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

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Abstract. Recent studies have suggested that calcium-activated potassium channel (Kca) 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 If (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.

Introduction

Small (SK1-3)- and intermediate (SK4)-conductance Ca2+-activated potassium channels bind intracellular Ca2+ 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.

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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 inguinal of the rats. The adipose tissue was cut into 1x1-mm³ 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 × g for 10 min at 25°C, and the cells were resuspended in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). Cells were cultured in an incubator at 37°C using a 5% CO₂ atmosphere, grown to 80-90% confluence and passaged using 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^ΔΔct 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 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 using 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.

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).
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-glucconate, 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 700A d/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_2, 1 M MgCl_2, 0.3 Na_2HPO_4, 10 HEPEG, 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

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

| mRNA | Primers 5'-3' | Product size (bp) |
|------|--------------|-------------------|
| SK4  | Sense: GTCGTACGCTGAGATTTTG | 126 |
|      | Antisense: TCTGGCATGGAAGACCAAT | |
| Oct-4| Sense: GTTCGGAGTGGGAGTGA | 223 |
|      | Antisense: AGAAGCGAGAACATAAGTCTCC | |
| Sox-2| Sense: GATGCACTGCTGAGCCTGC | 197 |
|      | Antisense: TGGAGTGGGAGGAGGAGTAAC | |
| Tbx18| Sense: GGAGACCTTGGATGAGACAGTTGAT | 282 |
|      | Antisense: TGGGCAAATGATTTCTTTCT | |
| Shox2| Sense: ATCCAGAGCCTTTATGCGC | 214 |
|      | Antisense: TCCCTCCTGAAAAGCCCTCT | |
| cTnI | Sense: TCGATGGGCTGGCTT | 286 |
|      | Antisense: CTCCTCTTACCTGCTTGA | |
| HCN4 | Sense: CACCAAAGCAACAAGAGACC | 281 |
|      | Antisense: GGTGTGGAAGAGCCTGAGTTG | |
| GAPDH| Sense: CGCTAACATCAATGCGGTT | 201 |
|      | Antisense: TGTCTGACAATCTTGGAGGAG | |

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, 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-glucconate, 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 700A d/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_2, 1 M MgCl_2, 0.3 Na_2HPO_4, 10 HEPEG, 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.
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 MOI of 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 expression 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 Tbx18 and 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.
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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 cTnnI 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 Ca²⁺-activated potassium channel; MOI, multiplicity of infection; cTnnI, cardiac troponin I.

Figure 4. Expression of HCN4 and pacemaker current Iᵢ 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) Iᵢ current detected by the patch clamp technique and blockade with CsCl (2 mM/l). (D) Mean current-voltage association of the Iᵢ current (n=8). SK4, intermediate-conductance Ca²⁺-activated potassium channel; MOI, multiplicity of infection; 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 SKCa 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 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 If, 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 I1. 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 SKCa 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 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.

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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, AW and FW performed the experiments. HZ., QZ and MY analyzed data. XY, 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.

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