Stearic acid methyl ester promotes migration of mesenchymal stem cells and accelerates cartilage defect repair

Yamei Liu a,b,*, Liangliang Xu c,d,*, Liuchao Hu a,e,*, Dongfeng Chen a,b, Lijuan Yu a,b, Xican Li f, Hongtai Chena,e, Junlang Zhua,e, Chen Chen a,b, Yiwen Luo a,e,⁎⁎, Bin Wang a,e,⁎⁎⁎, Gang Li g,h,*

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ABSTRACT

Background: Mesenchymal stem cells (MSCs) can be easily expanded without losing the ability of multilineage differentiation, including osteogenic, chondrogenic and adipogenic differentiation. These characters make MSCs a promising cell resource for cartilage defect repair. MSCs could be recruited by inflammatory stimulation, then home to the injury tissues. However, its capacity of homing is extremely limited. Thus, it has become extremely necessary to develop an agent or a method, which can be used to enhance the efficiency of MSCs homing. This study investigates the effect of stearic acid methyl ester (SAME) on MSCs mobilisation and cartilage regeneration. Methods: MSCs were isolated from femurs of Sprague-Dawley (SD) rats. MTT assay was used to detect effect of SAME on viability of MSCs. Transwell assay and wound healing assay were used to detect effect of SAME on migration of MSCs. RNA-seq, quantitative real-time PCR and western blot were performed to analyze the expression of RNAs and proteins. Colony forming assay and flow cytometry were used to evaluate the effect of SAME on MSCs mobilisation in vivo. A rat cartilage defect model was created to evaluate the effect of SAME on cartilage regeneration. Results: We found that SAME could promote the migration of MSCs. Interestingly, we found SAME significantly increased the expression levels of Vav1 in MSCs. On the other hand, the enhanced migration ability of MSCs induced by SAME was retarded by Vav1 small interfering RNA (siRNA) and Rho-associated protein kinase 2 (ROCK2) inhibitor. In addition, we also checked the effect of SAME on mobilisation of MSCs in vivo. The results showed that SAME increased the number of MSCs in peripheral blood and enhanced the capacity of colony formation. Finally, using a cartilage defect model in rats, we found SAME could improve cartilage repair. Conclusion: Our study demonstrates that SAME can enhance MSCs migration ability mainly through the Vav1/ROCK2 signalling pathway, which could contribute to the accelerated cartilage regeneration. The translational potential of this article: These findings provide evidence that SAME could be used as a therapeutic reagent for MSCs mobilisation and cartilage regeneration.

⁎ Corresponding author. Room 904, 9/F, Li Ka Shing Institute of Health Institute, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, SAR, Hong Kong China.
⁎⁎ Corresponding author. The Third Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou University of Chinese Medicine, Guangzhou, China.
⁎⁎⁎ Corresponding author. The Third Affiliated Hospital of Guangzhou University of Chinese Medicine, Guangzhou University of Chinese Medicine, Guangzhou, China.
E-mail addresses: gzhlyw@gzucm.edu.cn (Y. Luo), wangbin1973@163.com (B. Wang), gangli@cuhk.edu.hk (G. Li).
⁎ Contributed equally as the first authors.

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Introduction

Arthritis is a crippling disease which affects people's life and health. It is characterised by the progressive loss of articular cartilage and chondrocytes within synovial joints, leading to symptoms such as pain and stiffness. Not only are the elderly but also young people commonly injured by this disease [1]. The cartilage is difficult to self-repair because of lack of blood vessels, nerves and nutritional sources [2,3]. And the management of cartilage lesions is still a big challenge for surgeons because of its limited regenerative ability. Generally, several treatments have been attempted to restore joint surface defects, such as osteochondral autograft transplant system and autologous chondrocyte implantation and microfracture [4]. Unfortunately, the cartilage formed by these procedures is frequently fibrocartilage instead of functional hyaline cartilage. The development of tissue engineering provides a new approach for cartilage repair. Mesenchymal stem cells (MSCs) are regarded as the most promising seed cell because of its multidirectional differentiation and self-renewal potential [5,6]. Preclinical studies with MSCs transplantation provide evidence that they have capacity of tissue repair such as bone nonunion and osteoarthritis [7–9]. However, MSCs transplantation has been hampered by several key problems, lack of targeting or poor cell migrates to injury sites and apoptosis of the implanted cells [10,11].

MSCs migration is one of the important factors which influences tissue repair. When stimulated by specific signals such as cytokines or chemokines, MSCs can be released into circulation and recruited to the target tissues. Then, MSCs differentiate into specific cells in situ and contribute to tissue regeneration [12]. These cells can potentially be mobilised into the circulation in response to injury signals and exert their reparative effects at the site of injury. Trafficking of MSCs to the injury site and their subsequent participation in the regenerative process is thought to be a natural healing response that can be imitated or augmented by enhancing the endogenous MSCs pool with exogenously administered MSCs [13]. Cell migration is precisely regulated by multiple signalling pathways of which Rho-associated protein kinase (ROCK) pathway plays an important role. ROCK, also known as Rho kinase, is the downstream target effector of RhoA that has been studied in a detailed manner currently. Inhibition of ROCK signalling pathway results in the downstream target effector of RhoA that has been studied in a detailed manner currently. Inhibition of ROCK signalling pathway results in the inhibition of migration of haematopoietic stem cells, monocytes, neutrophils and myoblast [14–17]. ROCK signalling pathway regulates rearrangements of the actomyosin cytoskeleton, which constitute a conductive loop of the contractile force to conduct contractile force and result in cell migration [18].

Some researchers have reported that chemotactic factors such as platelet derived growth factor (PDGF), transforming growth factor β3 (TGF-β3) load on bioadhesive materials enhanced endogenous MSCs recruitment and facilitated in situ articular cartilage regeneration [19, 20]. These studies indicate that mobilisation of endogenous MSCs is beneficial to cartilage repair. Sufficient number of MSCs homing to sites of injury is an important factor in tissue repair. However, the capacity of MSCs homing to the injured site is extremely limited. Only a few MSCs can cross the endothelial cells and migrate to the injured site for repair [21]. To improve the homing efficiency, different strategies have been developed such as pretreatment of MSCs in culture, genetic modifications and cell surface engineering [22]. Results of the in vivo studies indicate that the combination of insulin-like growth factor 1 (IGF1) and Plerixafor octahydrochloride (AMD3100) could augment the bone growth by mobilisation of stem/progenitors from the bone marrow (BM) [23]. Drug therapy is a valuable method worthy spreading because of its simplicity, low toxicity and good effects compared with other treatments. Some studies have indicated that pretreatment of MSCs in culture by some herb extracts such as ligustrazine, icarin and ilexnon-A can enhance MSCs proliferation and migration [24–26]. Stearic acid methyl ester (SAME) can be extracted from some herbs such as safflower and plastrum testudinis, which has been proven to promote MSCs proliferation [27]. Safflower has long been used to promote blood circulation and remove blood stasis in China [28]. In China, the blood activating and stasis eliminating drugs are widely applied for the treatment of arthritis and obtain good clinical outcomes [29]. SAME is one of the main components in Safflower's volatile oil. Our previous study has shown that volatile oil of safflower can promote migration capacity of MSCs, while there is no report about the effect of SAME on MSCs migration. In this study, we found that SAME could promote the proliferation and migration of MSCs by activating the Vav1/ROCK signalling pathway. Furthermore, we demonstrated that SAME could mobilise endogenous MSCs to improve the healing of impaired cartilage in the knee cartilage defect model of Sprague-Dawley (SD) rats. These findings provide evidence that SAME can be used as a therapeutic reagent for MSCs mobilisation and cartilage regeneration.

Materials and methods

MSCs isolation, culture and characterisation

MSCs were harvested from femurs of SD rats (male, 4-weeks-old). In brief, the bone marrow of the bilateral femoral was flushed out and layered onto LymphoprepTM (1077 g/ml; Axis-Shield) for centrifugation at 400g for 25 min. The isolated mononuclear cells were suspended in low-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco, USA, serial number: C11885500BT) containing 10% foetal bovine serum (FBS, Gibco, USA, serial number: 10099141) and 1% penicillin-streptomycin (Gibco, USA, serial number: 15140122) and incubated in air containing 5% CO2 at 37°C. The nonadherent cells were removed by phosphate buffered saline (PBS) washing and changing the medium 6d later. Then, medium was changed once every 2 days. When the adherent cells reached 80 ~ 90% confluence, they were passaged at a ratio of 1:3. Cells from passages 3 to 5 were used in the study. The cell surface markers (cluster of differentiation, CD90, CD45, CD44 and CD34) were confirmed by flow cytometry. The cells were induced for osteogenic and adipogenic differentiation in vitro to determine multipotency of MSCs, as described previously [30].

MTT assay

MSCs were inoculated at 2 × 104/mL in 96-well culture plates (approximately 2000 cells in 100 μL of medium per well). The cells were incubated at different concentrations of SAME (0, 1, 5, 10, 50 and 100 μg/mL) in DMEM. 10 μL MTT (3-4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide, Beyotime, China) was added to each well after 24 h or 72 h, and cells were incubated for an additional 4 h at 37°C. The absorbance was recorded at a wavelength of 570 nm using a microplate reader to determine the level of proliferation in each well.

Cell migration assay

Cell migration ability was evaluated by transwell assay and wound healing assay. Transwell plates (Corning Costar, USA, serial number: 3422) with a pore size of 8 μm (Corning Costar) were used to transwell assay. Cells were pretreated by different condition including different concentrations of SAME, Y27632 (ROCK inhibitor) or transfection of cluster of differentiation, CD90, CD45, CD44 and CD34 were confirmed by flow cytometry. The cells were induced for osteogenic and adipogenic differentiation in vitro to determine multipotency of MSCs, as described previously [30].
determined by averaging five random fields per well.

For wound healing assay, MSCs were incubated in 6 cm dish and cultured until 95% confluence. Then the medium was changed with 1% FBS to exclude the influence of cell proliferation. A scratch wound was created with a micropipette tip. The cells were photographed and counted under a phase contrast microscope.

RNA-seq and data analysis

MSCs were treated with 10 μg/ml SAME for 24 h. Total RNA was obtained from the control or SAME-treated MSCs using TRIzol Reagent (Takara, Dalian, China). The quality and integrity of total RNA samples were assessed using a 2100 Bioanalyzer or a 2200 TapeStation (Agilent Technologies) according to the manufacturer’s instructions. The preparation of whole transcriptome libraries and deep sequencing were performed by the Annoroad Gene Technology Corporation (Beijing, China). The differentially expressed genes (above 2.0 folds) from RNAseq were analysed by DAVID bioinformatics tool for functional annotation enrichment and clustering.

Quantitative real-time PCR

Total RNA was extracted from MSCs using TRIzol reagent (Invitrogen, USA, serial number: 15596-026) according to the manufacturer’s instructions. Then RNAs were treated with DNase I to remove the DNA contamination. The concentration and purity of RNA were measured using NanoDrop 2000. cDNA was synthesised with PrimeScript RT Master Mix (TaKaRa, Japan, serial number: RR036Q). Real-time quantitative RT-PCR was performed using the SYBR Premix Ex Taq II (TaKaRa, Japan, serial number: RR820A). β-actin was used as an internal parameter to determine the relative expression.

Primer sequences were as follows:

Vav1-forward: 5′-GGAGTCCAGGACTGAGG-3′ and Vav1-reverse: 5′-CTGACTCCACCTGACTGCAA-3′;
ROCK2-forward: 5′-AACAAAGATTGCTTGATTGGCA-3′ and ROCK2-reverse: 5′-GCCGACGACACCTCTCCAT-3′;
β-actin-forward: 5′-CTGTAAGACCTCTATGCAAACA-3′and β-actin-reverse: 5′-GGGACTCTAGTACTCTGCT-3′.

Western blot analysis

MSCs were lysed with radio immunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors (Beyotime). The protein concentration was measured by a bicinchoninic acid (BCA) Protein Assay kit (Beyotime). Equal amounts of protein were separated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene fluoride (PVDF) membrane, blocked in 5% milk, and immunoblotted with primary antibodies overnight at 4°C. The membranes were washed in tris buffered saline with tween 20 (TBST) and incubated with a corresponding secondary antibody for 1 h at room temperature. Protein bands were visualised using an enhanced chemiluminescence kit (Pierce). The following primary antibodies were used: Vav1 (1:1,000, Abcam, UK, serial number: ab97574); β-actin (1:1000; CST, USA, serial number: 3700S).

Gene silencing of Vav1

siRNAs targeting Vav1 and negative mismatched control were designed and synthesised from Genepharma (China). The sequences for siRNAs targeting Vav1 were as follows: siRNAs targeting Vav1 (siVav1)-1: GUGGGAGGGAAGAAUACATT; siVav1-2: GUGGAGGU-CAAGCUAUUAAUTT; siVav1-3: GAGGAAGACAUUCUGAAUTT. The transfection was performed with lipofectamine 3000 solution (Invitrogen) according to the manufacturer’s protocol. After the terminal transfection for 48 h, the cells were collected analysed.

Colony forming assay of peripheral blood

Peripheral blood was collected before and at the 7th day after treatment with heparin anticoagulation. Then 1 ml blood was transferred into a 15 ml conical centrifuge tube and treated with fresh cold red blood cell lysing solution. Then, the tubes were inverted or rocked for 10 min at room temperature until liquid was clear red. The cells were then centrifuged and cultured in MesenCult medium supplemented with mesenchymal stem cell stimulatory factors. The cells were allowed to grow for 10 days, and numbers of adherent cell colonies were enumerated in a phase contrast microscope.

Determine the number of MSCs in peripheral blood

To confirm the number of MSCs mobilised to peripheral blood, peripheral blood was collected by orbital blood collection before and at 6th day after treatment. The cell surface markers of peripheral blood mesenchymal stem cells (PBMSCs) were determined by flow cytometry. The cells were incubated for 30 min at room temperature with monoclonal antibodies for the stem cell markers CD90 and the haematopoietic marker CD45. Irrelevant identical isotypic antibody served as negative control. Cells were analysed using a BD (Becton, Dickinson and Company) Biosciences FACSCanto II flow cytometer.

Rat cartilage defect model

A rat cartilage defect model was created as previously described [31]. Briefly, the osteochondral defects in the femoral trochlear groove of SD rats under anaesthesia as previously described. The 8-week-old SD rats were anaesthetised by intraperitoneal injection of ketamine and xylazine. Skin tissue around the knee was disinfected, and then the right knee joints were exposed through a medial parapatellar approach. The knee remains fully flexed to expose the femoral condyle.

Then a defect (diameter 1.5 mm and 1.5 mm in depth) was created in the centre of the groove using a dental drill. All debris was washed out of the defect. The joint capsule, subcutaneous tissues and skin were closed with sutures. The experimental group was divided into high-dose group (n = 3) and low-dose group (n = 3). High-dose group was injected with SAME at 0.05 mg/kg per day through intraperitoneal while low-dose group at 0.01 mg/kg per day. Control group (n = 3) received PBS supplemented with dimethylsulfoxide (DMSO). After 4 weeks, all rats were sacrificed, and femoral samples were collected for further study.

Safarin-O/Fast Green and immunohistochemical staining

Femoral samples were fixed in 10% buffered formalin and then decalcified in 10% buffered ethylenediaminetetraacetic acid (EDTA) (pH 7.4, Sigma-Aldrich, USA, serial number: E6758-500G) followed by the Safranin-O (Sigma-Aldrich, USA, serial number: 84120-25G) and Fast Green (Sigma-Aldrich, USA, serial number: F7252-5G) and immunohistochemical staining with collagen type II antibody. Bone callus samples were fixed in 10% formaldehyde solution for 12 h, demineralised in 5% ethylenediaminetetraacetic acid for 1 month, dehydrated through a graded ethanol series, cleared in xylene, embedded in paraffin, and sliced into 5 μm thick sections. Sections were subjected to Safranin-O/Fast Green staining to evaluate the morphological changes in osteoblasts and trabecular bone and to determine the content of cartilage and collagen matrix within the callus. Sections were also incubated with the corresponding primary antibody and stained by the standard method. Colour development was performed using a diaminobenzidine (DAB) kit.
following the manufacturer’s instructions. Each section was observed under a light microscope, and four visual fields were randomly selected for measurement of the rate of positive cells and the gray value, which were measured at the same light intensity. Histological sections of the repaired tissue were analysed using a grading system reported by other researchers [32]. Details of histological scores are provided in the Supplementary Table 1. The scoring was performed by three observers in a blinded manner.
Statistical analysis

Data are presented for each group as means ± standard deviation. Analysis was performed using SPSS16.0 software. Differences between groups were compared by t tests or one-way analysis of variance. A value of \( p < 0.05 \) was considered to be statistically significant.

Results

Characterisation of MSCs

MSCs were obtained from the femur bone marrow of SD rats. At passage 3, the cells were completely adherent and grew into uniform spindle-shaped (Figure 1A). Alizarin red staining showed that intracellular calcium nodule formation, indicating osteogenic differentiation ability (Figure 1B). Oil red staining showed the formation of red small droplets of oil in cell, indicating adipogenic differentiation ability (Figure 1C). MSCs surface markers were analysed by flow cytometry. FITC-CD44, FITC-CD90, FITC-CD45 and FITC-CD34 antibodies were used to stain MSCs and isotype IgG1 as negative control. The result showed that MSCs were positive expression of CD44 (99.24%), CD90 (97.03%) and negative for CD45 (0.06%), CD34 (0.20%) (Figure 1D).

Effect of SAME on the viability of MSCs

The molecular structure of SAME (methyl stearate) was shown in Figure 2A. To observe whether SAME affected the viability of MSCs, MSCs were treated with SAME at different concentration (1, 5, 10, 50 and 100 μg/ml) for 24 h and 72 h, followed by MTT assay. As shown in Figure 2B&C, SAME ranging from 1 μg/ml to 100 μg/ml did not cause any significant changes of the cell viability at 24 h and 72 h, which means that SAME had no cytotoxicity to MSCs.

Effect of SAME on migration of MSCs

To observe the effect of SAME on migration of MSCs, we performed transwell assay and wound healing assay. For transwell assay, MSCs were pretreated by different dosages of SAME (5, 10, 50 μg/ml) for 24 h. And then the equal number of MSCs was added into upper chamber. The lower chamber contained DMEM supplemented with 10% FBS. After 10 h, migrated cells were counted after staining and photographing. The result showed that SAME with the concentrations of 5, 10 and 50 μg/ml could increase the MSCs migratory capacity compared with the control group, especially in the 10 μg/ml SAME group (Figure 3A–E). For wound healing assay, we observed the cell migration at 0 h, 9 h, 16 h, 24 h and 36 h. We obtained similar results that the concentrations of 5, 10 and 50 μg/ml could increase the MSCs migratory capacity compared with the control group. The results are shown on Supplementary Figure 1.

RNAseq analysis of SAME-treated MSCs

To analyse the underlying mechanism leading to the enhanced migration ability of MSCs by SAME, RNAseq was further performed to check the gene expression profiles of these MSCs. The Heatmap and volcano map were shown in Figure 4A and 4B. The Kyoto Encyclopedia of Genes and Genomes analysis revealed that several signalling pathways were enriched as shown in Figure 4C, among which we found that leukocyte transendothelial migration signalling was most attractive for further study (Figure 4D). One of the main components of this signalling pathway, Vav1, was significantly increased by SAME. And this finding was further confirmed by quantitative RT-PCR (Figure 4E).

ROCK inhibitor represses SAME’s effect on migration

RhoA/ROCK signalling axis has been found to promote actin stress fibre formation and influence cell migration. To identify the mechanism underlying MSCs migration, we conducted kinase inhibition assays with the ROCK inhibitor Y27632. As the SAME in concentration 10 μg/ml had significant effects on MSCs migration ability with transwell chamber, we therefore chose the dose for the subsequent experiments. The combined use of SAME and Y27632 decreased the migration ability of MSCs, compared with the use of SAME alone. It demonstrated that inhibition of ROCK signalling pathway significantly suppressed the effect of SAME on migration capacity of MSCs (Figure 5).

Silencing endogenous Vav1 inhibits SAME’s effect on migration

In previous studies, we found that the expression of Vav1 was significantly increased. We wondered whether Vav1, as the upstream of RhoA/ROCK, was involved in MSCs migration. Therefore, we verified its...
function by silencing the expression of Vav1 by transfection siRNA.

Three siRNAs were designed to target endogenous Vav1. The silencing efficiency of siRNAs was checked and the most effective one (siVav1-3) was chosen to use in the following experiments (Figure 6C). Then we further confirmed the protein level of Vav1 in MSCs treated by SAME was significantly increased, as compared with the control group (Figure 6A and 6B). We found that the effect of SAME on Vav1 expression was significantly suppressed by siRNA-Vav1 (Figure 6D and 6E). And the transwell assay also demonstrated that silencing Vav1 significantly suppressed the effect of SAME on migration capacity of MSCs (Figure 6F–I).

**Effect of SAME on mobilisation of MSCs**

To confirm whether SAME can mobilise MSCs to peripheral blood, flow cytometry was used to detect the quantity of PBMCs (defined as CD45+/CD90- mononuclear cells). Compared with the control group (about 0.3%), the percentage of CD45+/CD90+ PBMCs in rats treated with SAME was significantly increased in both low- and high-dose group (p < 0.05), indicating SAME could significantly mobilise MSCs to peripheral blood (Figure 7A–D). The colony formation assay showed that the colonies in the low-dose group were much bigger and larger,
compared with the control group (Figure 7E). And the in vitro differentiation assay showed that circulating MSCs could be induced to differentiate into adipocytes and osteoblasts (Figure 7F and 7G).

**SAME accelerates cartilage defect repair**

Finally, to determine the effect of SAME on cartilage regeneration, rat cartilage defect model was created and injected with SAME or PBS through intraperitoneal. After 4 weeks of treatment, the animals were killed, and the cartilages were harvested to perform histological examinations. Safranin-O/Fast Green staining of bone sections showed that there was a significant difference in the structure of articular cartilage. Compared with the control group, the defects were healed, accompanied by local fibrous cartilage connection and smoothing of cartilage surface (Figure 8A–D). The histologic grading scale for cartilage repair showed that the score of SAME-treated group was significantly improved.
As shown in Figure 8D, there was a remarkable increase in positive regions of cartilage marker protein-collagen type II expression, suggesting the promoting role of SAME in cartilage regeneration.

Discussion

In present study, we have shown that treatment of MSCs with SAME enhances the migration ability of MSCs and demonstrated the underlying mechanisms that activation of Vav1/Rock signalling pathway is accounted for the effect of SAME. Most importantly, we found SAME could be used to mobilise MSCs and accelerate cartilage defect repair. This is very interesting because the cartilage itself cannot heal well without additional treatment. While, SAME as a natural chemical found in many Chinese herbs, such as plastrum testudinis and safflower, has never been reported to help cartilage defect repair. Our findings provide strong evidence that SAME can be used as a therapeutic reagent for cartilage regeneration.

MSCs transplantation has been applied in bone and cartilage repair. However, it has been hampered by the poor capacity of migration towards pathologic tissues. In recent years, it is reported that preconditioning of MSCs could be a beneficial strategy to improve the therapeutic potential of MSCs transplantation by improving cell survival and homing to the lesion sites [11]. Thus, it has been increasingly necessary to devise an agent, which can be used to promote MSCs homing to pathologic tissues. The role of ROCK pathway in regulation of cell migration is well recognised [33–35]. However, there are no simple and effective ways to enhance MSCs migration by activating ROCK signalling pathway. In this study, we investigated the optimal conditions required for SAME to upregulate Vav1 and Rho-associated protein kinase 2 (ROCK2) to affect MSCs migration. Results showed that treatment of MSCs with 50ug/ml for 48 h produced the most optimal effects. In addition, we found that SAME had...
no cytotoxicity to MSCs in a broad dosages. At the dosage of 10 μg/ml, SAME could promote the MSCs proliferation at 48 h.

RhoA/ROCK axis is the main signalling pathway controlling the formation of stress fibres which consist of actin filaments and play important roles in various cellular functions, including cell adhesion, isometric contraction, and cell motility [36,37]. F-actin cytoskeleton networks are believed to be essential for the force generation necessary for cell migration [38]. ROCK affects F-actin cytoskeleton networks via myosin light chain (MLC) phosphorylation. Phosphorylated MLC can promote actin–myosin interaction and generate F-actin-dependent cytoskeletal contractile tension [39]. ROCK inhibits the activity of MLC phosphatase and increases phosphorylated MLC through phosphorylation of MYPT1. The phosphorylation of MLC enhances the cross-linking of myosin and actin, which enhances the contraction of actin and induces cell migration [40,41]. Our founding showed that the effect of SAME on MSCs migration was significantly suppressed by ROCK inhibitor (Y27263). SAME also improved F-actin cytoskeleton, which shown more noticeable stress fibres and elongated morphology.

To further investigate the mechanism of SAME promoting cell migration, we focused on the upstream molecules of ROCK pathway. Rho guanosine triphosphate hydrolases (ROCK) is regulated by upstream Rho GTPases [42]. Activation of Rho GTPases can activate ROCK to participate in cell adhesion, migration, proliferation, and differentiation [43]. Vav protein is a catalyst for Rho family GTPases, which can activate Rho GTPases by catalysing the exchange of guanosine diphosphate (GDP) and GTP [44]. In present study, we found that SAME significantly increased Vav1 expression. We also found that the migration ability of MSCs decreased by RNA interference knockdown of Vav1 expression and the inhibition of ROCK signalling. The above results suggested that SAME could promote MSCs migration by regulation of Vav1. This finding also confirmed that the Vav1 is an upstream factor in the ROCK signalling pathway.

MSCs migration is a highly integrated multistep process that involves the release of chemokines and mobilisation of MSCs into peripheral blood. Then, the MSCs rolls along the wall of vascular endothelial cells and finally passes through the endothelial cells to the site of injury [45,46]. It is reported that increased numbers of MSCs have been isolated from peripheral blood cells of injured mice compared with noninjured controls, which is evidence that MSCs participate in the repair of injury [47]. In the present study, the results of flow cytometry analysis showed that SAME significantly increase the number of CD45-/CD90+ circulating MSCs. Colony formation assay suggested that SAME treatment group had a significantly higher number of cultured MSCs colonies. The above results indicated that SAME could mobilise MSCs to the peripheral blood in vivo.

The ultimate goal of promoting MSCs migration is to repair damaged tissues. Therefore, we established a cartilage defect model to study whether SAME could promote cartilage defect regeneration. In our study,
Safranin O & Fast green staining of bone sections showed that the defect surface of cartilage in the SAME treatment group was obviously repaired, accompanied by local fibrous cartilage connection and smoothing of cartilage surface, indicating that SAME improved cartilage healing.

To summarise, our study confirms that SAME can promote the migration of MSCs, and most importantly, it can mobilise MSCs to peripheral blood. Mechanistically, SAME robustly increased ROCK transcriptional level in MSCs via the Vav1/ROCK signalling pathway. Furthermore, we demonstrated that SAME could mobilise endogenous MSCs to improve the healing of impaired cartilage in knee cartilage defect model of SD rats. These findings provide evidence that SAME can be used as a therapeutic reagent for MSCs mobilisation and cartilage regeneration.

Conflict of interest

The authors have no conflicts of interest to disclose in relation to this article.

Ethics approval and consent to participate

This experiment was approved by the Animal Care and Use Committee of Guangzhou University of Chinese Medicine. The study has been carried out in accordance with the guidelines of the Chinese Medical Association.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jot.2019.09.008.

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