Enhancement of Spontaneous Activity by HCN4 Overexpression in Mouse Embryonic Stem Cell-Derived Cardiomyocytes - A Possible Biological Pacemaker

Yukihiro Saito1, Kazufumi Nakamura1*, Masashi Yoshida1, Hiroki Sugiyama1, Tohru Ohe2, Junko Kurokawa3, Tetsushi Furukawa3, Makoto Takano4, Satoshi Nagase1, Hiroshi Morita1,5, Kengo F. Kusano6, Hiroshi Ito1

1 Department of Cardiovascular Medicine, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan, 2 Sakakibara Heart Institute of Okayama, Okayama, Japan, 3 Department of Bio-informational Pharmacology, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan, 4 Department of Physiology, Kurume University School of Medicine, Kurume, Japan, 5 Department of Cardiovascular Therapeutics, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan, 6 Department of Cardiovascular Medicine, National Cerebral and Cardiovascular Center, Osaka, Japan

* ichibun@cc.okayama-u.ac.jp

Abstract

Background

Establishment of a biological pacemaker is expected to solve the persisting problems of a mechanical pacemaker including the problems of battery life and electromagnetic interference. Enhancement of the funny current (If) flowing through hyperpolarization-activated cyclic nucleotide-gated (HCN) channels and attenuation of the inward rectifier K+ current (I\textsubscript{K1}) flowing through inward rectifier potassium (Kir) channels are essential for generation of a biological pacemaker. Therefore, we generated HCN4-overexpressing mouse embryonic stem cells (mESCs) and induced cardiomyocytes that originally show poor I\textsubscript{K1} currents, and we investigated whether the HCN4-overexpressing mESC-derived cardiomyocytes (mESC-CMs) function as a biological pacemaker in vitro.

Methods and Results

The rabbit Hcn4 gene was transfected into mESCs, and stable clones were selected. mESC-CMs were generated via embryoid bodies and purified under serum/glucose-free and lactate-supplemented conditions. Approximately 90% of the purified cells were troponin I-positive by immunostaining. In mESC-CMs, expression level of the Kcnj2 gene encoding \textit{I}\textsubscript{K1} currents that are responsible for stabilizing the resting membrane potential, was lower than that in an adult mouse ventricle. HCN4-overexpressing mESC-CMs expressed about a 3-times higher level of the \textit{Hcn4} gene than did non-overexpressing mESC-CMs. Expression of the Cacna1h gene, which encodes T-type calcium channel and generates diastolic depolarization in the sinoatrial node, was also...
confirmed. Additionally, genes required for impulse conduction including Connexin40, Connexin43, and Connexin45 genes, which encode connexins forming gap junctions, and the Scn5a gene, which encodes sodium channels, are expressed in the cells. HCN4-overexpressing mESC-CMs showed significantly larger If currents and more rapid spontaneous beating than did non-overexpressing mESC-CMs. The beating rate of HCN4-overexpressing mESC-CMs responded to ivabradine, an If inhibitor, and to isoproterenol, a beta-adrenergic receptor agonist. Co-culture of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) with aggregates composed of mESC-CMs resulted in synchronized contraction of the cells. The beating rate of hiPSC-CMs co-cultured with aggregates of HCN4-overexpressing mESC-CMs was significantly higher than that of non-treated hiPSC-CMs and that of hiPSC-CMs co-cultured with aggregates of non-overexpressing mESC-CMs.

Conclusions
We generated HCN4-overexpressing mESC-CMs expressing genes required for impulse conduction, showing rapid spontaneous beating, responding to an If inhibitor and beta-adrenergic receptor agonist, and having pacing ability in an in vitro co-culture system with other excitable cells. The results indicated that these cells could be applied to a biological pacemaker.

Introduction
The establishment of a biological pacemaker is expected to solve the persisting problems of a mechanical pacemaker including the problems of battery life, lead breaks, infection, electromagnetic interference, appearance, and heart rate response during exercise.

Cardiac pacemaker activity originates in the sinus node. Spontaneous diastolic depolarization in phase 4 of the action potential is initiated in the sinus node, and the electrical impulse is conducted through the atria to the atrioventricular node. The sinus node can generate impulses faster than those generated in other areas. Different kinds of ionic currents are involved in the formation of spontaneous diastolic depolarization of the sinus node [1-6].

Among them, the funny current (If) flowing through hyperpolarization-activated cyclic nucleotide-gated potassium (HCN) channel is robustly present in the sinus node [7]. Since the HCN4 isoform is mainly expressed in the sinus node [8, 9] and contains the cAMP-binding domain, adrenergic stimulation followed by intracellular cAMP accumulation increases the heart rate through augmentation of If currents [7, 10]. We previously reported a mutation of HCN4 found in a patient suffering from sick sinus syndrome (SSS) [11]. Other groups also reported an association between SSS and mutation of HCN4 [12, 13]. Therefore, HCN4 is thought to be the key pacemaking ion channel [8].

In contrast, the inward rectifier K+ current (IK1), which maintains resting membrane potential in working myocytes and antagonizes spontaneous activity, is negligibly small in sinus node cells [14, 15]. Interestingly, genetic suppression of IK1 can give rise to pacemaker activity in ventricular myocytes [16]. Despite the presence of IK1, Purkinje fibers show If-dependent pacemaking, but the pacemaking is slow and not robust. Thus, one of the most important factors responsible for the rapid and robust pacemaking of the sinus node is the absence of IK1 [1, 17].

That is, enhancement of If flowing through HCN channels and attenuation of IK1 flowing through Kir channels are required for pacemaker cells to undergo spontaneous diastolic
depolarization. Therefore, we generated HCN4-overexpressing mouse embryonic stem cells (mESCs) and induced cardiomyocytes that originally show weak \( I_{K1} \) currents to achieve two prerequisites for forming spontaneous diastolic depolarization, large \( I_f \) currents and small \( I_{K1} \) currents, and investigated whether the HCN4-overexpressing mESC-derived cardiomyocytes (mESC-CMs) function as a biological pacemaker \textit{in vitro}.

**Materials and Methods**

**Plasmid construction**

Vertebrate HCN4 proteins are highly conserved [11] and we used the rabbit \( Hcn4 \) in mESCs due to the traceability. CAG promoter-IRES-\textit{EGFP} construct in pCAGIG (Addgene \#11159) and kanamycin/neomycin resistance gene (\( \text{Kan}^R/\text{Neo}^R \)) in pIRES2-AcGFP1 vector (Clontech) were amplified via the polymerase chain reaction (PCR) method using PrimeSTAR GXL DNA polymerase (TaKaRa), purified using a QIAquick PCR Purification Kit (Qiagen), and ligated using an In-fusion HD enzyme (Clontech), i.e., pCAGIG- \( \text{Kan}^R/\text{Neo}^R \). Rabbit \( Hcn4 \) cDNA in the pCI vector (previously reported and kindly provided by Dr Takano) [9, 11] was amplified via the PCR method and ligated with pCAGIG-\( \text{Kan}^R/\text{Neo}^R \), i.e., pCAGIG-rabbit \( Hcn4 \)-\( \text{Kan}^R/\text{Neo}^R \). PCR primers are shown in Table 1.

**Maintenance of mESCs**

Mouse ESCs (cell line CGR8; ECACC) were cultivated on 0.1% gelatin-coated plates in high-glucose Dulbecco’s Modified Eagle’s medium (DMEM; GIBCO) supplemented with 20% fetal bovine serum (Sigma), 50 \( \mu \)M \( \beta \)-mercaptoethanol (2-ME), MEM nonessential amino acids solution (NEAA, GIBCO), 1000 units/mL leukemia inhibitory factor (LIF; WAKO), and 100 \( \mu \)g/mL kanamycin (Sigma) in a humidified atmosphere containing 5% \( \text{CO}_2 \).

**Nucleofection**

CGR8 cells were harvested using 0.05% trypsin/EDTA. Two \( \mu \)g of a non-linearized vector was used for nucleofection (Amaxa Nucleofector II; A-023, which is optimized for a nucleofection program for mouse ESCs) and HCN4-overexpressing ESCs were selected using a medium containing 400 \( \mu \)g/ml G418 (Roche Applied Science) for 7 days. Three stable clones that were resistant to G418 and were EGFP-positive were selected and expanded.

**Electrophysiology**

The funny current (\( I_f \)) was recorded at room temperature by using the perforated patch-clamp technique. Cells were superfused with a bath solution containing (in mM): 132 NaCl, 4.8 KCl, 2.0 CaCl\(_2\), 1.2 MgCl\(_2\), 1.0 BaCl\(_2\), 2.0 MnCl\(_2\), 5.0 D-glucose, and 10 Hepes; pH 7.4. Pipettes (2–4 M\( \Omega \) resistances) were filled with a pipette solution containing (in mM): 110 K-aspartate, 5.0 K\(_2\)-ATP, 11 EGTA, 1.0 CaCl\(_2\), 1 MgCl\(_2\), and 5 Hepes; pH 7.2. Then 0.3 mg/mL Amphoteracin B was added to the pipette solution to achieve patch perforation (10–20 M\( \Omega \); series resistance). The \( I_f \) current was activated by a standard activation protocol. \( I_f \) currents through activated HCN4 channels were obtained during hyperpolarizing test pulses of 5 seconds between -45 and -125 mV in 20 mV increments from a holding potential of -35 mV.

Action potentials (APs) were also measured with the perforated patch-clamp technique. mESC-CMs were dissociated using 0.25% Trypsin/0.02% EDTA for 5 minutes and resuspended in high-glucose DMEM supplemented with 20% FBS, 50 \( \mu \)M 2-ME, and NEAA. Then 1 \( \times 10^6 \)/cm\(^2\) cells were replated on Matrigel-coated cover glasses and incubated for 48 hours. AP recordings were performed on monolayer cardiomyocytes. APs were measured respectively by the
perforated patch-clamp technique using an Axopatch 200B amplifier (Molecular Devices). Data acquisition of APs were performed with pClamp10.2/Clampfit (Axon Instruments). APs were measured using a modified Tyrode’s solution containing (in mM): 140 NaCl, 5.4 KCl, 1.8 CaCl2, 1.0 MgCl2, 5.5 glucose, and 5.0 HEPES; pH 7.4 (NaOH). The pipette solution contained (in mM) 110 DL-aspartic acid, 30 KCl, 1 CaCl2, 5 ATP-Mg, 5 Creatine P-Na, 5 HEPES, and

| Genes | Sequences | Annealing temperature | Cycles |
|-------|-----------|-----------------------|--------|
| Subcloning | CAG-iRES-EGFP forward | CGGTTCCCTCTAGTTATTAATAGTAATCAATTACG | 71 | 25 |
| | reverse | ATATTGGAAGTGTCAGGGGATCT | |
| | KanR/NeoR forward | CTGCAGTTCAATATGTAATCCGCTCA | 71 | 25 |
| | reverse | ATAACTAGAGGACTGCAATGACGCC | |
| | rabbit Hcn4 forward | CGAATTCACTAGGACAGCGTGGGCGTC | 71 | 25 |
| | reverse | CTCGAGTCACAGGTTGACGGCGAGT | |

**RT-PCR**

| Genes | Sequences | Annealing temperature | Cycles |
|-------|-----------|-----------------------|--------|
| Gapdh | forward | CATGGCCCTTCGTTTCTTTA | 58 | 25 |
| | reverse | TGCTGCTTACCACCTTCTTTT | |
| Oct4 | forward | AGATCACCTACGTCGCAAT | 57 | 25 |
| | reverse | AAGGTGCTGCTAGCCTTCTT | |
| Nanog | forward | GCAAAGACTTCCCTCCAT | 57 | 25 |
| | reverse | ATACCCACCTGGTGTGCTA | |
| Nkx2.5 | forward | CGACCGAAGCCAGCGTTGCT | 57 | 35 |
| | reverse | CCGCTGTCGCTTCGACTTG | |
| Tnnt2 | forward | CAGGAAAAGTCTAGAAGCA | 62 | 35 |
| | reverse | GCCTCCACTATCACAACAGG | |
| Scn5a | forward | CTTGGCCCAAGTCAACCTGCTT | 57 | 35 |
| | reverse | CGGACAGGGGCAAATACTCAAATG | |
| Cacna1h | forward | GCTGTTCGAGGAGCTAGAAT | 57 | 35 |
| | reverse | CGAAGGTGACGAGAACTAGACG | |
| rabbit Hcn4 | forward | GTACTCCTACGCGCTTGCTC | 57 | 30 |
| | reverse | GCTTCTCTCTGAGAATCTT CT | |
| mouse Hcn4 | forward | GGATTACCCACCCCTCAG | 60 | 30 |
| | reverse | GTCTCGGCAAGTCAAGTGGGGAAT | |
| Gja5 | forward | CCACGGAGAAGAAATGTCTTCA | 55 | 35 |
| | reverse | TGCTGCTGCAAGAGTCTAAG | |
| Gja1 | forward | TGGGGGAAAGGCGGTGAG | 55 | 35 |
| | reverse | CTACTCCTGGCCTGAGGAAGGT | |
| Gjc1 | forward | ATACCTCCTGCTGTCACCTT | 57 | 35 |
| | reverse | CTCTTCTGACGGGCTCCTCC | |

**quantitative PCR**

| Genes | Sequences | Annealing temperature | Cycles |
|-------|-----------|-----------------------|--------|
| β-Actin | forward | GGAGGAGGGGTAGATTTTCTT | 61 | 40 |
| | reverse | GTTGCTGACTTGTATTGGCTT | |
| Kcnj2 | forward | GCTGTCTGCAAGGCCAAGG | 61 | 40 |
| | reverse | TTCCCTCCTCCAGAGAGACATGCT | |
| Gapdh | forward | CATGGCCCTTCGTTTCTTTA | 55 | 40 |
| | reverse | TGCTGCTGCTGCTGCTGCTT | |
| total Hcn4 | forward | CCCATGCTGCAGGACTT | 55 | 40 |
| | reverse | GCTTCCCCGAGAGGTTTAT | |

doi:10.1371/journal.pone.0138193.t001
10 EGTA; pH 7.25 (KOH). To achieve patch perforation (series resistance: 10–20 MΩ), amphotericin B (0.3 mg/mL) (Nacalai Tesque, Inc., Kyoto) was added to the pipette solution. Temperature was maintained at 35–36°C by a TC-344B dual channel heating system (Warner Instruments).

Cardiac differentiation of mESCs
Emryoid bodies (EBs) were formed by cultivating 500 mESCs with 0.5 mmol/L 2-O-alpha-D-glucopyranosyl-L-ascorbic acid (AA-2G; Hayashibara Biochemical Labs) and without LIF in a hanging drop for 5 days (culture day 0 to day 5)[18]. On day 5, EBs were collected and plated on a 0.1% gelatin-coated dish with a medium containing 0.25 mM AA-2G and 10 μM IWR-1-endo (WAKO). On day 7, the medium was exchanged a medium consisting of modified Eagle’s medium (MEM; GIBCO), Insulin-Transferrin-Selenium-A supplement (100×; ITS-A supplement; GIBCO), and 100 μg/mL kanamycin. The medium was changed every other day after plating on the dishes.

On day 14, the medium was changed to no glucose DMEM (Gibco) with 4 mM L-sodium lactate (Sigma-Aldrich)[19]. Until day 21, the medium was changed every other day. On day 21, EBs were treated with 0.25% Trypsin/EDTA (invitrogen) at 37°C for 5 minutes and dissociated. Dissociated cells were resuspended in 3 ml of medium and loaded onto a discontinuous Percoll (GE Healthcare) gradient, containing 20 mM HEPES and 150 mM NaCl. The gradient consisted of 3 ml of a 40.5% Percoll layer over 3 ml of a 58.5% Percoll layer. After centrifugation at 1,500 xg for 30 minutes, cell layers were apparent. Cells at a 58.5% layer were collected [20]. The purified cells were resuspended in high-glucose DMEM supplemented with 20% FBS, 1% NEAA, and 100 μM 2-ME.

Generation of human induced pluripotent stem cells (hiPSCs)
To investigate pacing ability of HCN4-overexpressing mESC-CMs, we used a co-culture system with human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs).

Human dermal fibroblasts (HDFs) were obtained from abdominal skin of 41-year-old healthy Japanese male, the corresponding author of this article, by punch biopsy. The skin biopsy sample was dissected into 10 evenly sized pieces. The dissected pieces were plated in 6-well plates containing DMEM supplemented with 10% FBS and 0.1 mg/mL kanamycin and placed in a 37°C incubator. The medium was changed every 3 days. After reaching confluence, the cells were dissociated with trypsin/EDTA and passaged.

hiPSCs were generated from HDFs as previously reported [21]. HDFs were transfected with human OCT4, SOX2, KLF4 and c-MYC cDNA (plasmid #17217, #17218, #17219, and #17220, Addgene) twice using retroviral vectors produced by Platinum Retrovirus Expression System, Pantznic (VPK-302, Cell Biolabs, Inc). Two days after retrovirus transfection, 1 x 10^5 HDFs dissociated with 0.05% trypsin/0.02% EDTA were replated on 10 cm dishes with an SNL feeder layer (07032801, ECACC) and maintained in DMEM/F12 (GIBCO) supplemented with 20% knockout serum replacement (GIBCO), 100 μmol/L 2-ME, NEAA, 10 ng/mL basic fibroblast growth factor (bFGF; WAKO), 0.05 mmol/L AA-2G and 100 μg/mL kanamycin (Sigma). Twenty-five days after replating, hiPSC colonies were selected. The hiPSC colonies were expanded on the SNL feeder layer. For feeder-free culture, hiPSCs were maintained on human embryonic stem cell-qualified Matrigel (Corning) in TeSRE8 medium (Stemcell Technologies).

Cardiac Differentiation of hiPSCs
hiPSCs were dissociated into single cells with StemPro Accutase Cell Dissociation Reagent (GIBCO) and seeded on Matrigel-coated dishes at 1 x 10^3/cm² in TeSRE8 medium
supplemented with 10 μM Y27632 (WAKO). Five days after plating, at day 0, cells were treated with 6 μM CHIR99021 (Cayman) and 50 μM AA-2G in RPMI medium (GIBCO) supplemented with B27-insulin supplement (GIBCO) and kanamycin. After 24 hours, at day 1, the medium was changed to RPMI supplemented with B27-insulin and 50 μM AA-2G. On day 3, 5 μM IWR-1-endo (WAKO) was added and was removed during the medium change on day 5. Cells were maintained in RPMI supplemented with B27 supplement (GIBCO) starting from day 7, with the medium was changed every 2 or 3 days. On day 28, cardiomyocyte purification was performed by the Percoll gradient procedure mentioned above.

**Reverse Transcription-PCR (RT-PCR) and quantitative PCR**
Total RNA from mESC-CMs was extracted using a Trizol Plus RNA Purification Kit (Invitrogen). Complementary DNA was synthesized from 1 μg of total RNA using a QunatiTect Reverse Transcription Kit (QIAGEN) as prescribed in the manual and subjected to PCR amplification.

Taq DNA polymerase (Roche Applied Science) was used for RT-PCR and PCR products were subjected to electrophoresis in 2% agarose gels and stained with ethidium bromide. RT-PCR experiments were performed twice in each of the 3 cell lines. SYBR Green PCR Master Mix and Applied Biosystems 7300 Real-Time PCR Systems (Applied Biosystems) were used for quantitative PCR (q-PCR). The q-PCR data were processed with a standard curve method. PCR primers are shown in Table 1. The q-PCR experiments were performed in triplicate in each 3-cell line.

**Immunocytofluorescence**
mESCs, mESC-CMs and hiPSC-CMs were plated on matrigel-coated cover glasses and were fixed in 4% paraformaldehyde. Cells were stained with primary antibodies against OCT4 (1:50 dilution, Santa Cruz), EGFP (1:200 dilution, Frontier Institute), α-Actinin (1:800 dilution, Sigma EA-53), Troponin I (1:50 dilution, Santa Cruz), MLC-2v (1:50 dilution, ProteinTech Group) and MLC-2a (1:50 dilution, Synaptic Systems). Secondary antibodies were FITC-conjugated rabbit anti-goat IgG antibody, TRITC-conjugated rabbit anti-mouse IgG antibody, TRITC-conjugated swine anti-rabbit IgG (1:20 dilution, DAKO), and Alexa Fluor 488 goat anti-mouse IgG (1:200 dilution, Molecular Probes). Nucleus staining was performed with Hoechst 33342 (1:2500 dilution, Molecular Probes). F-actin staining was performed with Rho-damine Phalloidin (1:200 dilution, Molecular Probes).

**Counting beating rates of mESC-CMs**
The purified mESC-CMs were plated on a laminin (Sigma Aldrich)-coated 96-well plate at 4 x 10^4 cells/well in high-glucose DMEM supplemented with 20% FBS, 1% NEAA, and 100 μM 2-ME and were incubated for 4 days.

We counted spontaneous beating frequencies and examined responses to ivabradine and isoproterenol.

**Counting beating rates of hiPSC-CMs in a co-culture system with mESC-CMs**
Aggregates containing 1 x 10^4 mESC-CMs were made by using the hanging drop method for 3 days. The purified hiPSC-CMs were plated on a Matrigel-coated 24-well plate at 2 x 10^4 cells/well and maintained in RPMI supplemented with B27. Three days after plating of hiPSC-CMs,
the aggregate of mESC-CMs was plated on the well layered with hiPSC-CMs. After 7 days, synchronized beating rates of hiPSC-CMs away from aggregates were counted.

Statistical analysis
All data are expressed as means ± SD. Statistical analysis was performed by student’s t test for unpaired data or one-way ANOVA with comparison of different groups by Dunnett’s post hoc test. Values of P < 0.05 were considered to be significant.

Results
Generation of mESC lines stably overexpressing rabbit Hcn4
A CAG promoter-rabbit Hcn4-IRES-EGFP/SV40 promoter-neomycin resistance gene plasmid vector (HCN4/EGFP vector) (Fig 1A) or CAG promoter-IRES-EGFP/SV40 promoter-neomycin resistance gene plasmid vector (EGFP vector) was transfected in mESCs, and colonies of cells that were resistant to G418 and were EGFP-positive were selected (Fig 1B and S1 Fig). We generated 3 mESC lines in each group (HCN4/EGFP or EGFP-stably-transfected mESCs group). All 3 selected HCN4/EGFP-stably-transfected mESC lines expressed rabbit Hcn4 (S2 Fig).

As shown by immunostaining, RT-PCR and q-PCR, expression levels of Oct4 (Figs 1C and 2B and S2 and S3 Figs) and Nanog (Fig 2B and S2 and S3C Figs), which are essential for maintaining the self-renewing and undifferentiated state in mESCs, were not influenced by HCN4 overexpression in any of the 3 cell lines.

If currents through activated HCN channels could be obtained during a hyperpolarizing test in HCN4-overexpressing mESCs (in HCN4/EGFP mESCs-2 cell line, n = 4) but not in non-overexpressing mESCs (in EGFP mESCs-1 cell line, n = 4) (Fig 1D).

Fig 1. Generation of mESC lines stably overexpressing rabbit Hcn4. A. A transfection construct bearing the rabbit Hcn4-IRES-EGFP cassette. B. Representative living mESCs observed by phase contrast microscopy (a to c) and fluorescence microscopy (d to f). Hcn4/EGFP or EGFP-stably-transfected mESCs (HCN4/EGFP mESC-2 and EGFP mESC-1 cell lines) were positive for EGFP (green) fluorescence (e and f). C. Immunofluorescence staining of a pluripotency marker, OCT4, in Hcn4/EGFP-stably-transfected mESCs (a to c) and EGFP-stably-transfected mESCs (d to f). OCT4 was expressed in both mESC lines. Bar = 50 μm. D. Measurement of If currents in EGFP-stably-transfected mESCs (a) and Hcn4/EGFP-stably-transfected mESCs (b). Activation of the If current was demonstrated in Hcn4/EGFP-stably-transfected mESCs. If currents through activated HCN channels were obtained during hyperpolarizing test pulses of 5 seconds between -45 and -125 mV in 20 mV increments from a holding potential of -35 mV.

doi:10.1371/journal.pone.0138193.g001
Establishment of purified HCN4-overexpressing mESC-CMs

We generated 3 mESC lines in each group. In all mESCs, cardiac differentiation was performed well via EB formation with or without HCN4 overexpression. Cardiomyocytes were purified by changing culture media to glucose-free and lactic acid-supplemented media. Most of the collected cells spontaneously beat and expressed cardiac sarcomere proteins as assessed by immunostaining: non-overexpressing mESC-CMs, 91.6 ± 6.5% α-actinin positive and 90.2 ± 6.7% troponin I-positive; HCN4 overexpressing mESC-CMs, 86.2 ± 1.3% α-actinin positive and 88.8 ± 6.9% troponin I-positive. Proportions of α-actinin, troponin I, myosin light chain (MLC)-2v and MLC-2a-positive cells were not significantly different between the non-overexpression and HCN4 overexpression groups (Fig 2A).

RT-PCR showed that undifferentiated markers (Oct4 and Nanog) had disappeared and that cardiac markers (Nkx2.5 and Tnnt2) were positive in all 3 mESC-CM lines in each group (HCN4/EGFP or EGFP-stably-transfected mESC-CM group) (Fig 2B and S2 Fig). Expression of the Cacna1h gene, which encodes T type calcium channel and generates diastolic depolarization in the sinoatrial node, was also confirmed. Additionally, genes required for impulse conduction including Connexin40, Connexin43, and Connexin45 genes, which encode connexins forming gap junctions, and the Snc5a gene, which encodes sodium channels, are expressed in the cells (Fig 2B and S2 Fig).

q-PCR showed that rabbit Hcn4 mRNA levels were not significantly different among the 3 HCN4/EGFP mESC-CM lines (S2B Fig). HCN4-overexpressing mESC-CMs (HCN4/EGFP
Biological Pacemaker by HCN4-Overexpressing ESC-CMs

mESC-CMs) expressed a 3-times higher level of Hcn4 than did non-overexpressing mESC-CMs (EGFP mESC-CMs) (Fig 3A). Both HCN4-overexpressing and non-overexpressing mESC-CMs expressed lower levels of Kcnj2, which is involved in I_K1 maintaining resting membrane potential, than did an adult mouse ventricle (Fig 3B).

Furthermore, HCN4-overexpressing mESC-CMs (HCN4/EGFP mESC-CMs) showed a significantly larger I_f current than did non-overexpressing cells. I_f currents through activated HCN channels were obtained during hyperpolarizing test pulses of 5 seconds between -45 and -125 mV in 20 mV increments from a holding potential of -35 mV. B, I_f-V relationship curve in EGFP-(blue line) or Hcn4/EGFP- (red line) overexpressing mESC-CMs.

Fig 3. Quantitative PCR for total Hcn4 and Kcnj2 genes. A. Relative expression of total Hcn4 gene. HCN4-overexpressing mESC-CMs expressed about 3-times higher mRNA levels of total Hcn4 than did EGFP mESC-CMs. B. Relative expression of Kcnj2 gene. In mESC-CMs, the expression level of Kcnj2 was lower than that in an adult mouse ventricle.

doi:10.1371/journal.pone.0138193.g003

Fig 4. Measurement of I_f currents in mESC-CMs. A. Representative I_f currents in EGFP-(left) or Hcn4/EGFP- (right) overexpressing mESC-CMs. HCN4-overexpressing mESC-CMs showed a larger I_f current than did non-overexpressing cells. I_f currents through activated HCN channels were obtained during hyperpolarizing test pulses of 5 seconds between -45 and -125 mV in 20 mV increments from a holding potential of -35 mV. B, I_f-V relationship curve in EGFP-(blue line) or Hcn4/EGFP- (red line) overexpressing mESC-CMs.

doi:10.1371/journal.pone.0138193.g004
Rapid spontaneous beating in HCN4-overexpressing mESC-CMs

HCN4-overexpressing mESC-CMs showed significantly more rapid beating than did non-overexpressing mESC-CMs (Control mESC-CMs, 43.1 ± 4.8 beats/min; HCN4/EGFP mESC-CMs, 87.4 ± 11.9 beats/min; EGFP mESC-CMs, 44.3 ± 11.9 beats/min, n = 8 in each group, $P < 0.0001$) (Fig 5A and 5B).

The beating rate of HCN4-overexpressing mESC-CMs decreased in response to ivabradine, an agent with a selective and specific antagonistic effect on $I_f$ currents, in a dose-dependent manner (no treatment, 105 ± 18.9 beats/min; 3 μM ivabradine, 74.0 ± 14.7 beats/min; 30 μM ivabradine, 37.5 ± 4.1 beats/min, n = 6 in each group, $P < 0.005$) (Fig 5C) and increased in response to isoproterenol, a beta-adrenergic receptor agonist (no treatment, 93.0 ± 8.9 beats/min; 1 μM isoproterenol, 124 ± 6.5 beats/min, n = 6 in each group, $P < 0.0001$) (Fig 5D).

Rapid spontaneous beating in hiPSC-CMs synchronized with beating in HCN4-overexpressing mESC-CMs

We generated hiPSCs in which immunofluorescent staining showed nuclear accumulation of OCT4 and the expression pattern of cell surface markers: SSEA-1 negative, SSEA-4 positive, TRA-1-60 positive and TRA-1-81 positive (S4A Fig). Then we generated hiPSC-CMs that were positive for α-actinin and troponin I by immunofluorescent staining (S4B Fig).

Co-culture of mESC-CMs with aggregates composed of mESC-CMs (Fig 6A) resulted in synchronized contraction of the cells. The beating rate of hiPSC-CMs co-cultured with aggregates of HCN4/EGFP mESC-CMs was significantly higher than that of non-treated hiPSC-CMs and that of hiPSC-CMs co-cultured with aggregates of EGFP mESC-CMs (not shown).
treated, 2.8 ± 3.3 beats/15 sec; co-cultured with aggregates of HCN4/EGFP mESC-CMs, 13.8 ± 0.8 beats/15 sec; co-cultured with aggregates of EGFP mESC-CMs, 9.2 ± 2.3 beats/15 sec, n = 5 in each group (not treated, n = 5; HCN4/EGFP mESC-CMs-1, n = 1; HCN4/EGFP-2, n = 2; HCN4/EGFP-3, n = 2; EGFP mESC-CMs-1, n = 1; EGFP-2, n = 2; EGFP-3, n = 2)) (Fig 6B). These data showed that HCN4-overexpressing mESC-CMs could electronically couple and pace excitable cells in vitro and indicated that these cells could function as a biological pacemaker.

**Discussion**

We established HCN4-overexpressing mESC-CMs as a candidate for a biological pacemaker. We generated the cells in order to achieve two prerequisites for forming spontaneous diastolic depolarization: large $I_f$ currents and small $I_{K1}$ currents. The cells have five specific abilities to become an appropriate biological pacemaker. (1) HCN4-overexpressing mESC-CMs expresses high levels of $Hcn4$ and $Cacna1h$ genes and a low level of the $Kcnj2$ gene. (2) The cells show large $I_f$ currents and rapid spontaneous beating, in other words, rapid impulse generation. (3) Additionally, genes required for impulse conduction including $Connexin40$, $Connexin43$, and $Connexin45$ genes, which encode connexins forming gap junctions and the $Scn5a$ gene, which encodes sodium channels, are expressed in the cells. (4) Furthermore, the beating of the cells responds to an $I_f$ inhibitor and beta-adrenergic receptor agonist. (5) HCN4-overexpressing mESC-CMs have pacing ability in an in vitro co-culture system with other excitable cells.

Several investigators have reported overexpression of HCN channels as a strategy for generating a biological pacemaker. Injection of the $Hcn1-ΔΔ$, $Hcn2$ or $Hcn4$ gene [22–27] and transplantation of $Hcn2$- or Hcn4-overexpressing mesenchymal stem cells [28, 29] have been reported. Furthermore, Tbx18 gene transfer in mature ventricular cardiomyocytes increases HCN4 channel expression and yields pacemaker activity [30]. We also generated HCN4-overexpressing mESC-CMs. The cells show large $I_f$ currents. Enhancement of $I_f$ currents flowing...
through HCN channels is a core strategy for generating a biological pacemaker. Mouse ESC-derived myocytes expressed $I_f$ currents [31]. Since HCN4 expression gradually decreases in the late stage of cardiac differentiation [10, 32], there is concern about whether pacemaker function can be maintained in the long term. Therefore, we overexpressed HCN4 in ESC-CMs. Several investigators also isolated or induced sinoatrial node-like cells that express HCN4 from ESCs [33–35]. Although it is not clear which method is the most useful, our HCN4-overexpressing system is an effective and easy method to obtain a large $I_f$ current and a large amount of cells.

Not only enhancement of $I_f$ current through HCN channels but also attenuation of $I_{K1}$ current and presence of other currents through T-type Ca$^{2+}$ channels are required for diastolic depolarization [36, 37]. Additionally, subsequent propagation to the surrounding working myocardium through connexins and Na$_{1.5}$ channel is necessary as a pacemaker [38, 39]. Our HCN4-overexpressing mESC-CMs also expressed these channels.

Ivabradine is an $I_f$ inhibitor and reduces the firing rate of pacemaker cells [40, 41], and it has already been applied in a clinical setting [42]. HCN4-overexpressing mESC-CMs showed responses to ivabradine. This drug could regulate tachyarrhythmia caused by hyperexcitability of these cells. In addition, HCN4-overexpressing mESC-CMs showed responses to isoproterenol, a β1- and β2-adrenoreceptor agonist, and might be useful to achieve heart rate response during exercise.

A sufficient number of cells is necessary to engraft well and capture the surrounding myocardium [28]. Since pluripotent stem cells have a strong self-renewal property, the use of pluripotent stem cell-derived cardiomyocytes is reasonable. Furthermore, hiPSCs are thought to be able to solve the likelihood of immune rejection [43, 44]. Thus, this HCN4-overexpressing method might be applicable to hiPSC-CMs.

Recently, Inada et al reported that the spatial heterogeneous nature of the sinus node is important for its normal functioning and that the presence of Na channel and connexin 43 in the periphery may be essential for the node to be able to drive the atrial muscle [38, 39]. Mouse ESC-CMs include a heterogeneous population: nodal-like, atrial myocyte-like and ventricular myocyte-like cells. Working myocyte-like cells from ESCs originally express Na channel and connexins. However, it is not obvious that spatial sorting would occur if ESC-CMs were injected in vivo. Further studies are needed to clarify this point.

Transplantation of human ESC-CMs into the ventricle of a complete atrioventricular block model animal has been reported [45]. Our experiment was only an in vitro experiment, and we need to evaluate the efficacy of these cells in bradycardia model animals.

Conclusion

We generated HCN4-overexpressing mESC-CMs showing rapid spontaneous beating, responses to drugs and improved pacing ability in vitro. The results indicated that these cells could be applied to a biological pacemaker.

Supporting Information

S1 Fig. Three mESC lines with or without HCN4 overexpression. A. Representative living Hcn4/EGFP-transfected mESCs observed by phase contrast microscopy (a to c) and fluorescence microscopy (d to f). Hcn4/EGFP-transfected mESCs were positive for EGFP (green) fluorescence (d to f). B. Representative living EGFP-transfected mESCs observed by phase contrast microscopy (a to c) and fluorescence microscopy (d to f). EGFP-transfected mESCs were positive for EGFP (green) fluorescence (d to f). C. Representative living control mESCs observed by phase contrast microscopy (a) and fluorescence microscopy (b). Control mESCs were negative.
for EGFP (b). Bar = 50 μm.

(TIF)

S2 Fig. Expression of cardiac differentiation marker genes in mESC and mESC-CM lines.
A. In all cell lines with or without HCN4 overexpression, RT-PCR showed increases in mRNA expression for cardiac markers Nkx 2.5, Tnnt2, connexin, Scn5a, Cacna1h, and mouse endogenous Hcn4 (lanes 7 to 12). Rabbit exogenous Hcn4 was expressed in HCN4/EGFP mESCs (lanes 4 to 6) and mESC-CMs (lane 10 to 12). B. Rabbit Hcn4 mRNA levels in 3 HCN4/EGFP mESC-CM lines assessed by q-PCR.

(TIF)

S3 Fig. Expression of undifferentiated cell markers in HCN4/EGFP and EGFP mESCs.
A. Immunofluorescent staining of OCT4 (a and b) and nuclear DNA staining by Hoeclst (c and d) in HCN4/EGFP mESC-1 and 3 (immunofluorescent staining in HCN4/EGFP mESC-2 shown in Fig 1C). Bar = 50 μm. B. Immunofluorescent staining of OCT4 (a and b) and nuclear DNA staining by Hoeclst (c and d) in EGFP mESC-2 and 3 (immunofluorescent staining in EGFP mESC-1 shown in Fig 1C). Bar = 50 μm. C. q-PCR showed that Nanog and Oct4 mRNA levels were not significantly different in all mESC lines with or without HCN4 overexpression.

(TIF)

S4 Fig. Generation of hiPSCs and hiPSC-CMs.
A. Generated hiPSCs observed by phase contrast microscopy (a). Immunofluorescent staining showed nuclear accumulation of OCT4 (red) (b to d) and cell surface antigen expression (green) pattern of human pluripotent stem cells (e, SSEA-1 negative; f, SSEA-4 positive; g, TRA1-60 positive; and h, TRA-1-81 positive). Bar = 50 μm. B. Differentiated cardiomyocytes from hiPSCs were positive for α-actinin (green) (a) and troponin I (green) (d). Counter staining with f-actin (red) (band e) and merge (c and f). Bar = 50 μm.

(TIF)

Acknowledgments
The authors are grateful to Aya Miura, Yuko Ohno, Kaoru Akazawa, and Megumi Kondo for technical assistance.

Author Contributions
Conceived and designed the experiments: KN. Performed the experiments: YS MY HS JK SN. Analyzed the data: YS KN TO TF HM KFK HI. Contributed reagents/materials/analysis tools: MT. Wrote the paper: YS KN.

References
1. Dobrzynski H, Boyett MR, Anderson RH. New insights into pacemaker activity: promoting understanding of sick sinus syndrome. Circulation. 2007; 115(14):1921–32. Epub 2007/04/11. doi: 10.1161/circulationaha.106.616011 PMID: 17420362.
2. Nikmaram MR, Boyett MR, Kodama I, Suzuki R, Honjo H. Variation in effects of Cs+, UL-FS-49, and ZD-7288 within sinoatrial node. Am J Physiol. 1997; 272(6 Pt 2):H2782–92. Epub 1997/06/01. PMID: 9227558.
3. Kodama I, Nikmaram MR, Boyett MR, Suzuki R, Honjo H, Owen JM. Regional differences in the role of the Ca2+ and Na+ currents in pacemaker activity in the sinoatrial node. Am J Physiol. 1997; 272(6 Pt 2):H2793–806. Epub 1997/06/01. PMID: 9227559.
4. Hagiwara N, Irisawa H, Karneyama M. Contribution of two types of calcium currents to the pacemaker potentials of rabbit sino-atrial node cells. J Physiol. 1988; 395:233–53. Epub 1988/01/01. PMID: 2457676; PubMed Central PMCID: PMC1191991.
5. Lei M, Jones SA, Liu J, Lancaster MK, Fung SS, Dobrzynski H, et al. Requirement of neuronal- and cardiac-type sodium channels for murine sinoatrial node pacemaking. J Physiol. 2004; 559(Pt 3):835–48. Epub 2004/07/16. doi: 10.1113/jphysiol.2004.068643 PMID: 15254155; PubMed Central PMCID: PMCPMC1665172.

6. Rigg L, Terrar DA. Possible role of calcium release from the sarcoplasmic reticulum in pacemaking in guinea-pig sino-atrial node. Exp Physiol. 1996; 81(5):877–80. Epub 1996/09/01. PMID: 8889484.

7. Biel M, Schneider A, Wahl C. Cardiac HCN channels: structure, function, and modulation. Trends Cardiovasc Med. 2002; 12(5):206–12. Epub 2002/08/06. PMID: 12161074.

8. DiFrancesco D. The role of the funny current in pacemaker activity. Circ Res. 2010; 106(3):434–46. Epub 2010/02/20. doi: 10.1161/circresaha.109.208041 PMID: 20167941.

9. Ishii TM, Takano M, Xie LH, Noma A, Ohmori H. Molecular characterization of the hyperpolarization-activated cation channel in rabbit heart sinoatrial node. J Biol Chem. 1999; 274(18):12835–9. Epub 1999/04/23. PMID: 10212270.

10. Abi-Gerges N, Ji GJ, Lu ZJ, Fischmeister R, Hescheler J, Fleischmann BK. Functional expression and regulation of the hyperpolarization activated non-selective cation current in embryonic stem cell-derived cardiomyocytes. J Physiol. 2000; 523 Pt 2:377–89. Epub 2000/03/04. PMID: 10699082; PubMed Central PMCID: PMCPMC2269804.

11. Ueda K, Nakamura K, Hayashi T, Inagaki N, Takahashi M, Arimura T, et al. Functional characterization of a trafficking-defective HCN4 mutation, D553N, associated with cardiac arrhythmia. J Biol Chem. 2004; 279(26):27194–8. Epub 2004/05/05. doi: 10.1074/jbc.M311953200 PMID: 15123648.

12. Schulze-Bahr E, Neu A, Friederich P, Kaupp UB, Breithardt G, Pongs O, et al. Pacemaker channel dysfunction in a patient with sinus node disease. J Clin Invest. 2003; 111(10):1537–45. Epub 2003/05/17. doi: 10.1172/jci16387 PMID: 12750403; PubMed Central PMCID: PMC155041.

13. Milanesi R, Baruscotti M, Gnecci-Ruscone T, DiFrancesco D. Familial sinus bradycardia associated with a mutation in the cardiac pacemaker channel. N Engl J Med. 2006; 354(2):151–7. Epub 2006/01/13. doi: 10.1056/NEJMoa052475 PMID: 16407510.

14. Shinagawa Y, Satoh H, Noma A. The sustained inward current and inward rectifier K+ current in pacemaker cells dissociated from rat sinoatrial node. J Physiol. 2000; 523 Pt 3:593–605. Epub 2000/03/16. PMID: 10718740; PubMed Central PMCID: PMC2269831.

15. Cho HS, Takano M, Noma A. The electrophysiological properties of spontaneously beating pacemaker cells isolated from mouse sinoatrial node. J Physiol. 2003; 550(1 Pt 1):169–80. Epub 2003/07/26. doi: 10.1113/jphysiol.2003.040501 PMID: 12879867; PubMed Central PMCID: PMC2343002.

16. Miale J, Marban E, Nuss HB. Biological pacemaker created by gene transfer. Nature. 2002; 419(6903):132–3. Epub 2002/09/13. doi: 10.1038/419132b PMID: 12226654.

17. Irisawa H, Brown HF, Giles W. Cardiac pacemaking in the sinoatrial node. Physiol Rev. 1993; 73(1):197–227. Epub 1993/01/01. PMID: 8380502.

18. Boheler KR, Czyz J, Tweedie D, Yang HT, Anisimov SV, Wobus AM. Differentiation of pluripotent embryonic stem cells into cardiomyocytes. Circ Res. 2002; 91(3):189–201. Epub 2002/08/10. PMID: 12169644.

19. Tohyama S, Hattori F, Sano M, Hishiki T, Nagahata Y, Matsuura T, et al. Distinct metabolic flow enables large-scale purification of mouse and human pluripotent stem cell-derived cardiomyocytes. Cell Stem Cell. 2013; 12(1):127–37. Epub 2012/11/22. doi: 10.1016/j.stem.2012.09.013 PMID: 23161814.

20. E LL, Zhao YS, Guo XM, Wang CY, Jiang H, Li J, et al. Enrichment of cardiomyocytes derived from mouse embryonic stem cells. J Heart Lung Transplant. 2006; 25(6):664–74. Epub 2006/05/30. doi: 10.1016/j.healun.2005.12.007 PMID: 16730572.

21. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007; 131(5):861–74. Epub 2007/03/01. doi: 10.1016/j..cell.2007.02.010 PMID: 17382969.

22. Cai J, Yi FF, Li YH, Yang XC, Song J, Jiang XJ, et al. Adenoviral gene transfer of HCN4 creates a genetic pacemaker in pigs with complete atrioventricular block. Life Sci. 2007; 80(19):1746–53. Epub 2007/03/27. doi: 10.1016/j.lfs.2007.02.006 PMID: 17382969.

23. Hu YF, Dawkins JF, Cho HC, Marban E, Cingolani E. Biological pacemaker created by minimally invasive somatic reprogramming in pigs with complete heart block. Sci Transl Med. 2014; 6(245):245ra94. Epub 2014/07/18. doi: 10.1126/scitranslmed.3008681 PMID: 25031269.

24. Tse HF, Xue T, Lau CP, Siu CW, Wang K, Zhang QY, et al. Bioartificial sinus node constructed via in vivo gene transfer of an engineered pacemaker HCN Channel reduces the dependence on electronic pacemaker in a sick-sinus syndrome model. Circulation. 2006; 114(10):1000–11. Epub 2006/08/23. doi: 10.1161/circulationaha.106.615385 PMID: 16923751.
25. Plotnikov AN, Sosunov EA, Qu J, Shlapakova IN, Anyukhovsky EP, Liu L, et al. Biological pacemaker implanted in canine left bundle branch provides ventricular escape rhythms that have physiologically acceptable rates. Circulation. 2004; 109(4):506–12. Epub 2004/01/22. doi:10.1161/01.cir.0000114527.10764.cc PMID: 14734518.

26. Boink GJ, Duan L, Nearing BD, Shlapakova IN, Sosunov EA, Anyukhovsky EP, et al. HCN2/SkM1 gene transfer into canine left bundle branch induces stable, autonomically responsive biological pacing at physiological heart rates. J Am Coll Cardiol. 2013; 61(11):1192–201. Epub 2013/02/12. doi: 10.1016/j.jacc.2012.03.031 PMID: 23935072.

27. Buccin A, Plotnikov AN, Shlapakova I, Danilo P Jr., Kryukova Y, Qu J, et al. Wild-type and mutant HCN channels in a tandem biological-electronic cardiac pacemaker. Circulation. 2006; 114(10):992–9. Epub 2006/08/23. doi:10.1161/circulationaha.106.617613 PMID: 16923750.

28. Plotnikov AN, Shlapakova I, Szabolcs MJ, Danilo P Jr., Lorell BH, Potapova IA, et al. Xenografted adult human mesenchymal stem cells provide a platform for sustained biological pacemaker function in canine heart. Circulation. 2007; 116(7):706–13. Epub 2007/07/25. doi: 10.1161/circulationaha.107.703251 PMID: 17646577.

29. Lu W, Yaoming N, Boli R, Jun C, Changhai Z, Yang Z, et al. mHCN4 genetically modified canine mesenchymal stem cells provide biological pacemaking function in complete dogs with atrioventricular block. Pacing Clin Electrophysiol. 2013; 36(9):1138–49. Epub 2013/05/15. doi:10.1111/pace.12154 PMID: 23663261.

30. Kapoor N, Liang W, Marban E, Cho HC. Direct conversion of quiescent cardiomyocytes to pacemaker cells by expression of Tbx18. Nat Biotechnol. 2013; 31(1):54–62. Epub 2012/12/18. doi:10.1038/nbt.2465 PMID: 23242162.

31. Barbati A, Crespi A, Capilupo D, Mazzocchi N, Baruscotti M, DiFrancesco D. Molecular composition and functional properties of t-channels in murine embryonic stem cell-derived pacemaker cells. J Mol Cell Cardiol. 2009; 46(3):343–51. Epub 2009/01/13. doi: 10.1016/j.yjmcc.2008.12.001 PMID: 19135060.

32. Yanagi K, Takeko M, Narazaki G, Uosaki H, Hoshino T, Ishii T, et al. Hyperpolarization-activated cyclic nucleotide-gated channels and T-type calcium channels confer automaticity of embryonic stem cell-derived cardiomyocytes. Stem Cells. 2007; 25(11):2712–9. Epub 2007/07/28. doi:10.1634/stemcells.2006-0388 PMID: 17655646.

33. Kleger A, Seufferlein T, Malan D, Tischendorf M, Storch A, Wolheim A, et al. Modulation of calcium-activated potassium channels induces cardiogenesis of pluripotent stem cells and enrichment of pacemaker-like cells. Circulation. 2010; 122(18):1823–36. Epub 2010/10/20. doi:10.1161/CIRCULATIONAHA.110.971721 PMID: 20956206.

34. Scavone A, Capilupo D, Mazzocchi N, Crespi A, Zoia S, Campostrini G, et al. Embryonic stem cell-derived CD166+ precursors develop into fully functional sinoatrial-like cells. Circ Res. 2013; 113(4):389–98. Epub 2013/06/12. doi: 10.1161/circresaha.113.301283 PMID: 23753573.

35. Hashem SI, Claycomb WC. Genetic isolation of stem cell-derived pacemaker-nodal cardiac myocytes. Molecular and cellular biochemistry. 2013; 383(1–2):161–71. Epub 2013/07/24. doi:10.1007/s11010-013-1764-x PMID: 23877224.

36. Lieu DK, Chan YC, Lau CP, Tse HF, Siu CW, Li RA. Overexpression of HCN-encoded pacemaker current silences bioartificial pacemakers. Heart Rhythm. 2008; 5(9):1310–7. Epub 2008/08/12. doi: 10.1016/j.hrthm.2008.05.010 PMID: 18693074.

37. Chan YC, Siu CW, Lau YM, Lau CP, Li RA, Tse HF. Synergistic effects of inward rectifier (I) and pacemaker (I) currents on the induction of bioengineered cardiac automaticity. J Cardiovasc Electrophysiol. 2009; 20(9):1048–54. Epub 2009/05/23. doi: 10.1111/j.1540-8167.2009.01475.x PMID: 19460073; PubMed Central PMCID: PMC2739246.

38. Butters TD, Aslanidi OV, Inada S, Boyett MR, Hancox JC, Lei M, et al. Mechanistic links between Na+ channel (SCN5A) mutations and impaired cardiac pacemaking in sick sinus syndrome. Circ Res. 2010; 107(1):126–37. Epub 2010/05/08. doi: 10.1161/circresaha.110.219949 PMID: 20448214; PubMed Central PMCID: PMC2901593.

39. Inada S, Zhang H, Tellez JO, Shibata N, Nakazawa K, Kamiya K, et al. Importance of gradients in membrane properties and electrical coupling in sinoatrial node pacing. PLOS One. 2014; 9(4):e94565. Epub 2014/04/25. doi: 10.1371/journal.pone.0094565 PMID: 24759974; PubMed Central PMCID: PMCPMC3997424.

40. Thollon C, Cambarat C, Vian J, Prost JF, Pegion JL, Vilaine JP. Electrophysiological effects of S16257, a novel sino-atrial node modulator, on rabbit and guinea-pig cardiac preparations: comparison with UL-FS 49. Br J Pharmacol. 1994; 112(1):37–42. Epub 1994/05/01. PMID: 8032680; PubMed Central PMCID: PMCPMC1910295.
41. Suenari K, Cheng CC, Lin YK, Nakano Y, Kihara Y, et al. Effects of ivabradine on the pulmonary vein electrical activity and modulation of pacemaker currents and calcium homeostasis. J Cardiovasc Electrophysiol. 2012; 23(2):200–6. Epub 2011/09/15. doi: 10.1111/j.1540-8167.2011.02173.x PMID: 21914029.

42. Swedberg K, Komajda M, Bohm M, Borer JS, Ford I, Dubost-Brama A, et al. Ivabradine and outcomes in chronic heart failure (SHIFT): a randomised placebo-controlled study. Lancet. 2010; 376(9744):875–85. Epub 2010/08/31. doi: 10.1016/s0140-6736(10)61198-1 PMID: 20801500.

43. Kiskinis E, Eggan K. Progress toward the clinical application of patient-specific pluripotent stem cells. J Clin Invest. 2010; 120(1):51–9. Epub 2010/01/07. doi: 10.1172/jci40553 PMID: 20051636; PubMed Central PMCID: PMCPMC2798698.

44. Araki R, Uda M, Hoki Y, Sunayama M, Nakamura M, Ando S, et al. Negligible immunogenicity of terminally differentiated cells derived from induced pluripotent or embryonic stem cells. Nature. 2013; 494(7435):100–4. Epub 2013/01/11. doi: 10.1038/nature11807 PMID: 23302801.

45. Kehat I, Khimovich L, Caspi O, Gepstein A, Shofti R, Arbel G, et al. Electromechanical integration of cardiomyocytes derived from human embryonic stem cells. Nat Biotechnol. 2004; 22(10):1282–9. Epub 2004/09/28. doi: 10.1038/nbt1014 PMID: 15448703.