide microdomains for a variety of functional proteins in T-tubules [30–32]. The third protein is bridging integrator 1 (BIN1), one of the membrane scaffolding proteins, which interact between BAR domain and phospholipid acid in the cell membrane to deform the membrane bilayer. BIN1 is not only involved in the formation of the T-tubules, but also transports the calcium voltage-gated channel subunit alpha1 C (CACNA1C) to the cell membrane. A decrease in the expression of BIN1 resulted in decrease in trafficking of CACNA1C to the T-tubules [33]. Therefore, rescuing diminished cardiac BIN1 expression could both rescue CACNA1C trafficking and restore normal T-tubule membrane morphology [34]. BIN1 could become a therapeutic target for heart failure [35–36]. When the cytoplasmic free calcium is elevating, calcium binds to troponin C and triggers contraction, and cause the concentrations of intracellular free calcium to elevate from 0~100 nM to ~1 μ M compared with the diastole period [37]. After contraction, SERCA2a is a macromolecular complex on the SR that reuptakes the free calcium back into the SR (intracellular calcium pool), facilitating cardiac relaxation. A small portion of cytosolic calcium is extruded by the NCX (sodium–calcium exchanger), and only a small level of released calcium enters the mitochondria [38].

The increased structural components are usually associated with abnormal calcium handling in cardiac myocytes [39–40]. In our cell model, there was an increase of structural components in KO. Calcium transient test was performed on KO, the results showed that the peak value of KO was lower than the WT (Fig. 5). The peak value of calcium transient is related to the level of calcium in the SR. The calcium load in SR was measured using caffeine-evoked calcium release test to elucidate the effect of SR function. These results showed that the SR of calcium loading decreased in KO (Fig. 6). The contraction of KO was lower than the WT *in vitro*, which is consistent with the result of calcium transient test.

The calcium release is related to the calcium loading in the SR. CASQ2, ASPH, and TRDN form complexes, which is involved in the SR calcium loading [41–43]. We assessed the genes involved in calcium loading and calcium handling. The results demonstrated that the expression of CASQ2, BIN1, and SERCA2a in both the transcriptional and protein levels were significant. The expression level of CASQ2 in KO was lower than WT. CASQ2 is a calcium-binding protein, which exists in the SR of cardiac muscle. It is low-affinity and high-capacity for binding calcium. It binds to 40-50 calcium ions through 60-70 negatively charged amino acid residues [44–45]. CASQ2 is considered to be an essential protein for

the storage and release of calcium in the SR [46]. The point mutations in *CASQ2* can lead to ventricular tachycardia [47–48], cardiomyopathy [49], catecholamine ventricular tachycardia [50], and the other heart-related diseases. The *Casq2*-knockdown mice only showed obvious SR calcium leak [51] and displayed early mortality [52]. The decrease of *CASQ2* expression in KO led to the decrease of calcium loading in the SR during diastole. We speculated that decreased of *CASQ2* expression maybe disturbed by the disorder of calcium handing. The specific mechanism will be verified by follow-up research. BIN1 was another scaffold protein under the cellular membrane. We speculated the high expression of *BIN1* was a kind of cellular compensatory protection. In addition, the expression of *SERCA2a* was decreased in KO, which showed that the SR weakly reuptakes free calcium from cytoplasm. There was a decrease expression of *CASQ2*, an increase expression of *BIN1*, and a decrease expression of *SERCA2a* in the KO. There was an abnormal calcium homeostasis in our cell model.

A decrease SR calcium loading of cardiomyocytes has no effective remedy currently. The metabolic support therapy could help to relieve the symptoms of cardiomyocyte diseases [53–55]. The metabolic support efforts alleviate the abnormal energy supply of hypertrophic, promote the survival rate, and improvement of myocardial cell functions [56]. Ranolazine inhibited the action of late sodium current, improved diastolic calcium overload, and the relaxation of ventricular myocytes [57–58]. It also enhanced the NCX extroversion mode, indirectly promoted the intracellular calcium excretion, and reduced high free intracellular calcium in diastole [59–60]. We utilized 10 μ M ranolazine to improve phenotype of KO on the 20th day of cardiac differentiation. The results showed that the peak of calcium transient amplitude and the contraction force increased in KO. Overall, the early application of ranolazine improved the phenotype of KO.

Limitation

The cardiomyocytes we obtained had no T-tubules structure and that was unlike the mature cardiomyocytes. Our research was only performed in the two-dimensional (2D) cell culture *in vitro*.

Conclusions

In the study, human *SNTA1*-knockout cell model was established using CRISPR-Cas9 system. The cell model can be used to study the damage of myocardial structure and abnormal function caused by the *SNTA1* deficiency *in vitro*. It provides a disease model for intracellular calcium homeostasis of cardiomyocytes study. It confirms that abnormal intracellular calcium handling was the core disease mechanism of *SNTA1*-deficient cardiomyocytes. This suggests that the strategy of maintenance of the intracellular calcium homeostasis is a key target in treatment of *SNTA1*-deficient myocardial diseases.

Abbreviations

Symbol	Full Name
SNTA1	α -1-syntrophin
hESCs	human embryonic stem cells
H9SNTA1KO	<i>SNTA1</i> -deficient hESCs
WT	Wilde type
KO	<i>SNTA1</i> -deficient cardiomyocytes
PAM	protospacer adjacent motif
CRISPR-Cas9	clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9
TNNT2	troponin T2, cardiac type
SSEA4	stage-specific embryonic antigen-4
NANOG	Nanog homeobox
SOX2	SRY-box transcription factor 2
DPPA4	developmental pluripotency associated 4
OCT-4	POU class 5 homeobox 1
MYL2	myosin light chain 2
KEGG	Kyoto Encyclopedia of Genes and Genomes
MYH6	myosin heavy chain 6
MYH7	myosin heavy chain 7
MYL7	myosin light chain 7
p-CaMKII	p-Ca ²⁺ /calmodulin-dependent protein kinase II
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
FPD	short field potential duration
EC	excitation-contraction coupling
SR	sarcoplasmic reticulum
CACNA1C	calcium voltage-gated channel subunit alpha1 C
SERCA2a	ATPase sarcoplasmic/endoplasmic reticulum Ca ²⁺ transporting 2
BIN1	bridging integrator 1
CASQ2	CASQ2

RyR2	ryanodine receptor 2
CICR	calcium-induced-calcium-release
JPH2	junctophilin 2
CAV3	caveolin 3
NCX	sodium–calcium exchanger
ASPH	aspartate beta-hydroxylase
TRDN	triadin

Declarations

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Competing interests

The authors declare that they have no competing interests.

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

All data transparency

Code availability

The RNA-seq data associated with this manuscript can be inquired from biosys.bgi.com (Username: F20FTSNCWLJ4149_PEOxzmR).

Authors' contributions

DT conceived the idea and designed the experiments; DT and ZY performed the cell experiments and data analysis. DT and SL performed the manuscript preparation. JHF is responsible for the collection and assembly of data. ZY contributed to the molecular experiments. LC contributed to the function analysis. SLL has been helping with revisions. All authors read and approved the final manuscript.

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Figures

Figure 1

Establishment of homozygous *SNTA1*-deficient hESCs

A. Schematic of the sgRNA designed to the PH1 region in *SNTA1* demonstrates that one adenine nucleotide is inserted into *SNTA1* before the PAM sequence.

B. The DNA molecules of H9 and H9SNTA1KO cells were detected by DNA sequencing. The DNA was extracted and amplified by PCR. The result showed one adenine nucleotide was inserted into the *SNTA1* before the PAM sequence in H9SNTA1KO genome.

C. Utilizing tools from Synthego website to assess the gene editing ratio. The upper graph offered the abstract of indel ratio with the results showing a knockout-score of 99%. The relative contribution of sequence is a nucleotide was inserted before the PAM sequence. The graph below offered the analysis of sequence using the Synthego tools. The red dotted line is under the PAM sequence in control sample diagram. The vertical dotted line demonstrated one adenine inserted in the edited sample diagram. The homozygous SNTA1 knockout hESCs were established.

D. Immunofluorescence staining for pluripotency was performed. Both SSEA4 and NANOG were positive in H9SNTA1KO. *SNTA1*-knockout did not influence the pluripotency of hESCs.

Figure 2

H9SNTA1KO differentiated into cardiomyocytes

A. Schematic of hESCs induction into cardiomyocytes using small molecule inhibitors.

B. Image B1 to B3 were hESCs induction into cardiomyocytes using working solutions. Scale bar: 100 μm . Image B4 showed the mass of beating cardiomyocytes on the 10th day of differentiation. Scale bar: 100 μm .

C. The image of KO exhibited purified by metabolic selection using a glucose-free medium supplemented with lactate. Scale bar: 50 μm .

D. Immunostaining of TNNT2 (green) and α -actinin (red) in KO. Scale bar: 7.5 μm .

E. The left graph: Flow cytometry was used to detect a specific cardiac marker, TNNT2. The result demonstrated that the differentiation rate of H9SNTA1KO was similar to the WT without purification. The right graph: Quantification of TNNT2 based on flow cytometry (n = 3). ns; not significant, unpaired two-sided Student's t test.

F. The left graph: Flow cytometry was used to detect a specific ventricular muscle marker, MYL2. The results demonstrated that the yield of WT and KO was similar purified using metabolic selection. The right graph: Quantification of MYL2 of the flow cytometry (n = 3). ns; not significant, unpaired two-sided Student's t test.

Figure 3

Structural components increased in KO

A. The left panel: The RNA molecules of WT and KO were extracted and detected using RNA-seq. The results of KEGG pathway enrichment analysis of these genes showed highly enriched in hypertrophic cardiomyopathy on the 30th day of cardiac differentiation.

The right panel: Heatmap showed the discrepancies of genes involved in structural components in WT and KO as detected by qRT-PCR on the 45th day of cardiac differentiation.

B. Immunostaining of MYL2 (green) in WT and KO and semi-quantification analysis measured on the 45th day of cardiac differentiation. Scale bar: 10 μ m. n = 3 independent experiments. * P < 0.05.

C. Immunostaining of TNNT2 (green) in WT and KO and semi-quantification analysis measured on the 45th day of cardiac differentiation. Scale bar: 5 μ m. n = 3 independent experiments. ** P < 0.01.

D. Immunostaining of α -actinin (red) in WT and KO and semi-quantification analysis measured on the 45th day of cardiac differentiation. Scale bar: 5 μ m. n = 3 independent experiments. * P < 0.05.

Figure 4

The KO had normal diameter and shorter FPD

A. to C. The diameter size of WT and KO were evaluated using flow cytometry on the 15th, 35th, and 45th day of cardiac differentiation.

D. Schematic of the process of microelectrode array (MEA) test.

E. MEA showed the discrepancy of field potential duration (FPD) between WT and KO. n = 9 independent experiments, **** P < 0.0001; unpaired two-sided Student's t test.

Figure 5

The KO exhibited calcium transient abnormality

A. The left panel: The representative line-scan image of WT and KO stained with Fluo-4.

The right panel: Calcium transient profile derived from the left panel.

B. Space-averaged calcium transients showed parameters measured for analysis of calcium handling.

C. to G. Quantification of peak, time to peak, decay time, contraction duration, and beating rate in WT and KO, n=6. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; unpaired two-sided Student's t test.

Figure 6

Abnormal caffeine-evoked Ca^{2+} release in KO

A. The left panel: The representative line-scan image of caffeine-evoked Ca^{2+} release in WT and KO stained with Fluo-4.

The right panel: Ca^{2+} transients profile induced with 10 mM caffeine in Ca^{2+} -free conditions derived from the left panel.

B. to D. Quantification of peak, time to peak, and decay time in caffeine-evoked Ca^{2+} transients WT and KO, n = 6. *** $P < 0.001$; **** $P < 0.0001$; unpaired two-sided Student's t test.

E. Schematic of the process of the contractility assess.

F. Contractility graph of WT and KO.

G. Quantification of contractility in WT and KO based on panel F. n = 3 independent experiments. ** $P < 0.01$; unpaired two-sided Student's t test.

Figure 7

Impairment of calcium handling in KO

A. Heatmap showed the discrepancies about the expression of genes involved in calcium handling in WT and KO.

B.to D. Immunoblot analysis of SERCA2a, BIN1, and CASQ2 in WT and KO.

Figure 8

Early application of ranolazine improving the calcium handling of KO

A. The left panel: The representative line-scan image of the KO and KO+R (KO treated with 10 μM ranolazine) stained with Fluo-4 on the 20th day of cardiac differentiation.

The right panel: Calcium transient profile derived from the left panel.

B. to E. Quantification of time to peak, peak, contraction duration, and beating rate in KO and KO+R (KO treated with 10 μ M ranolazine), n = 6. ** $P < 0.01$; **** $P < 0.0001$; unpaired two-sided Student's t test.

F. Contractility graph of KO and KO+R.

G. Quantification of the panel F. n = 3 independent experiments. * $P < 0.05$. unpaired two-sided Student's t test.

Figure 9

Schematic illustrating the disease model of KO

Abnormal calcium handling in KO.

Supplementary Files

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