Electric pulse current stimulation increases electrophysiological properties of I_f current reconstructed in mHCN4-transfected canine mesenchymal stem cells

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Abstract. The 'funny' current, also known as the I_f current, play a crucial role in the spontaneous diastolic depolarization of sinoatrial node cells. The I_f current is primarily induced by the protein encoded by the hyperpolarization-activated cyclic nucleotide-gated channel 4 (*HCN4)* gene. The functional I_f channel can be reconstructed in canine mesenchymal stem cells (cMSCs) transfected with mouse *HCN4* (m*HCN4*). Biomimetic studies have shown that electric pulse current stimulation (EPCS) can promote cardiogenesis in cMSCs. However, whether EPCS is able to influence the properties of the I_f current reconstructed in m*HCN4*-transfected cMSCs remains unclear. The present study aimed to investigate the effects of EPCS on the I_f current reconstructed in m*HCN4*-transfected cMSCs. The cMSCs were transfected with the lentiviral vector pLentis-m*HCN4*-GFP. Following transfection, these cells were divided into two groups: m*HCN4*-transfected cMSCs (group A), and m*HCN4*-transfected cMSCs induced by EPCS (group B). Using a whole cell patch-clamp technique, the I_f current was recorded, and group A cMSCs showed significant time and voltage dependencies and sensitivity to extracellular Cs^+. The half-maximal activation ($V_{1/2}$) value was -101.2±4.6 mV and the time constant of activation was 324±41 msec under -160 mV. In the group B cells the I_f current increased obviously and activation curve moved to right. The absolute value of $V_{1/2}$ increased significantly to -92.4±4.8 mV ($P<0.05$), and the time constant of activation diminished under the same command voltage (251±44 vs. 324±41, $P<0.05$). In addition, the mRNA and protein expression levels of *HCN4*, connexin 43 (Cx43) and Cx45 were upregulated in group B compared with group A, as determined by reverse transcription-quantitative polymerase chain reaction and western blot analyses. Transmission electron micrographs also confirmed the increased gap junctions in group B. Collectively, these results indicated that reconstructed I_f channels may have a positive regulatory role in EPCS induction. The cMSCs transfected with m*HCN4* induced by EPCS may be a source of effective biological pacemaker cells.

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Introduction

The sinoatrial node (SAN) is located in the right dorsal wall of the right atrium, and serves as the primary pacemaker for initiating the heartbeat and controlling the rate and rhythm of contraction (1). Deficiencies in such function due to congenital defects, acquired diseases, gene mutation and aging may lead to sinus node dysfunction (sick sinus syndrome), necessitating pacemaking therapy (2). The present primary therapy is electronic pacemaker implantation. However, such devices are not optimal because of the limited battery life, biological responsiveness deficiency and other shortages (2). With respect to these concerns, on-going research is focused on developing a biological pacemaker that is able to integrate into the myocardium and provide a permanent cure (2-5).

The 'funny' current, also called the I_f current, is crucially involved in the spontaneous diastolic depolarization of SAN cells. The I_f current is generated by the hyperpolarization-activated cyclic nucleotide-gated channel (HCN) family. Among the four known HCN isoforms, isoform 4 (encoded by *HCN4*) strongly contributes to pacemaker function (3). In previous studies, the present authors showed that mouse *HCN4* (m*HCN4*)-transfected mesenchymal stem cells (MSCs) are able to generate a functional I_f current (3-5).

The heart is the largest source of bioelectricity in the human body (6,7). Electrical signals are correlated with cardiac depolarization and contraction, and electrical currents have been administered to cells in order to mimic native heart conditions in a number of prior biomimetic studies (7-9). Furthermore, an electrical stimulation system has been designed to deliver electrical signals mimicking those in the native heart by the present authors (10). This electric pulse current stimulation (EPCS) had been shown to be safe in canine MSCs (cMSCs),

and may be able to promote cardiogenesis in cMSCs in our previous study (10). However, it is not clear whether EPCS is able to influence the properties of I_f current reconstructed in *HCN4*-transfected cMSCs.

In the present study, cMSCs with high-level expression of m*HCN4* were produced using exogenous transfection. The kinetic characteristics of the resulting I_f current were analyzed. In addition, the mRNA and protein expression levels of *HCN4*, connexin 43 (Cx43) and Cx45 were evaluated using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot assays, and the ultrastructures of the cardiac connective proteins were characterized using transmission electron microscopy (TEM).

Materials and methods

cMSC culture. Under 30 mg/kg sodium pentobarbital (i.v.) and 1-2% isoflurane anesthesia (Abbott Laboratories Co., Ltd., Shanghai, China), cMSCs were harvested from bone marrow of three adult Chinese rural canines (two males and one female; Third Military Medical University (Chongqing, Chian), weighing 10-14 kg, as previously described (5) . cMDCs were cultured in α-minimum essential medium (α-MEM) with L-glutamine and ribo- and deoxyribonucleosides (SH30265.01B; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA). cMSC purity was evaluated using flow cytometry, as previously described (5), in order to detect the markers $CD29^+$, $CD44^+$, $CD34^-$ and $CD45^-$, and by the ability to differentiate into adipogenic, chondrogenic and osteogenic lineages, using Oil Red O, Alcian Blue and Toluidine Blue, and Alizarin Red staining, respectively (5). Third generation cMSCs were transfected with pLentis-m*HCN4*-GFP in the presence of 2 μg/ml polybrene (H9268; Sigma-Aldrich, St. Louis, MO, USA) at a multiplicity of infection (MOI) of 20 for 24 h. Following 48 h of transfection, 90% of the cMSCs cells exhibited green fluorescent protein (GFP) expression, which indicated successful transfection. These cMSCs were expanded using standard techniques (3-5,10). In order to produce consistent comparisons between m*HCN4*-transfected cMSCs with or without EPCS induction, the cells were divided into two groups: m*HCN4*-transfected cMSCs (group A), and m*HCN4*-transfected cMSCs induced with EPCS (group B). Ethical approval for the present study was obtained from the Committee on the Ethics of Animal Experiments of Third Military Medical University (SYXK2013-0012).

mHCN4 transfection. The lentiviral vector expressing m*HCN4* (pLentis-m*HCN4*-GFP) was constructed by inserting the m*HCN4* gene into a pLentis-GFP vector using *Bam*HI (FD0054) and *Eco*RI (N41890) restriction sites, using materials obtained from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The transcription of m*HCN4* was promoted by a Spleen Focus-Forming Virus promoter (Invitrogen). The lentiviral particles were prepared using a calcium phosphate method, as previously described (3-5).

EPCS induction. An electric stimulation device designed in our lab provided a constant electric signal to the cMSCs, which had been shown to be safe to cMSCs and to promote cardiogenesis in cMSCs. The property of the electrical signal was pulse polarity-altered positive and negative variations (rectangular, 2 msec; current intensity, 40 μA; current frequency, 2 Hz) (10). For EPCS, the transfected GFP-positive cells in group B were reseeded in α-MEM at a density of $2x10^5$ cells into 60-mm culture dishes (430166; Corning Incorporated, Corning, NY, USA) with platinum electrode (Chow Sang Sang Jewellery Corporation, Hong Kong, China). The EPCS treatment was initiated on the next day after seeding, lasting 3-6 h per day for 5 days until the cells reached ~95% confluence. α-MEM was changed once during this treatment period. Each experiment was repeated three times.

Patch clamp studies for I_f current detection in transfected cMSCs. Following EPCS induction for 5 days, the cMSCs in group B and parallel cMSCs in group A were subjected to whole-cell patch clamp detection. The I_f current was recorded using a voltage clamp (Axon Instruments. Foster City, CA, USA) with an Axopatch 200B amplifier (Molecular Devices, LLC, Sunnyvale, CA, USA) (3-5). The pipette solution contained the following (in mmol/l): 5.0 Na_2-phosphocreatine, 5.0 Mg_2-adenosine triphosphate, 110 K aspartate, 0.1 GTP (Sigma-Aldrich), 1.0 $MgCl_2$, 20 KCl, 0.05 EGTA and 10 HEPES (Sangon Biotech Co., Ltd., Shanghai, China); pH was adjusted to 7.2 with KOH (Zhengzhongyi Biotech Co., Ltd., Beijing, China). The extracellular solution contained the following (in mmol/l): 0.33 NaH_2PO_4, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 5.4 KCl, 136 NaCl, 10 glucose and 10 HEPES; pH was adjusted to 7.4 with NaOH (Sangon Biotech Co., Ltd.). When required, 4 mmol/l CsCl was added into the bath solution. Cs^+ is a specific pacemaker current blocker to block I_f current and has been used to confirm the I_f current. The sealing resistance was >4 mΩ and the temperature was maintained at 25±1˚C.

In preparation for patch clamping, cells were trypsinized (SH30042.01; Hyclone; GE Healthcare Life Sciences) for 10-20 sec, while remaining attached to the coverslips. The membrane capacity was measured by the application of a voltage clamp step, and current density was shown as the value of the peak current per capacity. I_f current was detected using custom voltage clamp methods, as previously described (3-5). Normalized tail currents were recorded to construct the fully activated current-voltage relation. The slope factor (k) and the half-maximal activation voltage ($V_{1/2}$) were obtained by fitting the activation curves to the Boltzmann equation, as follows: $I_{tail}/I_{max} = A/\{1.0 + \exp[(V-V_{1/2})/k]\}$. The time constants of activation were determined by fitting current traces to a single exponential function.

Immunofluorescence analysis. Immunofluorescence was performed according to previously reported methods (10). Transfected cMSCs (1.5×10^4 cells/cm^2) were fixed with 4% paraformaldehyde (Wuhan Boster Biological Technology, Ltd., Wuhan, China) for 15 min at room temperature, washed in phosphate-buffered saline (PBS; AR0030; Wuhan Boster Biological Technology, Ltd.), then treated with 0.2% Triton X-100 (T8787; Sigma-Aldrich) for 15 min. Cells were incubated with rabbit polyclonal anti-*HCN4* antibody (1:100; ab69054; Abcam, Cambridge, UK) overnight at 4˚C. Following washing three times with PBS for 5 min, the cells were incubated with Alexa Fluor™ 647-conjugated donkey anti-rabbit IgG (1:200; A-31573; Invitrogen) for 60 min at 25±1˚C. After further washing with PBS, the cells were mounted with Antifade Mounting Medium

(Beyotime Institute of Biotechnology, Shanghai, China). Nuclei were stained with 4',6-diamidino-2-phenylindole (D9542; Sigma-Aldrich) as a location control. Fluorescent images were obtained using an inverted laser confocal microscope (LSM 710; Carl Zeiss Microscopy GmbH, Cologne, Germany). The results were analyzed using ZEN lite software, 2011 edition (Carl Zeiss Microscopy GmbH).

TEM. Cells were fixed in cacodylate-buffered 1% osmium tetroxide, then dehydrated and embedded in Epon 812 (both Sangon Biotech Co., Ltd.) for ultra-thin sectioning. The ultra-structures of connection protein were examined by TEM using a Tecnai^{TM}-10 electron microscope (FEI Company, Hillsboro, OR, USA) and operated at a high-tension setting of 80 kV with a magnification of x135,000.

RT-qPCR analysis. RT-qPCR was conducted according to previously described methods (11). Briefly, total mRNA was extracted using TRIzol (Invitrogen) and purified. For RNA purification, the RNA was treated with chloroform, centrifuged at 12,000 x g for 15 min at 4˚C to collect the supernatant prior to treatment with isoamylalcohol and vortexing for 10 min at room temperature. Following centrifugation at 12,000 x g for 15 min at 4˚C, the supernatant was discarded and the subsequent sediment RNA was reverse transcribed into cDNA at 37˚C for 15 min and 98˚C for 5 min using a ReverTra Ace qPCR RT kit (FSQ-101; Toyobo Co., Ltd., Osaka, Japan). RT-qPCR amplification was performed using a 12.5 μl SYBR® Green (2X) Realtime PCR Master Mix kit (QPK-201; Toyobo Co., Ltd.), 1 μl forward primer, 1 μl reverse primer, 5 μl cDNA and 5.5 μl water on a Stratagene Mx3000P qPCR system (Agilent Technologies, Inc., Santa Clara, CA, USA), according to the manufacturer's instructions. Thermal cycling was performed as follows: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 20 sec, and 72˚C for 10 min prior to holding at 4˚C. The primer sequences used were designed as follows: *HCN4* forward, 5'-AGTTGCGTT TCGAGGTCTT-3' and reverse, 5'-CTTTGTTGCCCTTAG TGAGC-3'; *Cx43* forward, 5'-TGCTATGACAAATCCTTC CCAATC-3' and reverse, 5'-GCCGTGCTCTTCAATTCC ATACTT-3'; *Cx45* forward, 5'-CAGCAGACTTCCTTGCCC TCATA-3' and reverse, 5'-CTTAGCATTGGACAGTTC GGTGT-3'; *GAPDH* forward, 5'-GAGATCCCGCCAACA TCAAA-3' and reverse, 5'GGCATCAGCAGAAGGAGCAG3' (Invitrogen). Quantitative measurements were determined using the comparative Ct ($2^{-\Delta\Delta Cq}$) method (12). All samples were normalized against the endogenous level of GAPDH. All results were repeated in triplicate and were analyzed using MxPro-Mx3000P software (Agilent Technologies, Inc.).

Western blot analysis. Western blot assays were performed according to previously described methods (10,11,13). Briefly, cMSCs were lysed with RIPA buffer containing phenylmethylsulfonyl fluoride (Sangon Biotech Co., Ltd.) , then 50 μg total protein was quantitated using a BCA protein assay kit (P0009; Beyotime Institute of Biotechnology) and subjected to 6-10% SDS-PAGE. After being resolved by electrophoresis, the proteins were transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, CA, USA), blocked for 3 h at room temperature in Tris-buffered saline (TBS) with 5% bovine serum albumin (10099-141; Gibco; Thermo Fisher Scientific, Inc.), and incubated with anti-*HCN4* (1:100; ab69054; Abcam), anti-Cx43 (1:200; sc-9059; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-Cx45 (1:200; sc-25716) rabbit and GAPDH (1:200; sc-48166; Santa Cruz Biotechnology, Inc.) goat polyclonal antibodies overnight at 4˚C, with gentle agitation. After washing three times with TBS with Tween-20, the membranes were incubated with corresponding horseradish peroxidase-conjugated mouse anti-rabbit polyclonal IgG (1:5,000; sc-2357; Santa Cruz Biotechnology, Inc.) at room temperature for 2 h. The specific bands of the target proteins were visualized using an enhanced chemiluminescence detection kit (P0018A; Beyotime Institute of Biotechnology), according to the manufacturer's recommendations. Finally, the target signals were normalized against the GAPDH signal and analyzed using Quantity One software (version 4.62; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Standard curve concentrations were 0.025, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 μg/μl, respectively with the wavelength set to 562 nm in UV. Experiments were performed >3 times to verify quantification values.

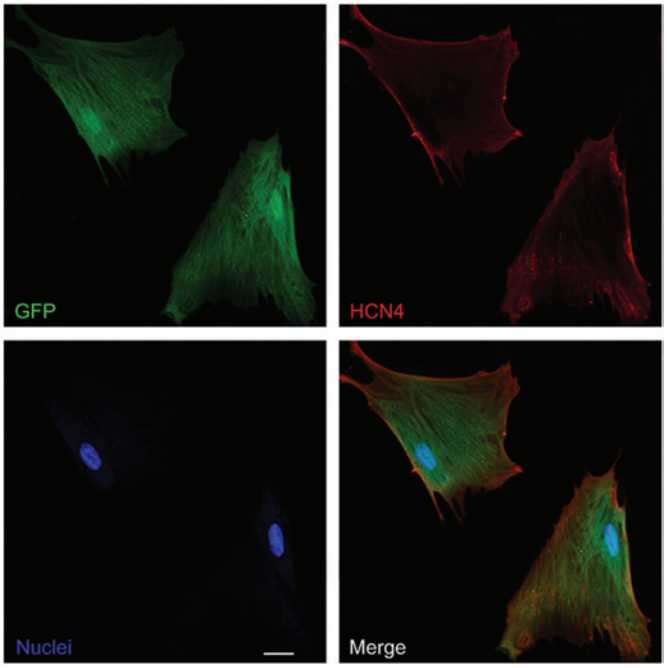

Figure 1. Laser confocal microscopy images of canine mesenchymal stem cells following successful transfection of pLentis-mHCN4-GFP, as evidenced by the expression of HCN4 with GFP. Nuclei were stained with 4',6-diamidino-2-phenylindole (scale bar, 20 μm). GFP, green fluorescent protein; HCN4, hyperpolarization-activated cyclic nucleotide-gated channel 4.

Statistical analysis. All values are presented as the mean ± standard error of the mean. Statistical comparisons were analyzed using Student's unpaired t-test with SPSS 19.0 software (IBM SPSS, Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Demonstration of gene transfer. At an MOI of 20, the transfection rate of the cMSCs was 95±3.7%, which was confirmed by confocal laser microscope images in at least

Table I. Comparative data measured for I_f in mHCN4-transfected canine mesenchymal stem cells in groups A and B.

Parameter	Group A	Group B
Detection rate	76% (16/21)	77% (17/22)
Current amplitude (pA, -160 mV)	-1,081.5±15.4	-1, 343.4±18.6[a]
Cell membrane capacitance (pF)	24.3±4.8	25.0±5.6
Current density (pA/pF, -160 mV)	-45.6±7.7	-53.7±4.0[a]
Half-maximal activation (mV)	-101.2±4.6	-92.4±4.8[a]
Slope factor	-16.0±1.6	-18.8±1.8[a]
Time constant of activation (msec, -160 mV)	324±41	251±44[a]
Reversal potential (mV)	-26.4±3.8	-28.9±3.0[a]

Data are presented as the mean ± standard error. [a]P<0.05 vs. group A.

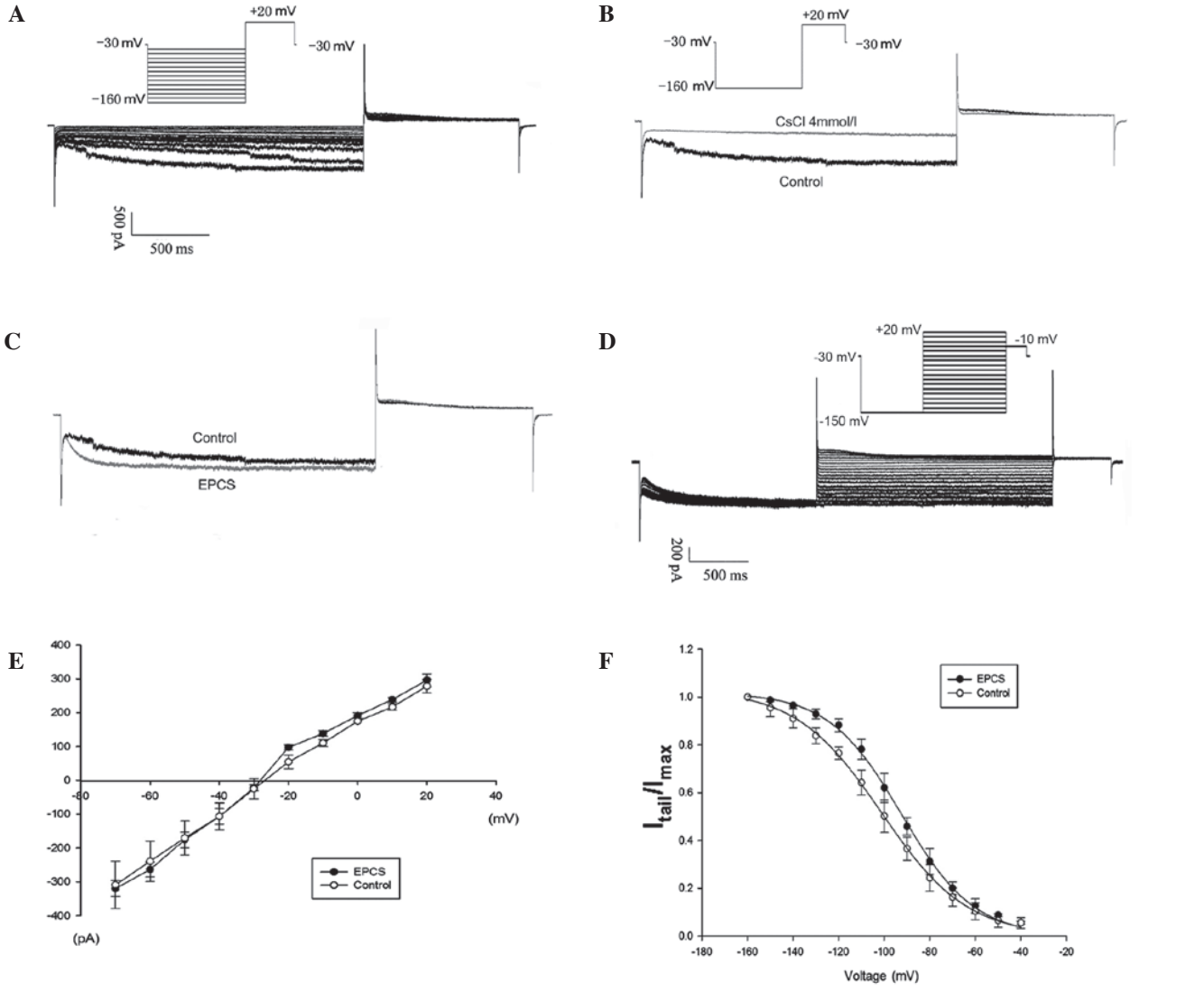

Figure 2. Characteristics of functional expression of I_f in mouse hyperpolarization-activated cyclic nucleotide-gated channel 4 (mHCN4)-transfected canine msenchymal stem cells (cMSCs). (A) I_f current recorded in group A. (B) Effect of extracellular Cs^+ on I_f. (C) Effect of EPCS on HCN4-transfected cMSCs. (D) Voltage protocol enables the mensuration of the (E) reversal potentials. (F) Representative tail currents used to construct the I_f activation curves. Data are presented as the mean ± standard error of the mean. EPCS, electric pulse current stimulation.

two different random fields. The m*HCN4*-GFP-transfected cMSCs in groups A and B expressed GFP and *HCN4* protein (Fig. 1).

Characteristics of I_f in transfected cMSCs. The I_f characteristics were recorded in whole cell patch-clamp mode. The m*HCN4*-GFP transfected cMSCs in group A showed a time

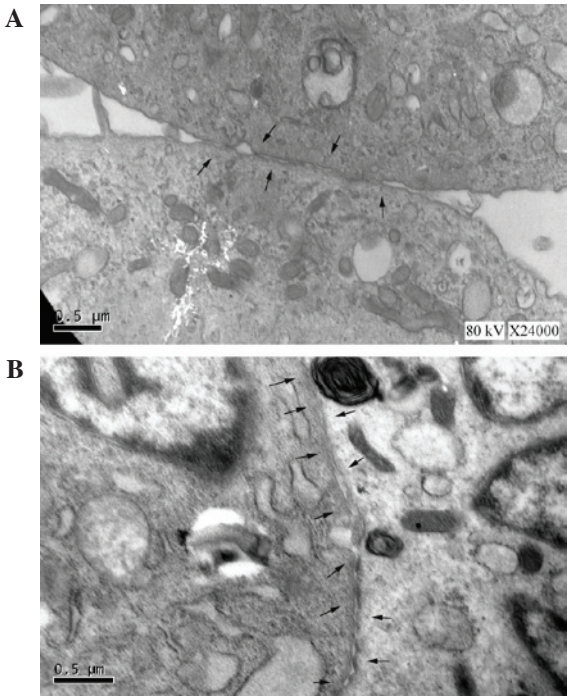

Figure 3. Transmission electron micrographs of gap junctions in adjacent cells. (A) Gap junctions in hyperpolarization-activated cyclic nucleotide-gated channel 4 (mHCN4)-transfected canine mesenchymal stem cells (cMSCs) (group A). (B) Gap junctions were increased in mHCN4-cMSCs induced by EPCS (group B). Arrows indicate the gap junctions.

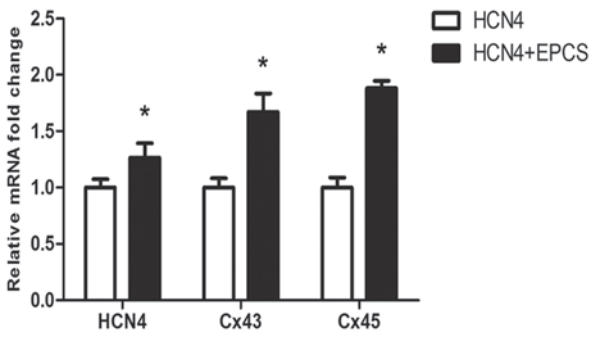

Figure 4. HCN4, Cx43 and Cx45 gene expression were examined using reverse transcription-quantitative polymerase chain reaction. Data are presented as the mean ± standard error. *P<0.05 vs. HCN4 (group A). HCN4, hyperpolarization-activated cyclic nucleotide-gated channel 4; EPCS, electric pulse current stimulation; Cx, connexin.

and voltage dependent inward current (Fig. 2A). The current amplitude was -1,081.5±15.4 pA at -160 mV command voltage, and the current density was -45.6±7.7 pA/pF at the same voltage. The $V_{1/2}$ was -101.2±4.6 mV, the time constant of activation was 324±41 msec at -160 mV, similar to values for *HCN4* expression in cardiac myocytes (14). The voltage protocol (Fig. 2D) enabled the measurement of the reversal potential (Fig. 2E), which was -26.4±3.8 mV. The detection rate of this inward current was 76% (16/21). It was also investigated whether Cs^+, a specific pacemaker current blocker, was able to block this expressed current. This inward current was blocked following the external addition of 4 mmol/l Cs^+ (Fig. 2B), consistent with Cs^+ inhibition of I_f as previously reported (3-5).

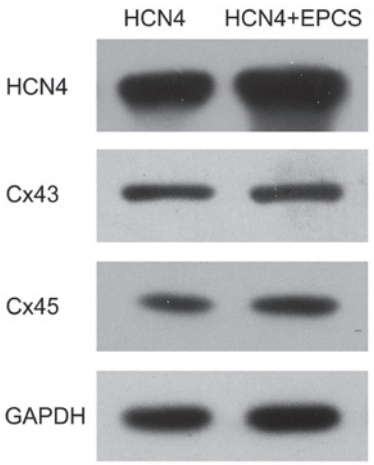

Figure 5. HCN4, Cx43 and Cx45 protein expression was detected using western blot analysis. HCN4, hyperpolarization-activated cyclic nucleotide-gated channel 4; EPCS, electric pulse current stimulation; Cx, connexin.

Under the same recording conditions, the detection rate of the I_f current was similar in the EPCS inductive group B (77%, 17 of 22). The channel activation curve shifted to right (Fig. 2F), which indicated that the current curve depicted a positive forward reaction. The $V_{1/2}$ changed to -92.4±4.8 from -101.2±4.6 mV (P<0.05; Table I), while the absolute values of I_f channel reversal potential increased (-28.9±3.0 vs. -26.4±3.8 mV, P<0.05). The time constant of activation decreased to 251±44 msec at -160 mV (P<0.05).

Expression of gap junction proteins. For consistent comparisons in m*HCN4* transfected cMSCs with or without EPCS induction, the gap junctions between adjacent cells were analyzed using TEM (Fig. 3). Following EPCS induction, the cMSCs showed an increased expression of gap junctions as Fig. 3B. In addition, the mRNA and protein expression levels of Cx43 and Cx45 in each group were evaluated using RT-qPCR and western blot analyses. As shown in Fig. 4, the mRNA expression levels of *HCN4*, Cx43 and Cx45 were significantly increased in EPCS inductive group B compared with group A (P<0.05). The protein expression levels of *HCN4*, Cx43 and Cx45 were also upregulated in group B, as shown in Fig. 5.

Discussion

Due to the limited ethical restrictions and immune privilege, MSCs are currently the optimal candidate for biological pacemaker reconstruction (3-5). In our previous studies, we demonstrated that m*HCN4*-transfected cMSCs can express *HCN4* protein stably and produce an I_f pacemaker current *in vitro* and *in vivo* (3,4). Wen *et al* previously reported that cMSCs are able to differentiate into cardiomyocytes by treatment with EPCS (10). The aim of the present study was to investigate the effects of EPCS on the I_f channel reconstructed in m*HCN4*-transfected cMSCs.

The HCN gene family is responsible for the generation of the pacemaker I_f current. The isoform *HCN4* has been demonstrated to be important for the proper function of pacemaking (3). The heart is the largest source of bioelectricity in

the human body, and numerous studies have investigated the effects of applying electrical stimulation signals to heart (6-10). In cardiac tissue engineering studies, biomimetic systems mimicking electrical signals in native heart have been used to enhance functional coupling of the cells and to increase the amplitude of synchronous construct contractions (6-10). An electric stimulation system had been designed in the Department of Cardiology at Southwest Hospital, Third Military Medical University to deliver electrical signals mimicking those in the native heart. This EPCS system had been shown to be safe for use in cMSCs, and successfully promoted the cardiogenesis of cMSCs in our previous study (10). The present study demonstrated that m*HCN4*-expressing cMSCs are capable of generating a time- and voltage- dependent inward current. This current is quite sensitive to extracellular Cs^+ and has similar channel kinetic characteristics as physiological *HCN4* I_f current (14). Following EPCS conduction, the channel activation curve shifted to the right and the time constant of activation decreased. TEM images showed more gap junctions in the EPCS inductive group. Therefore, it was hypothesized that these changes in the I_f current may be associated with the variation of gap junctions.

Gap junctions can establish communication channels between adjacent cells and allow direct intercellular transfer of ions, small molecules and electrical coupling (15). Gap junctions are composed of Cx protein subunits, and there are 21 members in the Cx gene family in the human genome and 20 members in the mouse to date (16). Cx40, Cx43 and Cx45 are expressed in cardiac tissues with distinct distributions. Cx45 is predominantly expressed in SAN, and is able to assemble gap junction channels with low conduction. By contrast, Cx40 and Cx43 are primarily expressed in working myocardium and can form gap junction channels with high conduction. Notably, a number of studies have suggested that Cx43 may also be expressed in SAN (16,17). Mutations in Cx43 may result in cardiac malformations and related to atrial fibrillation and sudden infant death syndrome (18,19). Recently, Yamada *et al* reported that Cx45 expression was markedly increased in patients with heart failure, and the upregulation of Cx45 in cardiac myocytes may lead to increased arrhythmias in the failing heart, which may impact the downregulation of Cx43 (20,21).

In the present study, the expression fold changes of Cx43 and Cx45 were evaluated in m*HCN4* genetically modified cMSCs *in vitro*. The mRNA and protein expression levels of Cx43 and Cx45 were detected in m*HCN4*-transfected cMSCs. Following EPCS induction, the expression of Cx43, Cx45 and *HCN4* were significantly increased. Under the same conditions, reduced Cx43 expression and barely detectable Cx45 expression were detected in GFP-transfected cMSCs, indicating that m*HCN4* is able to promote the expression of gap junction proteins, and that EPCS enhanced this effect. Collectively, the present results indicate that the activation and amplification of m*HCN4* may promote the cardiac differentiation progress of cMSCs. In our previous study, we performed preliminary experiments using fluorescence recovery after photo bleaching to determine whether m*HCN4*-transfected cMSCs could form functional gap junctions (22). The average fluorescence recovery rate of m*HCN4*-transfected cMSCs co-cultured with neonatal rat atrial cells was obviously increased compared with those of GFP-transfected cMSCs co-cultured with neonatal rat atrial cells, and it was same as cultured neonatal rat atrial cells (data not shown). These results suggest that m*HCN4*-transfected cMSCs are able to assemble functional gap junctions, which is consistent with the findings of Valiunas *et al* (23).

In conclusion, the present results suggest that m*HCN4*-transfected cMSCs are able to generate pacemaker I_f current, and that EPCS enhanced this effect. In addition to the increased gap junctions and upregulated Cx43 and Cx45 expression, we infer that EPCS may promote the I_f current reconstructed in m*HCN4* genetically modified cMSCs via the incremental gap junctions assembled by Cx43 and Cx45. In addition, this study may provide a reference for the future studies involving biological pacemaker reconstruction in canines, and may assist in the future development of gene-targeted and regenerative therapeutic remedies for SAN dysfunction in humans.

Acknowledgements

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References

1. Van Mierop LH and Gessner IH: The morphologic development of the sinoatrial node in the mouse. Am J Cardiol 25: 204-212, 1970.
2. Miake J, Marbán E and Nuss HB: Biological pacemaker created by gene transfer. Nature 419: 132-133, 2002.
3. Tong S, Yao Q, Wan Y, Zhou J, Shu M, Zhong L, Li Y, Zhang Q, Yindai J and Song Z: Development of functional I f channels in mMSCs after transfection with mHCN4: Effects on cell morphology and mechanical activity in vitro. Cardiology 112: 114-121, 2009.
4. Nong Y, Zhang C, Wei L, Zhang Z, Cheng J, Wen L and Song Z: In situ investigation of allografted mouse HCN4 gene-transfected rat bone marrow mesenchymal stromal cells with the use of patch-clamp recording of ventricular slices. Cytotherapy 15: 905-919, 2013.
5. Jun C, Zhihui Z, Lu W, Yaoming N, Lei W, Yao Q and Zhiyuan S: Canine bone marrow mesenchymal stromal cells with lentiviral mHCN4 gene transfer create cardiac pacemakers. Cytotherapy 14: 529-539, 2012.
6. Hart RA and Gandhi OP: Comparison of cardiac-induced endogenous fields and power frequency induced exogenous fields in an anatomical model of the human body. Phys Med Biol 43: 3083-3099, 1998.
7. Tandon N, Cannizzaro C, Chao PH, Maidhof R, Marsano A, Au HT, Radisic M and Vunjak-Novakovic G: Electrical stimulation systems for cardiac tissue engineering. Nat Protoc 4: 155-173, 2009.
8. Radisic M, Marsano A, Maidhof R, Wang Y and Vunjak-Novakovic G: Cardiac tissue engineering using perfusion bioreactor systems. Nat Protoc 3: 719-738, 2008.
9. Radisic M, Park H, Chen F, Salazar-Lazzaro JE, Wang Y, Dennis R, Langer R, Freed LE and Vunjak-Novakovic G: Biomimetic approach to cardiac tissue engineering: Oxygen carriers and channeled scaffolds. Tissue Eng 12: 2077-2091, 2006.
10. Wen L, Zhang C, Nong Y, Yao Q and Song Z: Mild electrical pulse current stimulation upregulates S100A4 and promotes cardiogenesis in MSC and cardiac myocytes coculture monolayer. Cell Biochem Biophys 65: 43-55, 2013.
11. Zhao Q, Wang H, Yang M, Yang D, Zuo Y and Wang J: Expression of a tumor-associated gene, LASS2, in the human bladder carcinoma cell lines BIU-87, T24, EJ and EJ-M3. Exp Ther Med 5: 942-946, 2013.
12. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408, 2001.

13. Gonen-Korkmaz C, Sevin G, Gokce G, Arun MZ, Yildirim G, Reel B, Kaymak A and Ogut D: Analysis of tumor necrosis factor α-induced and nuclear factor κB-silenced LNCaP prostate cancer cells by RT-qPCR. Exp Ther Med 8: 1695-1700, 2014.
14. Stieber J, Herrmann S, Feil S, Löster J, Feil R, Biel M, Hofmann F and Ludwig A: The hyperpolarization-activated channel HCN4 is required for the generation of pacemaker action potentials in the embryonic heart. Proc Natl Acad Sci USA 100: 15235-15240, 2003.
15. Söhl G and Willecke K: Gap junctions and the connexin protein family. Cardiovasc Res 62: 228-232, 2004.
16. Kwong KF, Schuessler RB, Green KG, Laing JG, Beyer EC, Boineau JP and Saffitz E: Differential expression of gap junction proteins in the canine sinus node. Circ Res 82: 604-612, 1998.
17. Verheule S, van Kempen MJ, Postma S, Rook MB and Jongsma HJ: Gap junctions in the rabbit sinoatrial node. Am J Physiol Heart Circ Physiol 280: H2103-H2115, 2001.
18. Van Norstrand DW, Asimaki A, Rubinos C, Dolmatova E, Srinivas M, Tester DJ, Saffitz JE and Duffy HS: Connexin43 mutation causes heterogeneous gap junction loss and sudden infant death. Circulation 125: 474-481, 2012.
19. Salameh A, Blanke K and Daehnert I: Role of connexins in human congenital heart disease: The chicken and egg problem. Front Pharmacol 4: 70, 2013.
20. Yamada KA, Rogers JG, Sundset R, Steinberg TH and Saffitz J: Up-regulation of connexin45 in heart failure. J Cardiovasc Electrophysiol 14: 1205-1212, 2003.
21. Betsuyaku T, Nnebe NS, Sundset R, Patibandla S, Krueger CM and Yamada KA: Overexpression of cardiac connexin45 increases susceptibility to ventricular tachyarrhythmias in vivo. Am J Physiol Heart Circ Physiol 290: H163-H171, 2006.
22. Qin Y, Zhiyuan S, Shifei T and Zewen W: Study of differentiation of rat mesenchymal stem cells with mHCN4 gene into spontaneous cardiomyocyte-like cells in vitro. Zhong Guo Xin Zang Qi Bo Yu Xin Dian Sheng Li Za Zhi 21: 55-58, 2007.
23. Valiunas V, Doronin S, Valiuniene L, Potapova I, Zuckerman J, Walcott B, Robinson RB, Rosen MR, Brink PR and Cohen IS: Human mesenchymal stem cells make cardiac connexins and form functional gap junctions. J Physiol 555: 617-626, 2004.