Adipose-derived stem cells overexpressing SK4 calcium-activated potassium channel generate biological pacemakers

MEI YANG[1-3*], QINGYAN ZHAO[1-3*], HONGYI ZHAO[1-3], ANKANG YANG[1-3], FENGYUAN WANG[1-3], XI WANG[1-3], YANHONG TANG[1-3] and CONGXIN HUANG[1-3]

[1]Department of Cardiology, Renmin Hospital of Wuhan University; [2]Cardiovascular Research Institute, Wuhan University; [3]Hubei Key Laboratory of Cardiology, Wuhan, Hubei 430060, P.R. China

Received May 17, 2019; Accepted September 11, 2019

DOI: 10.3892/ijmm.2019.4374

Abstract. Recent studies have suggested that calcium-activated potassium channel (K_{Ca}) agonists increase the proportion of mouse embryonic stem cell-derived cardiomyocytes and promote the differentiation of pacemaker cells. In the present study, it was hypothesized that adipose-derived stem cells (ADSCs) can differentiate into pacemaker-like cells via overexpression of the SK4 gene. ADSCs were transduced with a recombinant adenovirus vector carrying the mouse SK4 gene, whereas the control group was transduced with GFP vector. ADSCs transduced with SK4 vector were implanted into the rat left ventricular free wall. Complete atrioventricular block (AVB) was established in isolated perfused rat hearts after 2 weeks. SK4 was successfully and stably expressed in ADSCs following transduction. The mRNA levels of the pluripotent markers Oct-4 and Sox-2 declined and that of the transcription factor Shox2 was upregulated following SK4 transduction. The expression of α-actinin and hyperpolarization-activated cyclic nucleotide-gated potassium channel 4 (HCN4) increased in the SK4 group. The hyperpolarizing activated pacemaker current I_f (8/20 cells) was detected in ADSCs transduced with SK4, but not in the GFP group. Furthermore, SK4 transduction induced the expression of p-ERK1/2 and p-p38 MAPK. In the *ex vivo* experiments, the heart rate of the SK4 group following AVB establishment was significantly higher compared with that in the GFP group. Immunofluorescence revealed that the transduced ADSCs were successfully implanted and expressed HCN4 in the SK4 group. In conclusion, SK4 induced ADSCs to differentiate into cardiomyocyte-like and pacemaker-like cells via activation of the extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase pathways. Therefore, ADSCs transduced with SK4 may be used to generate biological pacemakers in *ex vivo* rat hearts.

Correspondence to: Dr Congxin Huang, Department of Cardiology, Renmin Hospital of Wuhan University, 238 Jiefang Road, Wuchang, Wuhan, Hubei 430060, P.R. China
E-mail: huangcongxin@vip.163.com

*Contributed equally

Key words: calcium-activated potassium channel, intermediate-conductance, biological pacemaker, adipose-derived stem cells, stem cells

Introduction

Small (SK1-3)- and intermediate (SK4)-conductance Ca^{2+}-activated potassium channels bind intracellular Ca^{2+} to produce K^+ outflow and transform calcium signaling into changes in membrane potential. SK4 expression was found to be 9 times higher compared with SK1-3 expression during the development of the atrioventricular node (AVN) (1). SK2 and SK4 are expressed in the atrial and pulmonary vein regions in the mature heart and regulate the late phase of cardiac repolarization (2,3). These channels are also expressed in the AVN and sinoatrial node (SAN) (4-6). Previous studies have demonstrated that SK4 inhibitors can lower the frequency of action potentials of the SAN (5-7), whereas mathematical models have predicted that the upregulation of SK4 would increase the automaticity of SAN cells (5,8).

It was recently reported that SK4 inhibitors exert a suppressive effect on the pacemaker function of cardiomyocytes derived from human embryonic stem cells (ESCs) (9), and the SK1-4 agonist EBIO, which markedly slows the channel deactivation process (10), promoted the differentiation of mouse and human ESCs and induced pluripotent stem cells (iPSCs) into pacemaker cells (11,12). In addition, downregulation of SK4 by RNA interference inhibits this inducing effect and produces no spontaneously beating cardiomyocytes, whereas SK1-3 knockdown does not alter EBIO induction (11). It was previously demonstrated that overexpression of SK4 plasmids in mouse ESCs also enhanced the generation of cardiac and pacemaker cells (13). Another recent study reported that EBIO can modify the cardiac subtype of human ESCs and iPSCs (14).

Adipose-derived stem cells (ADSCs) have the advantages of convenient accessibility, low immunogenicity, and autologous and allogeneic transplantation (15). ADSCs differentiated into pacemaker-like cells in a semisolid methylcellulose medium or by transfecting transcription factor Tbx18 (16,17), which supports the use of ADSCs as seed cells for biological pacemakers.

The aim of the present study was to examine whether ADSCs are capable of differentiating into pacemaker-like cells *in vitro* by overexpressing SK4 following transduction with an adenovirus vector carrying the SK4 gene, and to investigate the mechanisms underlying this differentiation.

Materials and methods

Ethical approval. All animal procedures were performed in agreement with the Wuhan University institutional guidelines and in compliance with suggestions from the panel of Euthanasia of the American Veterinary Medical Association and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study was approved by the Ethics Committee of Renmin Hospital of Wuhan University (Wuhan, China).

Isolation and culture of ADSCs. Adult male Sprague Dawley (SD) rats (n=2; 4 weeks old, weighing 80-100 g) were housed in an environmentally controlled room at a temperature of 22±1˚C and relative humidity 40-60% with a standard 12-h light/dark cycle. Food and water were provided in the cages. The rats were anesthetized with 3% sodium pentobarbital (30 mg/kg) by intraperitoneal injection. Following cessation of pain reflexes, adipose tissue was obtained from the inguen of the rats. The adipose tissue was cut into 1x1-mm^3 pieces and digested with 1 mg/ml collagenase type I (Sigma-Aldrich; Merck KGaA) for 1 h at 37˚C. The homogenate was centrifuged at 300 x g for 10 min at 25˚C, and the cells were resuspended in Dulbecco's modified Eagle's medium/F12 supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). Cells were cultured in an incubator at 37˚C with a 5% CO_2 atmosphere, grown to 80-90% confluence and passaged using 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.). Cell passages 3-5 were used for subsequent experiments. Euthanasia was conducted via sedation by CO_2 followed by cervical dislocation.

Adenovirus construction and purification. CMV-MCS-EGFP (Genechem) was digested using *Bam*HI and *Age*I. Polymerase chain reaction (PCR) was used to amplify the open reading frame sequence of the mouse SK4 gene (GenScript), which was linearized and inserted into a vector to construct CMV-MCS-EGFP-SK4. The recombinant plasmid was transformed into DH5a-competent cells (Tiangen) to obtain positive clones, which were identified using enzyme digestion and sequencing. Large-scale preparations of recombinant plasmid were created using the Plasmid Midi Preparation kit (Promega Corporation). The concentration and purity of the plasmid DNA were determined by UV absorption. The A260/A280 of the plasmid DNA was between 1.8 and 2.0. When the density of the 293 cells (ATCC, cat. no. CRL-1573) reached 50-60%, they were transfected with CMV-MCS-EGFP-SK4 and the backbone vector pBHG using Lipofectamine 2000 (both from Genechem) for 6 h in an incubator at 37˚C with a 5% CO_2 atmosphere. The supernatant was harvested after virus amplification. Ad-GFP and Ad-SK4 were measured as $2x10^1$ plaque-forming units/ml and preserved at -80˚C.

ADSCs transduced with Ad-SK4 and flow cytometric analysis. Cells were grown to 70-80% confluence, and Ad-SK4 in transduction enhancer Polybrene (Yeasen) was added to ADSCs at different multiplicity of infection (MOI) values (0, 20, 50, 100, 150 and 300). Polybrene was added at the same concentration for 4 h for different MOI values. The control group was transduced with Ad-GFP. The medium was replaced after 4 h. Cells were observed using light and fluorescence microscopy (BX51 systems, Olympus Corporation). ADSCs with different MOI values were digested with 0.25% trypsin at 48 h after transduction. The cell suspension was washed twice with phosphate-buffered saline (PBS). Non-transduced cells served as a negative control. The percentage of GFP-positive cells was detected using flow cytometric analysis (Becton, Dickinson and Company).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. qPCR was performed to evaluate the mRNA expression of mouse SK4, rat SK4, Oct-4, Sox-2, cardiac troponin I (cTnI), hyperpolarization-activated cyclic nucleotide-gated potassium channel 4 (HCN4) and transcription factors Tbx18 and Shox2. The primers used were synthesized by Invitrogen; Thermo Fisher Scientific, Inc. (Table I). Total RNA was extracted from the transduced ADSCs after 1 week using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and converted into cDNA using the First Strand cDNA Synthesis kit (Toyobo Life Science). The PCR conditions were 45 cycles at 95˚C for 15 sec and at 58˚C for 1 min. RT-qPCR was performed using the StepOne™ Real-Time PCR system (Thermo Fisher Scientific, Inc.). Semilog amplification curves were analyzed using the $2^{-\Delta\Delta Cq}$ comparative quantification method (18), and the expression of each gene was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Western blot analysis. The transduced ADSCs were plated in 6-well culture dishes. Cells were harvested using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology). The protein concentration was detected by the BCA protein concentration assay kit (AS1086, ASPEN). Equal amounts of protein (40 μg) were loaded onto a 10% gel for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were incubated with primary antibodies against HCN4 (rat monoclonal antibody, 1:1,000, ab32675, Abcam), SK4 (rabbit anti-rat monoclonal antibody, 1:500, ab215990, Abcam), phosphorylated extracellular signal-regulated kinase (p-ERK; rabbit anti-rat monoclonal antibody, 1:1,000, cat. no. 4370, Cell Signaling Technology, Inc.), phosphorylated c-jun N-terminal kinase (p-JNK; rabbit anti-rat monoclonal antibody, 1:1,000, cat. no. 4668, Cell Signaling Technology, Inc.) and p-p38 (rabbit anti-rat monoclonal antibody, 1:1,000, cat. no. 4511, Absin) overnight at 4˚C. Primary antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:10,000, AS1107, ASPEN) and goat anti-rat secondary antibodies (1:10,000, AS1093, ASPEN). Signal intensities were normalized to GAPDH levels. qPCR was performed with the ABI Prism 7500 sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The SYBR-Green real-time PCR Master Mix kit (Takara) was utilized in subsequent PCR assays in accordance with the manufacturer's instructions.

Table I. Sequences of primers for reverse transcription-quantitative polymerase chain reaction analysis.

mRNA	Primers 5'-3'	Product size (bp)
SK4	Sense: GTTCTGCACGCTGAGATGTTG Antisense: CTTGGCATGGAAGACCACAAT	126
Oct-4	Sense: GTGTCGGAGTGGGGATTGA Antisense: AGAAACGGAGAACTACATAGGTCC	223
Sox-2	Sense: GATGCACCGCTACGACGTC Antisense: TGGAGTGGGAGGAAGAGGTAAC	197
Tbx18	Sense: GGAGACTTGGATGAGACAAGTGAT Antisense: TTGGCAAATGGATTCCTGTCT	282
Shox2	Sense: ATCCAGACGCTTTTATGCGC Antisense: TCCTGCTGAAATGGCATCCT	214
cTnI	Sense: TTGGATGGGCTGGGCTT Antisense: CCTCCTTCTTCACCTGCTTGA	286
HCN4	Sense: CACTAAGGGCAACAAGGAGACC Antisense: GGTAGTTGAAGACGCCTGAGTTG	281
GAPDH	Sense: CGCTAACATCAAATGGGGTG Antisense: TTGCTGACAATCTTGAGGGAG	201

SK4, intermediate-conductance Ca^{2+}-activated potassium channel; HCN4, hyperpolarization-activated cyclic nucleotide-gated potassium channel 4; cTnI, cardiac troponin I.

Immunostaining studies. Transduced ADSCs were cultured on gelatin-coated coverslips in 6-well culture dishes, washed with PBS, and fixed using 4% paraformaldehyde. Cells were incubated with primary antibody against α-actinin (rabbit anti-rat monoclonal antibody, 1:200, ab13734, Abcam) overnight at 4˚C. Secondary antibody FITC-conjugated goat anti-rabbit (1:50, AS1110, ASPEN) was used to detect α-actinin. The nuclei were visualized with 4',6-diamidino-2-phenylindole. The cells were observed under a fluorescence microscope (Leica Microsystems GmbH). Three visual fields in three different cell isolates were randomly selected to observe positive cells.

Electrophysiological recordings. Transduced ADSCs were plated on gelatin-coated coverslips in 24-well culture dishes. A whole-cell patch clamp was used to record the I_f current 5-7 days after transduction. The bath solution included the following components (in mmol/l): 140 NaCl, 5.4 KCl, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 1.0 $BaCl_2$, 5.5 HEPES, and 5.0 glucose (pH 7.3). The pipette solution contained the following components (in mmol/l): 20 KCl, 125 K-gluconate, 1.0 $MgCl_2$, 5.0 NaCl, 10 HEPES, and 5 K_2ATP (pH 7.3). The impedance of the fluid-filled electrode was 6-8 MΩ. Experiments were performed using an Axon patch-clamp amplifier 700B (Molecular Devices, LLC). A digital 700AD/DA converter and 6.0.4 pClamp (both from Axon Instruments) were used for data recording and analysis. The I_f current was recorded using a voltage clamp. The holding potential was -40 mV, which was decreased to -140 mV with each 10-mV sweep and returned to the resting potential. CsCl (2 mmol/l) was added to detect changes in I_f.

Transplantation of transduced ADSCs in rats. Male SD rats (weight, 200-250 g) were randomly divided into GFP and SK4 groups (n=8/group). Transduced ADSCs were cultured for 5-7 days *in vitro*. The rats were anesthetized with 3% sodium pentobarbital (30 mg/kg) by intraperitoneal injection, and a ventilator and electrocardiograph were connected. An injection site at the free wall of the left ventricle was indicated using a suture. Each rat was injected subepicardially around the suture with 10^6 transfected ADSCs in 0.1 ml PBS using a micropipettor (30 G, Hamilton). Subsequently, the chest was closed in layers. All animals were monitored carefully for 2 weeks post-injection.

Establishment of a complete atrioventricular block (AVB) model in ex vivo rat hearts. The rats received an intraperitoneal injection of heparin 2 weeks after cell injection. The rats were anesthetized with an intraperitoneal (IP) injection of 0.2 ml Telazol and subjected to isoflurane inhalation. Following cessation of pain reflexes, the heart was removed and connected to a Langendorff cardiac perfusion device (AD Instruments) that contained Tyrode's solution (in mmol/l): 135 NaCl, 5.4 KCl, 1.8 CaCl, 1 $MgCl_2$, 0.3 Na_2HP0_4, 10 HEPES, and 10 glucose (pH 7.4). The isolated hearts were perfused for 20 min prior to further experimentation. The perfused heart was placed in a Sylgard-coated plate filled with warm Tyrode's solution. Electrocardiographic leads I and II were placed at appropriate sites. After a 20-min equilibration period, an AVB model was established in *ex vivo* hearts via injection of 70% ethanol within the AVN region using a micropipettor (30 G, Hamilton). The electrode pacing was performed at the site of the transgene injection at 200-msec

intervals. All measured signals were amplified and filtered using a PowerLab system (AD Instruments).

Statistical analysis. The reported data are expressed as means ± standard deviation. The data on the effect of SK4 on cell numbers were analyzed using a general linear model. Tukey's post hoc tests were used to identify pairwise changes between groups on different days. SK4 expression on different days was analyzed with two-way analysis of variance (ANOVA) and Bonferroni's multiple comparison test. The statistical significance of the differences between two groups was examined using the unpaired and two-tailed t-test. One-way ANOVA and Bonferroni's multiple comparison test were used to compare differences among the three groups. A P-value of <0.05 was considered to indicate a statistically significant difference.

Results

Transfection efficiency and SK4 expression after transfection. ADSCs were transfected with SK4 at different MOI values (20, 50, 100, 150 and 300). The control group was transfected with GFP at MOI=50. Flow cytometric analysis revealed that the SK4 transfection efficiency was >70% at MOI≥100. The transfection efficiencies were 76.8±4.5 and 80.0±6.3% at MOI=100 and MOI=150, respectively (P>0.05; Fig. 1A). Most cells appeared to float and die at MOI=300. PCR analysis revealed that the level of mouse SK4 was significantly elevated at 48 h and 7 days after transfection (P<0.05; Fig. 1B). Western blotting also demonstrated increased SK4 expression 7 days after SK4 vector transduction (Fig. 1C and D), whereas the level of SK4 in the control group was low. These results confirmed that SK4 was successfully and stably expressed in ADSCs.

Effect of SK4 on the expression of pluripotent markers Oct-4 and Sox-2 and transcription factors Tbx18 and Shox2 after SK4 vector transduction. Oct-4 and Sox-2 are embryonic SC markers that may play important roles in the differentiation potential of ADSCs (19,20). ADSCs express Oct-4 and Sox-2 (20,21). The expression of Oct-4 mRNA in the SK4 group was significantly lower compared with that in the control group and declined with increasing MOI values (P<0.05; Fig. 2A). Sox-2mRNA was significantly downregulated in the SK4 group at MOI=150 (P<0.05; Fig. 2B). Therefore, increased expression of SK4 appeared to promote the differentiation of ADSCs.

A number of transcription factors, including Tbx18 and Shox2, regulate the development of SAN (22). PCR analysis revealed that, although no significant difference in Tbx18 mRNA expression was observed between the two groups (Fig. 2C), Shox2 mRNA was significantly increased following SK4 transduction (P<0.05; Fig. 2D).

SK4 induces differentiation of ADSCs into cardiomyocyte-like cells. The expressions of the myocardial-specific markers α-actinin and cTnI were detected using PCR and immunofluorescence 7 days after transduction. α-Actinin expression was observed after SK4 transduction at MOI=100 and MOI=150 using immunofluorescence. The morphology of differentiated cardiomyocyte-like cells changed to columnar or polygonal shapes compared with the fibroblast-like shape of ADSCs transduced with GFP vector (Fig. 3A-C). The expression of α-actinin was negative in the GFP group. Quantitative analyses demonstrated that the α-actinin positivity rate in the SK4 group was significantly higher at MOI=150 compared with MOI=100 (60.0±10.6% vs. 25.4±7.6%, respectively; P<0.05; Fig. 3D). cTnI mRNA was significantly upregulated after SK4 transduction, and its expression was greater at MOI=150 compared with MOI=100 (P<0.05; Fig. 3E). The number of non-transduced and GFP-transduced ADSCs gradually increased, which indicated that GFP did not affect the proliferation of ADSCs. The cell number of ADSCs transduced with SK4 at MOI=100 increased after 3 days and reached 86±3.0% after 7 days. The cell number was reduced by nearly ~50% at 1-5 days after transduction with MOI=150 (61±4.6% vs. 31±2.4%, P<0.05), and did not change significantly at 5-7 days (31±2.4% vs. 40±3.5%, P>0.05) (Fig. 3F). These results indicate that SK4 induces ADSC differentiation into cardiomyocyte-like cells, and the proportion of cardiomyocyte-like cells increases with increasing MOI values.

SK4 induces ADSCs to differentiate into pacemaker-like cells. The expression of the pacemaker channel HCN4 was significantly upregulated following SK4 transduction, and it was positively associated with the MOI value using western blot detection (Fig. 4A and B). The hyperpolarizing activated pacemaker current I_f (8/20 cells) was detected in ADSCs transduced with SK4 after 5-7 days at a of MOI=150, but not in the GFP group (Fig. 4C). CsCl (2 mmol/l) inhibited the I_f current. The maximum current density of active voltage was -10.6±0.5 pA/pF (n=8) (Fig. 4D).

SK4 activates the ERK and p38 signaling pathways. The mitogen-activated protein kinase (MAPK) family plays an important role in cell differentiation and proliferation, as well as organ development. ERK 1/2, c-jun N-terminal kinase (JNK), and p38 MAPK are members of the MAPK family. Western blot analysis demonstrated that p-ERK 1/2 and p-p38 MAPK expression increased significantly in the SK4 group, but there was no significant difference in p-JNK and total ERK 1/2, p-JNK or p38MAPK proteins between the SK4 and GFP groups (Fig. 5A-D).

ADSCs transduced with SK4 generate biological pacemakers in ex vivo rat hearts. The AVB model was successfully established, and electrocardiograms revealed AV separation. The heart rate of the SK4 group was significantly faster compared with that of the GFP group (145±14 bpm vs. 103±5 bpm, respectively; P<0.05) (Fig. 6B). Stimulation of the cell injection site revealed an electrocardiographic morphology that was identical to the spontaneous rhythm in the SK4 group (Fig. 6A). These results indicated that the ectopic pacing site was indeed the injection site. The GFP group exhibited the opposite morphology following stimulation (Fig. 6A). These results demonstrated that transplantation of ADSCs transduced with SK4 produced an ectopic rhythm and assumed the function of a biological pacemaker. Immunofluorescence confirmed that transduced ADSCs were successfully implanted and expressed HCN4 in the SK4 group (Fig. 6C).

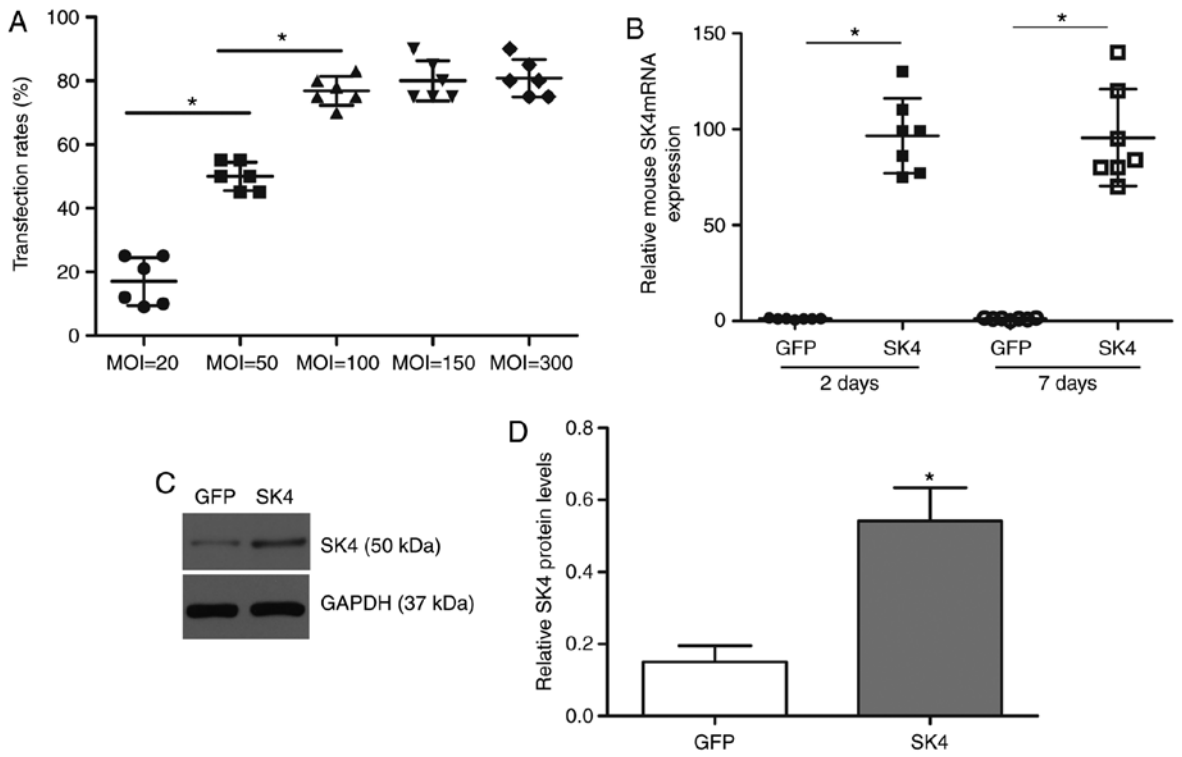

Figure 1. Expression of SK4 after transduction. (A) Transduction rate of different MOI values detected by flow cytometric analysis. The SK4 transduction efficiency was >70% at MOI≥100. (B) SK4 mRNA was stably expressed in ADSCs at 2 and 7 days. (C and D) Western blot assays revealed significantly increased SK4 expression compared with the GFP group. *P<0.05. SK4, intermediate-conductance Ca^{2+}-activated potassium channel; MOI, multiplicity of infection; ADSCs, adipose-derived stem cells.

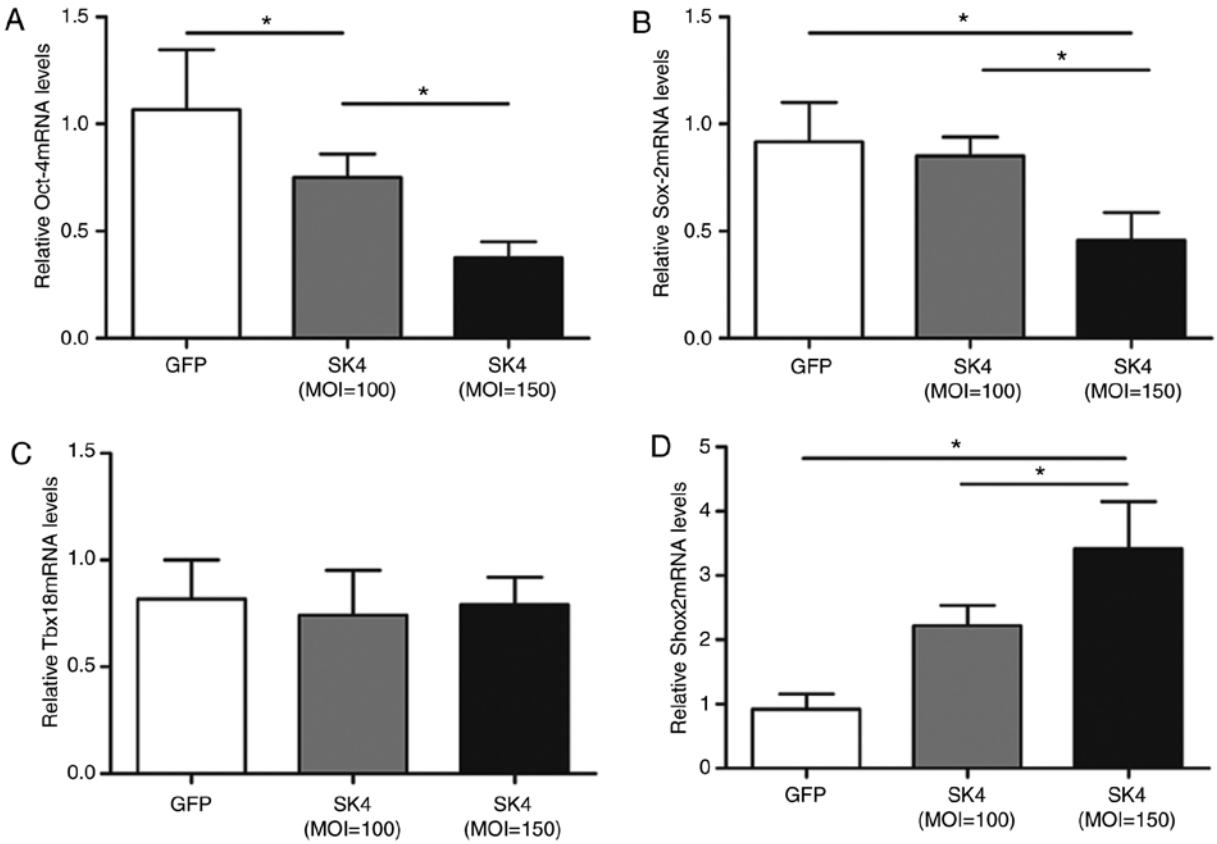

Figure 2. Expression of pluripotent markers Oct-4 and Sox-2 and transcription factors Tbx18 and Shox2 following SK4 adenovirus transduction with different MOI values. (A and B) Relative Oct-4 and Sox-2 mRNA levels were detected by RT-qPCR. (C and D) Relative Tbx18and Shox2 mRNA levels were detected by RT-qPCR. *P<0.05. SK4, intermediate-conductance Ca^{2+}-activated potassium channel; MOI, multiplicity of infection; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

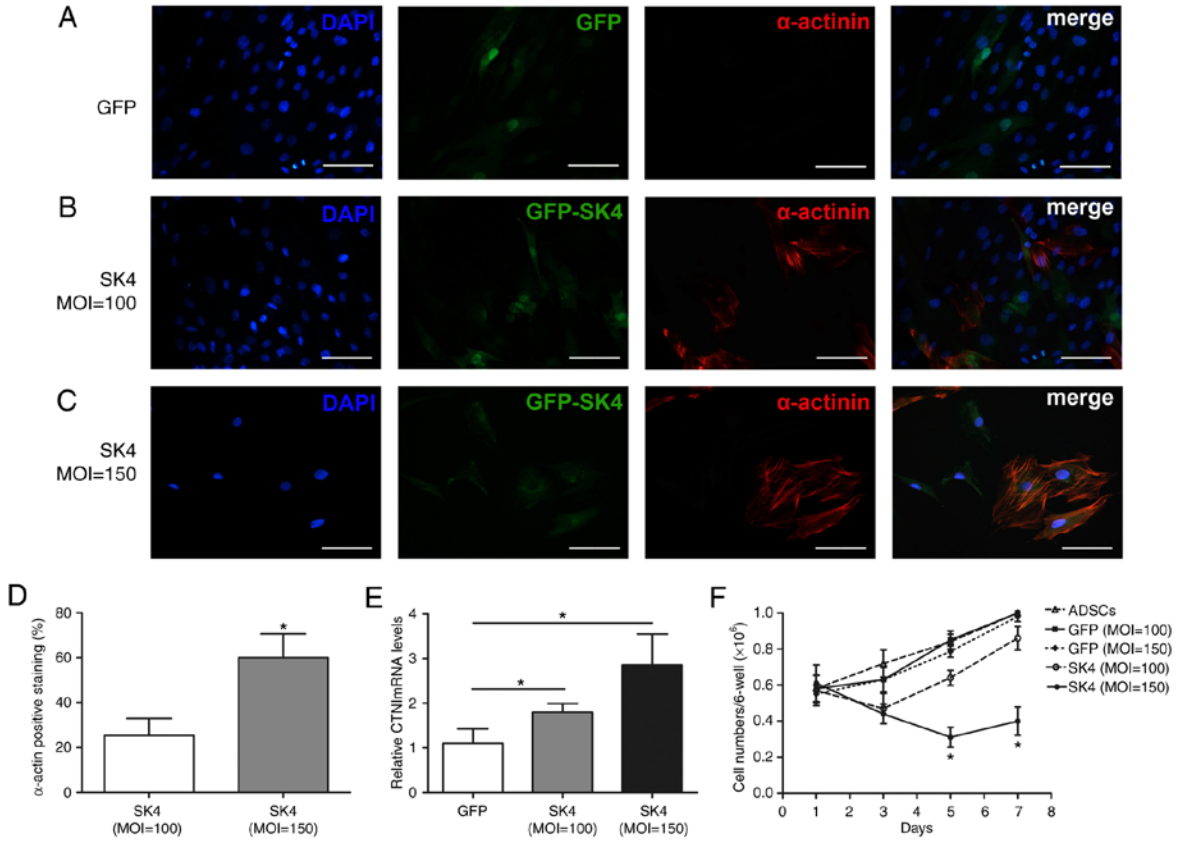

Figure 3. Expression of myocardial-specific markers after transduction. (A-C) Expression of α-actinin detected using immunofluorescence assay with different MOI values. Scale bar, 100 μm. (D) Quantitative analysis demonstrated that the α-actinin positivity rate in the SK4 group was significantly higher at MOI=150 compared with MOI=100. (E) Expression of cTnI mRNA after transduction with different MOI values. (F) The cell numbers were significantly reduced at 1-5 days after SK4 transduction with MOI=150, and were increased at 3 days after GFP transduction, SK4 transduction with MOI=100, and in the control group. *P<0.05. SK4, intermediate-conductance Ca^{2+}-activated potassium channel; MOI, multiplicity of infection; cTnI, cardiac troponin I.

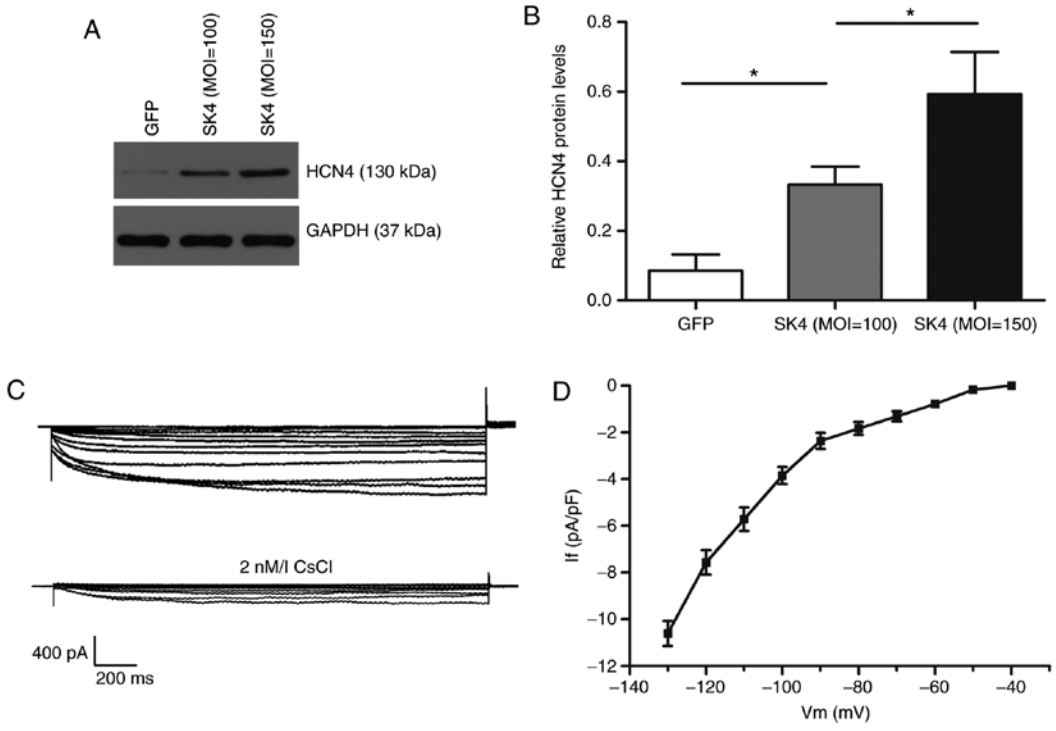

Figure 4. Expression of HCN4 and pacemaker current I_f after transduction. (A and B) Western blot analysis of HCN4 expression in the GFP and SK4 groups with MOI=100 and MOI=150 (*P<0.05). (C) I_f current detected by the patch clamp technique and blockade with CsCl (2 mM/l). (D) Mean current-voltage association of the I_f current (n=8). SK4, intermediate-conductance Ca^{2+}-activated potassium channel; MOI, multiplicity of infection; HCN4, hyperpolarization-activated cyclic nucleotide-gated potassium channel 4.

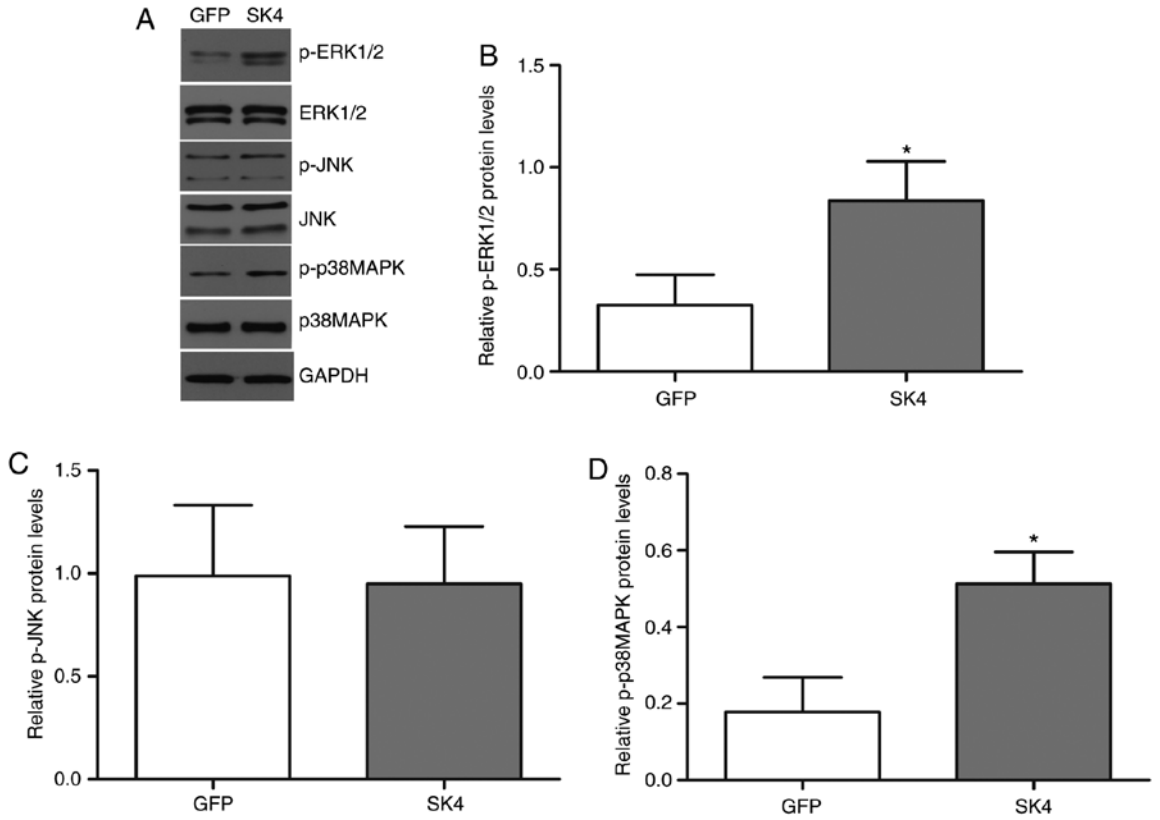

Figure 5. Effects of SK4 on the expression of p-ERK 1/2, p-JNK, and p-p38MAPK and total protein detected by western blot analysis. (A-D) The levels of p-ERK 1/2 and p-p38 MAPK increased significantly after SK4 transduction. *P<0.05. SK4, intermediate-conductance Ca^{2+}-activated potassium channel; ERK, extracellular signal-regulated kinase; JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase.

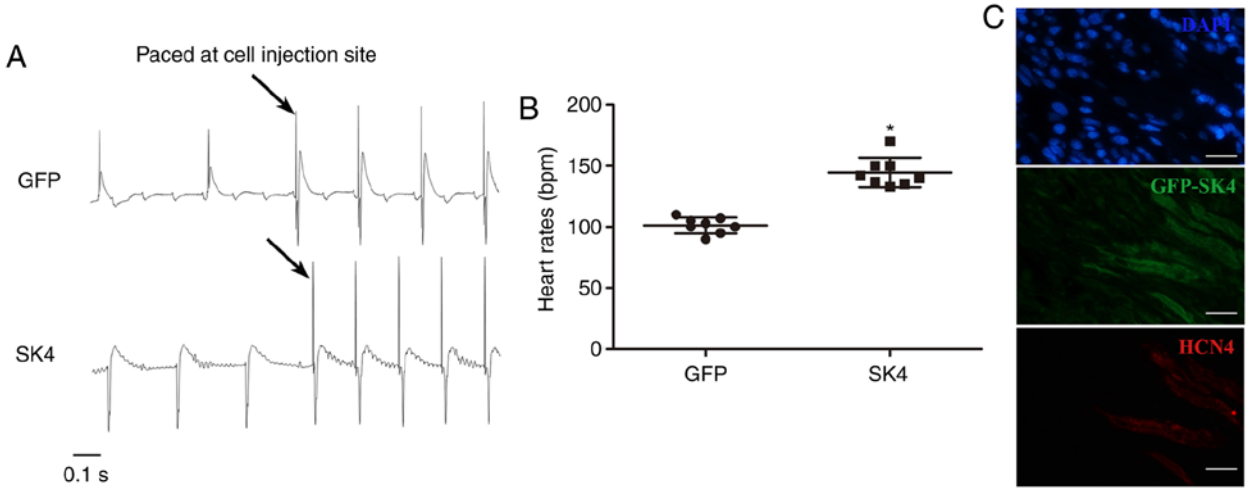

Figure 6. ADSCs transduced with SK4 generated biological pacemakers in *ex vivo* hearts. (A) After AVB model establishment, the electrocardiographic morphology was the same as the spontaneous rhythm in the SK4 group following stimulation of the cell injection site, whereas the GFP group exhibited the opposite morphology after stimulation. (B) Heart rate of the SK4 and GFP groups after AVB model establishment in *ex vivo* hearts (n=8/group) (*P<0.05). (C) Immunofluorescence detected that transduced ADSCs were successfully implanted and expressed HCN4 in the SK4 group. Scale bar, 100 μm. SK4, intermediate-conductance Ca^{2+}-activated potassium channel; ADSCs, adipose-derived stem cells; AVB, atrioventricular block; HCN4, hyperpolarization-activated cyclic nucleotide-gated potassium channel 4.

Discussion

The present study combined gene-based and SC-based therapies to create a biological pacemaker in *ex vivo* rat hearts. The results demonstrated that recombinant adenoviral vectors carrying the ion channel SK4 induced ADSCs to differentiate into cardiomyocyte-like and pacemaker-like cells in a dose-dependent manner *in vitro*. The underlying mechanism may be associated with the upregulation of the ERK1/2 and p38 MAPK signaling pathways. In addition, it was observed that SK4-induced pacemaker-like cells generated a pacemaker function in *ex vivo* rat hearts.

Biological pacemaker construction involves SCs and gene therapy. Adult SCs primarily include ADSCs and bone marrow (BM) SCs. Previous studies used BMSCs to construct biological pacemakers via overexpression of the ion channels of the HCN family (23-26). However, BMSCs were only used as ion channel-carrying tools in those previous studies and did not differentiate. The realization of the pacemaker function requires the hyperpolarization of the adjacent host myocardium to activate HCN (27). The present study demonstrated that ADSCs overexpressing the ion channel SK4 differentiated into pacemaker-like cells. In addition, the induced pacemaker-like cells expressed HCN4, which may interact with SK4 to generate the pacemaker function.

Recent research has demonstrated that the ion channel SK_{Ca} affects the differentiation of SCs (11-13). SK4 agonists were shown to enhance the differentiation of human ESCs and iPSCs into pacemaker-like cells (11,12). Conversely, SK4 inhibitors suppressed the pacemaker function of ESC-derived cardiomyocytes (9). Another recent study reported that EBIO can modify the cardiac subtype of human ESCs and iPSCs (14); the study suggested that the SK2/SK3 channel modulator exerted a similar EBIO-mediated effect, but the effect of the SK4 activator was not the same. This phenomenon may be attributed to the fact that SK4 current is not recorded in hiPSCs (28), and the expression of SK4 is low compared with that of SK2 and SK3 during hESC differentiation (14). In the present study, immunofluorescence and western blot analysis detected low SK4 expression in undifferentiated ADSCs. The expression of SK4 in ADSCs was previously investigated (29). Our results further confirmed that an exogenous adenovirus vector carrying the SK4 gene was successfully transduced into ADSCs to induce SK4 overexpression.

The present study demonstrated that SK4 reduced the expression of pluripotent markers and increased the expression of the myocardial markers cTnI and α-actinin, and the pacemaker channel HCN4 in ADSCs. HCN4 plays an important role in phase 4 of the automatic depolarization of SAN cells (30). Embryos cannot mature in HCN4 knockout mice due to pacemaker dysfunction (31). The results demonstrated that ADSCs can differentiate into cardiomyocyte-like and pacemaker-like cells via overexpression of SK4. The transcription factors Tbx18 and Shox2, which regulate the development of the SAN, were also investigated in the present study. Previous studies demonstrated that Tbx18 and Shox2 increased HCN4 expression and induced ESCs, ADSCs and BMSCs to differentiate into pacemaker-like cells (17,32,33). The expression of Shox2 in the conduction system is higher compared with that in the working myocardium during the early stages of mouse embryonic development (33). The present study demonstrated increased expression of Shox2, but the expression of Tbx18 did not significantly change following SK4 transduction. It was hypothesized that upregulation of Shox2 after SK4 transduction may be one of the mechanisms underlying increased HCN4 expression. More importantly, induced pacemaker-like cells were further confirmed by the pacemaker current I_f that was recorded in SK4-transduced ADSCs, but not in GFP-transduced ADSCs, and the maximum current density of the active voltage was greater than ADSCs transduced with Tbx18 in a previous study (-10.6±0.5 pA/pF vs. -5.43±1.36 pA/pF, respectively) (17). These findings suggest that SK4 and Tbx18 induce cell differentiation via different mechanisms.

It was previously demonstrated that SK4 plays an important role in the pacemaker function of SC-derived cardiomyocytes (9,34). SK4 greatly activates HCN channels in pacemaker cells, shortens the duration of the action potential, and increases the slope of automatic depolarization (34). The opening of SK4 hyperpolarizes the membrane potential and contributes to the activation of the excitatory diastolic current I_f. These results suggest that SK4 promotes the differentiation of ADSCs and increases the current amplitude of the HCN channel in induced pacemaker-like cells to strengthen the pacemaker function.

SCs differentiate toward the myocardial direction, and these differentiated cells comprise cardiomyocytes and non-cardiomyocytes. The former include the working myocardium and pacemaker cells. Electrical heterogeneity affects the pacemaker function and leads to arrhythmic potential (35,36). The SK_{Ca} agonist EBIO reduces the number of non-cardiac progenitor cells and ventricular myocytes, and increases the purity of pacemaker cells (14). The present study found that the number of cells markedly decreased at MOI=150 in the SK4 group, whereas the levels of cTnI, α-actinin and HCN4 were all upregulated at MOI=100. These results suggest that the differentiation efficiency of cardiomyocyte-like and pacemaker-like cells improved with increasing MOI values. This phenomenon may be attributed to the decrease in the number of differentiated non-cardiomyocytes, or the increase in the amount of SK4, which directly promotes the differentiation of pacemaker-like cells. These two activities may jointly promote the increased differentiation efficiency. Alleviation of cellular heterogeneity may facilitate the realization of the pacemaker function in *ex vivo* hearts. The transduced ADSCs were successfully implanted into the heart, and the beating frequency markedly increased following implantation of the SK4-transduced ADSCs. Accordingly, it may be inferred that SK4 is a potential target for biological pacemaker gene therapy.

Previous studies have demonstrated the role of ERK1/2 and p38 MAPK in cell proliferation and differentiation (37,38). p38 MAPK induced pluripotent SCs to differentiate towards the cardiac lineage at an early stage of differentiation, and inhibited differentiation towards the neural lineage (39). It was also demonstrated that p38 MAPK and ERK1/2 were activated in tandem to promote the expression of relevant markers in cardiac progenitor cells, such as MEF2c, GATA2 and ATF2 in P19CL6 cells (40). These studies demonstrated that p38-MAPK and ERK1/2 play important roles in the differentiation of pluripotent SCs towards the myocardial lineage. A recent study demonstrated that EBIO induced ESCs to produce a cardiac lineage via activation of the ERK signaling pathway (11). The present study demonstrated that the levels of p-p38 MAPK and p-ERK1/2 were significantly increased following SK4 transduction, which suggests that the effect of SK4 on differentiation is associated with the activation of the p38 MAPK and ERK1/2 signaling pathways.

There were several limitations to the present study. First, patch clamps should be used to confirm that SK4 can

sufficiently activate the I_f current, and the potential mechanism requires further study. Second, the duration of the pacemaker function was not examined. Third, the pacemaker function was only verified in *ex vivo* hearts. Further *in vivo* studies are required to continuously monitor biological pacemaker function and detect possible latent arrhythmias, and evaluate safety and effectiveness.

In conclusion, the present study demonstrated that SK4 induced ADSCs to differentiate into pacemaker-like cells via upregulation of Shox2 and activation of the p38 MAPK and ERK1/2 pathways, which may provide a new approach to the construction of adult SC-associated biological pacemaker.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Fundamental Research Funds for the Central Universities (grant no. 2018413000185) and the National Natural Science Foundation of China (grant no. 81670303).

Availability of data and materials

All the datasets generated and analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

MY and CH contributed to the conception and design of the study. MY, HZ, AY and FW performed the experiments. HZ., QZ and MY analyzed data. XW, YT, HH and CH interpreted the results of the experiments. MY, QZ and CH drafted and revised the manuscript. All authors have approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All animal procedures were performed in agreement with the Wuhan University institutional guidelines and in compliance with suggestions from the panel of Euthanasia of the American Veterinary Medical Association and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The study was approved by the Ethics Committee of Renmin Hospital of Wuhan University (Wuhan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Horsthuis T, Buermans HP, Brons JF, Verkerk AO, Bakker ML, Wakker V, Clout DE, Moorman AF, 't Hoen PA and Christoffels VM: Gene expression profiling of the forming atrioventricular node using a novel tbx3-based node-specific transgenic reporter. Circ Res 105: 61-69, 2009.
2. Mangoni ME and Nargeot J: Properties of the hyperpolarization-activated current (I(f)) in isolated mouse sino-atrial cells. Cardiovasc Res 52: 51-64, 2001.
3. Vinogradova TM, Zhou YY, Bogdanov KY, Yang D, Kuschel M, Cheng H and Xiao RP: Sinoatrial node pacemaker activity requires Ca(2+)/calmodulin-dependent protein kinase II activation. Circ Res 87: 760-767, 2000.
4. Zhang Q, Timofeyev V, Lu L, Li N, Singapuri A, Long MK, Bond CT, Adelman JP and Chiamvimonvat N: Functional roles of a Ca^{2+}-activated K^+ channel in atrioventricular nodes. Circ Res 102: 465-471, 2008.
5. Attali B, Weisbrod D, Bueno H, Behar J, Haron-Khun S and Yadin D: SK4 Ca^{2+}-activated K^+ channels regulate sinoatrial node firing rate and cardiac pacing in vivo. Biophys J 112: 35a, 2017.
6. Haron-Khun S, Weisbrod D, Bueno H, Yadin D, Behar J, Peretz A, Binah O, Hochhauser E, Eldar M, Yaniv Y, *et al*: SK4 K^+ channels are therapeutic targets for the treatment of cardiac arrhythmias. EMBO Mol Med 9: 415-429, 2017.
7. Oliván-Viguera A, Valero MS, Coleman N, Brown BM, Laría C, Murillo MD, Gálvez JA, Díaz-de-Villegas MD, Wulff H, Badorrey R and Köhler R: A novel pan-negative-gating modulator of KCa2/3 channels, fluoro-di-benzoate, RA-2, inhibits endothelium-derived hyperpolarization-type relaxation in coronary artery and produces bradycardia in vivo. Mol Pharmacol 87: 338-348, 2015.
8. Kharche S, Yu J, Lei M and Zhang H: A mathematical model of action potentials of mouse sinoatrial node cells with molecular bases. Am J Physiol Heart Circ Physiol 301: H945-H963, 2011.
9. Weisbrod D, Peretz A, Ziskind A, Menaker N, Oz S, Barad L, Eliyahu S, Itskovitz-Eldor J, Dascal N, Khananshvili D, *et al*: SK4 Ca^{2+} activated K^+ channel is a critical player in cardiac pacemaker derived from human embryonic stem cells. Proc Natl Acad Sci USA 110: E1685-E1694, 2013.
10. Devor DC, Singh AK, Frizzell RA and Bridges RJ: Modulation of Cl- secretion by benzimidazolones. I. Direct activation of a Ca(2+)-dependent K^+ channel. Am J Physiol 271: L775-L784, 1996.
11. Kleger A, Seufferlein T, Malan D, Tischendorf M, Storch A, Wolheim A, Latz S, Protze S, Porzner M, Proepper C, *et al*: Modulation of calcium-activated potassium channels induces cardiogenesis of pluripotent stem cells and enrichment of pacemaker-like cells. Circulation 122: 1823-1836, 2010.
12. Müller M, Stockmann M, Malan D, Wolheim A, Tischendorf M, Linta L, Katz SF, Lin Q, Latz S, Brunner C, *et al*: Ca^{2+} activated K channels-new tools to induce cardiac commitment from pluripotent stem cells in mice and men. Stem Cell Rev Rep 8: 720-740, 2012.
13. Liebau S, Tischendorf M, Ansorge D, Linta L, Stockmann M, Weidgang C, Iacovino M, Boeckers T, von Wichert G, Kyba M and Kleger A: An inducible expression system of the calcium-activated potassium channel 4 to study the differential impact on embryonic stem cells. Stem Cells Int 2011: 456815, 2011.
14. Jara-Avaca M, Kempf H, Rückert M, Robles-Diaz D, Franke A, la Roche J, Fischer M, Malan D, Sasse P, Solodenko W, *et al*: EBIO does not induce cardiomyogenesis in human pluripotent stem cells but modulates cardiac subtype enrichment by lineage-selective survival. Stem Cell Reports 8: 305-317, 2017.
15. Taha MF and Hedayati V: Isolation, identification and multipotential differentiation of mouse adipose tissue-derived stem cells. Tissue Cell 42: 211-216, 2010.
16. Planat-Bénard V, Menard C, André M, Puceat M, Perez A, Garcia-Verdugo JM, Pénicaud L and Casteilla L: Spontaneous cardiomyocyte differentiation from adipose tissue stroma cells. Circ Res 94: 223-229, 2004.
17. Yang M, Zhang GG, Wang T, Wang X, Tang YH, Huang H, Barajas-Martinez H, Hu D and Huang CX: TBX18 gene induces adipose-derived stem cells to differentiate into pacemaker-like cells in the myocardial microenvironment. Int J Mol Med 38: 1403-1410, 2016.
18. 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.

19. Reményi A, Lins K, Nissen LJ, Reinbold R, Schöler HR and Wilmanns M: Crystal structure of a POU/HMG/DNA ternary complex suggests differential assembly of Oct4 and Sox2 on two enhancers. Genes Dev 17: 2048-2059, 2003.
20. Izadpanah R, Trygg C, Patel B, Kriedt C, Dufour J, Gimble JM and Bunnell BA: Biologic properties of mesenchymal stem cells derived from bone marrow and adipose tissue. J Cell Biochem 99: 1285-1297, 2006.
21. Zhu Y, Liu T, Song K, Fan X, Ma X and Cui Z: Adipose-derived stem cell: A better stem cell than BMSC. Cell Biochem Funct 26: 664-675, 2008.
22. Christoffels VM, Smits GJ, Kispert A and Moorman AF: Development of the pacemaker tissues of the heart. Circ Res 106: 240-254, 2010.
23. Potapova I, Plotnikov A, Lu Z, Danilo P Jr, Valiunas V, Qu J, Doronin S, Zuckerman J, Shlapakova IN, Gao J, *et al*: Human mesenchymal stem cells as a gene delivery system to create cardiac pacemakers. Circ Res 94: 952-959, 2004.
24. Plotnikov AN, Shlapakova I, Szabolcs MJ, Danilo P Jr, Lorell BH, Potapova IA, Lu Z, Rosen AB, Mathias RT, Brink PR, *et al*: Xenografted adult human mesenchymal stem cells provide a platform for sustained biological pacemaker function in canine heart. Circulation 116: 706-713, 2007.
25. Zhang H, Li S, Qu D, Li B, He B, Wang C and Xu Z: Autologous biological pacing function with adrenergic-responsiveness in porcine of complete heart block. Int J Cardiol 168: 3747-3751, 2013.
26. Li Y, Li B, Li Z, Zhang J and Zeng M: Adipose tissue-derived adult stem cells transfected with the gene of hyperpolarization-activated cyclic nucleotide-gated ion channel 2 differentiated into pacemaker-like cells. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 29: 901-904, 909, 2013 (In Chinese).
27. Chauveau S, Brink PR and Cohen IS: Stem cell-based biological pacemakers from proof of principle to therapy: A review. Cytotherapy 16: 873-880, 2014.
28. Jiang P, Rushing SN, Kong CW, Fu J, Lieu DK, Chan CW, Deng W and Li RA: Electrophysiological properties of human induced pluripotent stem cells. Am J Physiol Cell Physiol 298: C486-C495, 2010.
29. Bai X, Ma J, Pan Z, Song YH, Freyberg S, Yan Y, Vykoukal D and Alt E: Electrophysiological properties of human adipose tissue-derived stem cells. Am J Physiol Cell Physiol 293: C1539-C1550, 2007.
30. Shi W, Wymore R, Yu H, Wu J, Wymore RT, Pan Z, Robinson RB, Dixon JE, McKinnon D and Cohen IS: Distribution and prevalence of hyperpolarization-activated cation channel (HCN) mRNA expression in cardiac tissues. Circ Res 85: e1-e6, 1999.
31. 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.
32. Li Y, Yang M, Zhang G, Li L, Ye B, Huang C and Tang Y: Transcription factor TBX18 promotes adult rat bone mesenchymal stem cell differentiation to biological pacemaker cells. Int J Mol Med 41: 845-851, 2018.
33. Feng Y, Luo S and Song Z: GW24-e3884 Canine bone marrow mesenchymal stromal cells modified with Shox2 gene rebuild biological pacemakers in vitro. Heart 99: A46, 2013.
34. Weisbrod D, Khun SH, Bueno H, Peretz A and Attali B: Mechanisms underlying the cardiac pacemaker: The role of SK4 calcium-activated potassium channels. Acta Pharmacol Sin 37: 82-97, 2016.
35. He JQ, Ma Y, Lee Y, Thomson JA and Kamp TJ: Human embryonic stem cells develop into multiple types of cardiac myocytes: Action potential characterization. Circ Res 93: 32-39, 2003.
36. Zhang YM, Hartzell C, Narlow M and Dudley SC Jr: Stem cell-derived cardiomyocytes demonstrate arrhythmic potential. Circulation 106: 1294-1299, 2002.
37. Sun Y, Liu WZ, Liu T, Feng X, Yang N and Zhou HF: Signaling pathway of MAPK/ERK in cell proliferation, differentiation, migration, senescence and apoptosis. J Recept Signal Transduct Res 35: 600-604, 2015.
38. Roux PP and Blenis J: ERK and p38 MAPK-activated protein kinases: A family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev 68: 320-344, 2004.
39. Wu J, Kubota J, Hirayama J, Nagai Y, Nishina S, Yokoi T, Asaoka Y, Seo J, Shimizu N, Kajiho H, *et al*: p38 Mitogen-activated protein kinase controls a switch between cardiomyocyte and neuronal commitment of murine embryonic stem cells by activating myocyte enhancer factor 2C-dependent bone morphogenetic protein 2 transcription. Stem Cells Dev 19: 1723-1734, 2010.
40. Eriksson M and Leppä S: Mitogen-activated protein kinases and activator protein 1 are required for proliferation and cardiomyocyte differentiation of P19 embryonal carcinoma cells. J Biol Chem 277: 15992-16001, 2002.