EXPERIMENTAL STUDY

HCN4-Overexpressing Mouse Embryonic Stem Cell-Derived Cardiomyocytes Generate a New Rapid Rhythm in Rats with Bradycardia

Yukihiro Saito,1 MD, Kazufumi Nakamura,1 MD, Masashi Yoshida,2 MD, Hiroki Sugiyama,3 MD, Makoto Takano,1 MD, Satoshi Nagase,4 MD, Hiroshi Morita,5 MD, Kengo F. Kusano,1,4 MD and Hiroshi Ito,1 MD

Summary

A biological pacemaker is expected to solve the persisting problems of an artificial cardiac pacemaker including short battery life, lead breaks, infection, and electromagnetic interference. We previously reported HCN4 overexpression enhances pacemaking ability of mouse embryonic stem cell-derived cardiomyocytes (mESC-CMs) in vitro. However, the effect of these cells on bradycardia in vivo has remained unclear. Therefore, we transplanted HCN4-overexpressing mESC-CMs into bradycardia model animals and investigated whether they could function as a biological pacemaker. The rabbit Hcn4 gene was transfected into mouse embryonic stem cells and induced HCN4-overexpressing mESC-CMs. Non-cardiomyocytes were removed under serum/glucose-free and lactate-supplemented conditions. Cardiac balls containing 5 × 10^3 mESC-CMs were made by using the hanging drop method. One hundred cardiac balls were injected into the left ventricular free wall of complete atrioventricular block (CAVB) model rats. Heart beats were evaluated using an implantable telemetry system 7 to 30 days after cell transplantation. The result showed that ectopic ventricular beats that were faster than the intrinsic escape rhythm were often observed in CAVB model rats transplanted with HCN4-overexpressing mESC-CMs. On the other hand, the rats transplanted with non-overexpressing mESC-CMs showed sporadic single premature ventricular contraction but not sustained ectopic ventricular rhythms. These results indicated that HCN4-overexpressing mESC-CMs produce rapid ectopic ventricular rhythms as a biological pacemaker.

(Key words: Biological pacemaker, Cell therapy

Cardiac pacemaker implantation is the established therapy for patients with bradycardia. A mechanical pacemaker is extremely useful, but it has several problems: battery life, lead breaks, infection, electromagnetic interference, and appearance. Efforts have been made to create a biological pacemaker, which is expected to solve these problems, but practical application of a biological pacemaker has never been achieved.1-10)

We previously reported that HCN4 overexpression improved pacemaking ability of mouse embryonic stem cell-derived cardiomyocytes (mESC-CMs) in vitro.11 HCN4 encodes a hyperpolarization-activated cyclic nucleotide-gated (HCN) channel producing If current, which is responsible for diastolic depolarization in the sinus node.11,12) Additionally, embryonic stem cell-derived cardiomyocytes have pacemaking ability that might be insufficient for treatment of bradycardia.1,13) Thus, HCN4-overexpressing ESC-CMs might be a candidate for a biological pacemaker; however, the effect of these cells on bradycardia has not been clarified in vivo. The purpose of this study was to investigate whether HCN4 overexpression can improve the pacemaking ability of mESC-CMs in bradycardia model animals.

Methods

Maintenance of mouse ESCs: 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 μmol/L β-mercaptoethanol (2-ME), MEM nonessential amino acids solution (NEAA, From the 1Department of Cardiovascular Medicine, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan, 2Department of Chronic Kidney Disease and Cardiovascular Disease, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan, 3Department of Physiology, Kurume University School of Medicine, Kurume, Japan, 4Department of Cardiovascular Medicine, National Cerebral and Cardiovascular Center, Osaka, Japan and 5Department of Cardiovascular Therapeutics, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan.

This study was supported by JSPS KAKENHI Grant Number 16K19407.

Address for correspondence: Yukihiro Saito, MD, Department of Cardiovascular Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan. E-mail: saitou-y@cc.okayama-u.ac.jp or Kazufumi Nakamura, MD. E-mail: ichibun@cc.okayama-u.ac.jp

Received for publication April 27, 2017. Revised and accepted July 21, 2017. Released in advance online on J-STAGE April 6, 2018.

doi: 10.1536/ihj.17-241

All rights reserved by the International Heart Journal Association.

601
Gibco), 1000 units/mL leukemia inhibitory factor (LIF; Wako), and 100 μg/mL kanamycin (Sigma) in a humidified atmosphere containing 5% CO₂.

**Plasmid vectors:** Plasmid vectors used in this study were constructed as previously reported (Figure 1).⁹ CAG promoter-IRES-EGFP construct in pCAGIG (Addgene #11159) and kanamycin/neomycin resistance gene (Kan/rf) in pIRES2-AcGFP1 vector (Clontech) were amplified using PrimeSTAR GXL (Takara) and ligated using an In-fusion HD enzyme (Clontech), i.e., pCAGIG-IRES-EGFP-Kan/rf. Rabbit Hcn4 cDNA in the pCI vector (provided by Dr. Takano) was amplified and ligated with pCAGIG-rabbit Hcn4-IRES-EGFP-Kan/rf.

**Transfection of rabbit Hcn4 gene in mESCs:** CG8 cells were harvested using 0.05% trypsin/ethylenediaminetetraacetic acid (EDTA). Two micrograms of a non-linearized vector (pCAGIG-rabbit Hcn4-IRES-EGFP-Kan/rf or pCAGIG-IRES-EGFP-Kan/rf) 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 μg/mL G418 (Roche Applied Science) for 7 days. Three stable clones that were resistant to G418 and were EGFP-positive were selected and expanded.

**Differentiation of mouse ESC-derived cardiomyocytes:** Differentiation of mouse ESC-derived cardiomyocytes was performed as previously described.⁹ Embryoid bodies (EBs) were formed by cultivating 500 mESCs with 0.5 mM 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). 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 with a medium consisting of MEM, 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). 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 the 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 × g for 30 minutes, cell layers were apparent. Cells at a 58.5% layer were collected.⁹ The purified cells were resuspended in high-glucose DMEM supplemented with 20% FBS, 1% NEAA, and 100 μM 2-ME.

**Immunocytofluorescence:** The purified mESC-CMs were plated on matrigel-coated cover glasses and were fixed in 4% paraformaldehyde. The cells were stained with primary antibodies against EGFP (1:200 dilution, Frontier Institute), α-actinin (1:800 dilution, Sigma EA-53), and Troponin I (1:50 dilution, Santa Cruz). 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), Alexa Fluor 488 goat anti-mouse IgG (1:200 dilution, Molecular Probes), and Alexa Fluor 488 chicken anti-goat IgG (1:200 dilution, Molecular Probes). Nucleus staining was performed with Hoechst 33342 (1:2500 dilution, Molecular Probes).

**Histological evaluation:** Hearts transplanted with mESC-CMs were harvested, fixed using 10% formalin overnight, and embedded in paraffin. The paraffin-embedded tissues were sectioned at 5 μm in a microtome (Microm) and mounted on APS-coated slide glasses (Matsunami Glass).
The sectioned tissues were deparaffinized, incubated in heated HistoVT One (Nacalai Tesque) for 20 minutes at 90°C followed by incubation with Blocking One Histo (Nacalai Tesque) for 30 minutes, and stained with primary antibodies against α-actinin (1:200 dilution, Sigma EA-53) and GFP (1:400 dilution, Novus). Secondary antibodies were Alexa Fluor 555 goat anti-mouse IgG1 and Alexa Fluor 488 chicken anti-rabbit IgG (1:200, Molecular Probes).

Model of complete atrioventricular block: Six Wistar rats aged 10-12 weeks (CLEA Japan, Inc) were anesthetized with isoflurane (Abbott Japan) and then intubated and mechanically ventilated (at a rate of 100 cycles/minute, tidal volume of 2.5 mL). A thoracotomy incision was made in the right side of the sternum. The pericardium was opened and 70% ethanol (50-100 μL) was injected into the myocardium using a 30-gauge syringe while recording an electrocardiogram. The injection site was located between the aortic root and the right atrial medial wall using the epicardial fat pad as a landmark. After 1 week, we confirmed persistent complete atrioventricular block and implanted a telemetry transmitter (DataSciences, TA11ETA-F10).

Cardiac ball formation: Purified mESC-CMs were dissociated by 0.25% trypsin/0.05% EDTA for 5 minutes, and cardiac balls (CBs) were made from 5 × 10^3 cells/CB by the hanging drop method for 4 days. More than 2 weeks after producing an atrioventricular block, from day 2 relative to cell transplantation, the rats were administered cyclosporine A (15 mg/kg/day, subcutaneous, for 7 days; thereafter reduced to 7.5 mg/kg/day) and methylprednisolone (2 mg/kg/day, intraperitoneal). The rats were anesthetized with isoflurane and then intubated and mechanically ventilated (at a rate of 100 cycles/minute, tidal volume of 2.5 mL). A thoracotomy incision was made in the left side of the sternum and a rib retractor was inserted. The pericardium was opened and 100 CBs suspended in 70 μL of medium were injected into one spot of the left ventricular free wall with 29-gauge needles. CBs with or without HCN4 overexpression were injected in three rats each. Heart beats were evaluated using an implantable telemetry system (DataSciences, TA11ETA-F10 and Dataquest ART) 7 to 30 days after cell transplantation. All animal protocols were approved by and conducted according to the recommendations of the Okayama University Animal Care and Use Committee.

Results

Generation of purified Hcn4-overexpressing mouse ES cell-derived cardiomyocytes: HCN4-overexpressing and non-overexpressing mESCs that we previously established were used for cardiac induction (Figure 1A and B). Cardiomyocytes were purified by no glucose/lactate-supplemented medium and density gradient centrifugation. Approximately 90% of the cells were α-actinin- and troponin I-positive (Figure 2A).

Ectopic ventricular rhythm occurring in rats transplanted with HCN4-overexpressing mESC-CMs: CBs were made by using the hanging drop method for 4 days (Figure 2B), and 100 cardiac balls were injected into the
left ventricular free wall. We confirmed retention of transplanted cells by immunostaining using anti-GFP antibody (Figure 3A). Heart rates of rats with CAVB ranged from 120 to 150 beats a minute. In CAVB model rats transplanted with HCN4-overexpressing mESC-CMs, ectopic ventricular beats that were faster than the intrinsic escape rhythm, ranging from 240 to 320 beats a minute, were often observed (Figure 3B). On the other hand, a sustained ectopic ventricular rhythm did not occur in rats transplanted with non-overexpressing mESC-CMs, whereas premature ventricular contractions were sporadically observed (Figure 3C). However, HCN4-overexpressing mESC-CMs could not replace the escape rhythm completely (Figure 3D).

**Discussion**

Transplantation of HCN4-overexpressing mESC-CMs resulted in a new ectopic ventricular rhythm in CAVB rats, that is, HCN4 overexpression improved the pacemaking ability of mESC-CMs in vivo. The pacemaking ability of pacemaker-like cells derived from pluripotent stem cells has been evaluated in vitro and ex vivo but not in vivo.\(^8,10,17-19\)

However, ectopic ventricular beats resulting from transplantation of HCN4-overexpressing mESC-CMs did not always appear. Additional ideas might be needed to increase exogenous HCN4 expression or susceptibility to excitability of sympathetic neurons. Probably, a transgene was randomly integrated in this study and HCN4-overexpressing mESC-CMs expressed not more than five-fold Hcn4 mRNA than non-overexpressing mESC-CMs did.\(^9\) In pluripotent stem cells and stem cell-derived cells, a randomly integrated transgene could be suppressed easily.\(^20-22\) Then site-specific DNA transfer through a homologous recombination technique might be useful for inhibiting the decrease in exogenous HCN4 expression.\(^23,24\) Miwa et al. and Fu et al. reported that a glial cell line-derived neurotrophic factor (GDNF) is useful for sympathetic innervations in engrafted cardiomyocytes.\(^25,26\) These methods might enable ectopic beats, that is, a pacemaker rhythm, to be sustained.

Fortunately, we did not find ventricular tachyarrhythmias exceeding the sinus rate in the transplanted rats. However, Chong et al. and Shiba et al. reported that non-human primate models of myocardial infarction transplanted with human pluripotent stem cell-derived cardiomyocytes showed ventricular arrhythmias.\(^27,28\) They presumed that conduction delay occurred in immature ESC-CM grafts and that a large graft size could favor the formation of re-entrant loops. A smaller number of cells and a smaller graft size would be required for pacing than those required for improving pump dysfunction. Furthermore, tachyarrhythmias did not appear 80 days or later.
post-transplantation. We previously showed that iv-abradine, an HCN channel inhibitor, could suppress the excitability of HCN4-overexpressing mESC-CMs.\(^9,29\) Iv-abradine has already been applied in a clinical setting, and it is expected to regulate tachyarrhythmia caused by hyperexcitability of these cells.\(^{30}\)

Since the sample size in our study was small due to the difficulty in making CAVB model rats, more detailed investigations are needed to evaluate the usefulness of HCN4-overexpressing pluripotent stem cell-derived cardiomyocytes. Additionally, there are several limitations in this study. We did not analyze survival rates and exercise capacity in CAVB model rats after transplantation with HCN4-overexpressing ESC-CMs. Furthermore, we analyzed heart rate variability up to 30 days after transplantation with HCN4-overexpressing ESC-CMs. Therefore, it was not clear whether the biological pacemaker cells originating from a mouse stably function in the rat heart after more than 30 days. Further studies are needed to clarify these points.

Recently, human induced pluripotent stem cells (hiPSCs) are expected to be useful for regenerative medicine and disease model study,\(^{10,20,31}\) and hiPSCs are thought to be able to solve the likelihood of immune rejection.\(^{31}\) Thus, this HCN4-overexpressing method might be applicable to hiPSC-derived cardiomyocytes. Additionally, reliable cell delivery systems also need to be developed as with other cell therapy.\(^{10,31}\)

In summary, HCN4-overexpressing mESC-CMs produced a new ectopic rhythm. The results suggest that HCN4 overexpression can improve the pacemaking ability of mESC-CMs in vivo and that HCN4-overexpressing mESC-CMs can partially function as a biological pacemaker.

**Acknowledgments**

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

**Disclosures**

**Conflicts of interest:** None.

**References**


