

***Catharmus tinctorius* volatile oil promote the migration of mesenchymal stem cells via ROCK2/Myosin light chain signaling**

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[ABSTRACT] MSC transplantation has been explored as a new clinical approach to stem cell-based therapies for bone diseases in regenerative medicine due to their osteogenic capability. However, only a small population of implanted MSC could successfully reach the injured areas. Therefore, enhancing MSC migration could be a beneficial strategy to improve the therapeutic potential of cell transplantation. *Catharmus tinctorius* volatile oil (CTVO) was found to facilitate MSC migration. Further exploration of the underlying molecular mechanism participating in the pro-migratory ability may provide a novel strategy to improve MSC transplantation efficacy. This study indicated that CTVO promotes MSC migration through enhancing ROCK2 mRNA and protein expressions. MSC migration induced by CTVO was blunted by ROCK2 inhibitor, which also decreased myosin light chain (MLC) phosphorylation. Meanwhile, the siRNA for ROCK2 inhibited the effect of CTVO on MSC migration ability and attenuated MLC phosphorylation, suggesting that CTVO may promote BMSC migration via the ROCK2/MLC signaling. Taken together, this study indicates that *C. tinctorius* volatile oil could enhance MSC migration via ROCK2/MLC signaling *in vitro*. *C. tinctorius* volatile oil-targeted therapy could be a beneficial strategy to improve the therapeutic potential of cell transplantation for bone diseases in regenerative medicine.

[KEY WORDS] *Catharmus tinctorius*; Volatile oil; MSC; Migration; ROCK2/MLC signaling

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Introduction

Stem cell-based therapy in recent years has gained much

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attention as the modern therapeutic approach to treat diseases. Mesenchymal stem cells (MSCs) are of particular interest to stem cell-based therapies for bone diseases in regenerative medicine^[1-3], not only due to their intrinsic capability to differentiate into bone and cartilage forming cell phenotype, but also because of their migration to sites of injury after intravenous infusion, and their ability to regulate the repair process or promote recovery of damaged tissues^[4-5]. MSC migration has been defined as the arrest of MSCs within the vasculature of a tissue followed by transmigration across the endothelium^[6]. Successful MSC transplantation requires that stem cells cross the endothelium and migrate to the target tissues and organs^[7-8]. However, the systemic administration of MSCs through cir-

cultation has some limitations; this is because only a small population of implanted MSCs could successfully reach the injured areas, most of which are trapped and dead within a short duration in small blood vessels in the target tissues^[9-10]. Therefore, enhancing the directed MSC migration capacity is essential for optimizing the therapeutic outcome^[11].

Approaches to modify MSCs or to enhance expression of surface markers of MSCs have been explored to enhance MSC migration capacity, such as pretreatment with cytokines, over-expression of some genes and proteins^[8, 12-13]. As traditional herbal extracts are composed of naturally occurring medicinal herbs, these may result in less toxic, affordable, and highly available natural alternative therapeutics than synthetic cytokines and genetic modification^[14-15]. Some studies have indicated that preconditioning of bone marrow-derived mesenchymal stem cells by tetramethylpyrazine, tanshinone IIA and astragaloside IV can enhance cell migration, originally isolated from the Chinese herb *Rhizoma Ligusticum walliichi*, *S. miltiorrhiza* Bunge and *A. membranaceus* respectively^[16-17]. Therefore, pharmacological preconditioning to enhance MSC migration could be a beneficial strategy to improve the therapeutic potential of cell transplantation.

Catharmus tinctorius (*C. tinctorius*), known as Flos Carthami, is a traditional Chinese herbal that has been prescribed since centuries for treating various ailments such as gynecological, cardiovascular, and cerebrovascular diseases as well as blood stasis and osteoporosis^[18]. Modern pharmacological experiment have demonstrated that *C. tinctorius* possesses wide-reaching biological activities, including anti-coagulation and anti-thrombosis, anti-oxidation, modulating immune system, anti-hypoxia, anti-inflammation, anti-tumor, etc.^[19]. In China, the water extract of *C. tinctorius* has been developed as an intravenous injection, which is extensively applied to treat clinical diseases; and more investigations on safflower have been conducted, and *C. tinctorius* has gradually been paid more attention to due to its medicinal value^[20-21]. However, little was known about the role of *C. tinctorius* volatile oil in MSCs. Whether volatile oil preconditioning could enhance the MSCs migration is still unclear.

Dynamism of the cell cytoskeleton allows for the adaptation, in space and time, to environmental factors, generating migratory responses^[22]. The regulation of migratory responses involves the integration of intrinsic molecular signal from the various extrinsic stimuli^[23]. Although a great variety of signals and receptors affecting migration exist, accumulating evidence suggests that they all ultimately converge on the same migration pathway, the RhoA-ROCK-myosin II axis. Rho-associated coiled-coil kinase (ROCK), a major downstream effector of the small GTPase RhoA, is thought to aid in the cell contraction process and may regulate transmigration through other cell layers^[24-25]. It can promote the light chain of myosin (MLC) phosphorylation, which is believed to be essential for the force generation necessary for cell migration^[26-28]. In this study, we studied the effect of *C. tinctorius* volatile oil

(CTVO) on promote MSCs migration *in vitro* followed by exploration of the relevant mechanisms. This research may further our understanding of pharmacology-targeted therapy to improve the efficiency of MSCs engraftment in clinical applications, which could significantly improve MSC-based cell therapy and regenerative medicine outcomes.

Materials and Methods

Samples

C. tinctorius flowers were collected from Xijing province, China. The sample was obtained from Faculty of Pharmacy, the Third Affiliated Hospital of Traditional Chinese Medicine (Guangzhou, China) and authenticated by Professor LI Xi-Can from Guangzhou University of Traditional Chinese Medicine (Guangzhou, China). Methyl hexadecanoate (C₁₇H₃₄O₂; CAS: 112-39-0), palmitic acid (C₁₆H₃₂O₂; CAS: 57-10-3), methyl stearate (C₁₉H₃₈O₂; CAS: 112-61-8) and linoleic acid (C₁₈H₃₂O₂; CAS: 60-33-3) were purchased from Sichuan Victory Biological Technology Co., Ltd. (Chengdu, China).

Extraction of volatile oil

Put 150 g of the dried flower-power of the *C. tinctorius* into the round-bottomed flask and add suitable amount of distilled water into it; and then extract the volatile oil with a cleverger apparatus according to the 2015 version of The Pharmacopoeia of China. Ethyl acetate (1.5 mL) was used as a collector solvent. The obtained volatile oil was subsequently dried over anhydrous MgSO₄ and store at 4 °C. The volatile oil dissolved to a concentration of 3 mg·mL⁻¹ with petroleum ether was subjected to GC-MS analysis.

Gas chromatography-mass spectrometry analysis

The identification of volatile oil extracted from *C. tinctorius*, methyl hexadecanoate, palmitic acid, methyl stearate and linoleic acid was performed by Gas chromatography-mass spectrometry (HP6890GC/5973MS, Agilent, USA), which was equipped with RTX-5MS (Restek, Bellefonte, PA, USA) fused-silica capillary column (30 m × 0.32 mm i.d., film thickness 1.0 μm). Carrier gas was Helium at a flow rate of 1.0 mL·min⁻¹ and the injection volume was 0.1 μL with a split ratio equal to 1/40. The injector temperature was 250 °C and the column pressure was 3.4 k pa. Temperature programming was performed from 40 °C (5 min) to 250 °C at a rate of 5 °C·min⁻¹ and held for 5 min. The temperature at the sample-feeding gate was 250 °C. The mass spectrum used 70 eV EI ion source, 280 °C ion source temperature and a mass range of *m/z* 50–450. The individual components were identified by comparing the mass spectra and GC retention data with those of authentic compounds previously analyzed and stored in the database from the National Institute of Standards and Technology (NIST).

Animals

Twenty 4-week-old specific pathogen-free male Sprague-Dawley (SD) rats, weight 80–90 g, were obtained from the animal center of Guangzhou University of Traditional Chi-

nese Medicine (Guangzhou, China; certificate No. 440059 00001722) and were housed with free access to food and water on a 12 h light/dark cycle and humidity of $55\% \pm 5\%$ at a constant temperature of 24 ± 1 °C in climate-controlled conditions. All animals received human care in accordance with the guideline set by the Care of Experiment Animals Committee of Guangzhou University of Chinese Medicine.

Isolation, culture and identification of MSCs

MSCs were isolated from the bone marrow of male Sprague-Dawley rats as previously reported with minor modification^[10]. Briefly, bone marrow was flushed from the femurs and tibia with L-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 1% penicillin/streptomycin in a sterile petri dish. The marrow washouts were collected, centrifuged and then cultured in L-DMEM supplemented with 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and a mixture of 1% penicillin/streptomycin in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. For identification of MSCs, osteogenic induction assay and adipogenic induction assay were performed by using Ori Cell TM Sprague-Dawley Rat Mesenchymal Stem Cell Osteogenic Differentiation Medium (RAXMX-09011; Cyagen Bioscience Inc., Guangzhou, China) and Adipogenic Differentiation Medium (RASM-90031; Cyagen Bioscience Inc., Guangzhou, China) according to the manufacturer's instructions. The quality of rBMSCs was ensured by flow cytometry as previously reported. BMSCs from passage 3 were used in all experiments. The ROCK2 inhibitor Y-27632 was used in this study: Y-27632 ($10 \mu\text{mol}\cdot\text{L}^{-1}$; #SCM075, Merck Millipore, USA) for 1 h, followed by the treatments of volatile oil of *C. tinctorius*.

Cell viability assay

Cell viability was assessed by detecting the optical density^[29]. MSCs were cultured in 96-well culture at a density of $1 \times 10^5\cdot\text{mL}^{-1}$. When MSCs reached 80% confluence, the cells were then pretreated with gradient concentrations of volatile oil of *C. tinctorius* (0, 1, 10, 25, 50, $100 \mu\text{g}\cdot\text{mL}^{-1}$) for 24 h under a 37 °C and 5% CO₂ environment. Cell viability was analyzed with a cell counting kit (CCK-8, Dojindo Laboratories, Kumamoto, Japan) following the manufacturer's protocol. Briefly, $10 \mu\text{L}$ CCK-8 solution was added into each well. Following 2 h incubation, the absorbance at a wave length of 450 nm was detected using a Bio-kinetics reader (PE-1420; Bio-Kinetics Corporation, Sioux Center, IA, USA). Cell viability was expressed as a percentage of the control culture value. The results were obtained from three independent experiments performed in triplicate.

Transwell migration assay

The migration assay was assessed using a Transwell Boyden Chamber with pore size of 8 μm (Corning Costar, Cambridge, MA, USA) as previously reported^[16]. The upper chambers were loaded with 3×10^4 MSCs in $150 \mu\text{L}$ of L-DMEM containing 1% FBS. The lower chambers were

loaded with $600 \mu\text{L}$ of L-DMEM containing 10% FBS, with or without volatile oil of *C. tinctorius*. The chambers were incubated at 37 °C and 5% CO₂ for 24 h. Non-migratory cells on the upper surface of the membrane was gently scraped and washed with PBS three times. Migrated cells on the under-sides of the membranes were fixed in 4% paraformaldehyde for 10 min followed by staining with 0.5% crystal violet (Beiyotime, Haimen, China) for 40 min. The number of migrated cells was counted under a by averaging five random fields per well. The experiments were performed in quadruplicate for each group.

Scratch wound healing assay

Migration was also evaluated by a scratch wound healing assay. MSCs at passage 3 were cultured in six-well plates until reached a confluent monolayer. A scratch was generated by scraping the cell monolayer with a pipette tip and then wells were washed with PBS to remove the cell debris. The scratched areas were photographed at 0 and 24 h at the same area after wounding. Means were taken from five fields in the scratched area using Image J software (NIH, Bethesda, MD, USA). Experiments were performed three separate times.

Western blotting analysis

Western blot was performed as previously described^[30]. In brief, cell lysates were prepared by using a cell lysis buffer supplemented with protease and phosphatase inhibitors (Beiyotime, Haimen, China). The protein concentration was quantified by BCA protein assay kit (Keygen, Nanjing, China), and $30 \mu\text{g}$ of total protein were subjected to SDS-PAGE, electrophoretically transferred onto PVDF membranes (Millipore, Bedford, USA) and blocked with 5% non-fat milk in PBST (PBS containing 0.05% Tween-20) for 1 h at room temperature. The membranes were then incubated with primary antibodies: rabbit poly-clonal ROCK2 antibody (1 : 1000; #9029, Cell Signaling Technology, Danvers, MA, USA), rabbit poly-clonal MLC2 antibody (1 : 1000, #3672, Cell Signaling Technology, Danvers, MA, USA), rabbit poly-clonal p-MLC2 antibody (Ser19) (1 : 1000, #3671, Cell Signaling Technology, Danvers, MA, USA) and rabbit poly-clonal GAPDH (1 : 1000; #5174, Cell Signaling Technology, Danvers, MA, USA) for overnight at 4 °C, followed by sequential incubation with horseradish peroxidase-conjugated secondary antibodies (1 : 5000; 4030-05, SouthernBiotech, Birmingham, Alabama, USA) for 1 h at room temperature. The proteins on PVDF membranes were detected with enhanced chemiluminescence (GE Healthcare Life Sciences, Chalfont, UK). The blots were quantified using Quantity One software (version 4.62; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each experiment was repeated at least three times.

Real-time quantitative polymerase chain reaction analysis

Total RNA from cells was extracted using TRIZOL reagent (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA). RNAs were reverse transcribed into cDNA using reverse Transcriptase M-MLV (Takara Biomedical Technology Co., Ltd., Japan) and real-time quantitative polymerase chain

reaction was performed using a SYBR® Premix Ex Taq™ (Takara, Japan) under the following conditions: 30 sec at 95 °C, followed by 45 cycles of 95 °C for 3 sec and 60 °C for 34 sec using ViiA7 software (Thermo Fiser Scientific, Waltham, MA, USA). Primers were used as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH); GAPDH-forward: 5'-AGGGCATCCTGGGCTACT-3', GAPDH-reverse: 5'-TCCACCACCCTGTTGCTGTAG-3'; ROCK2-forward: 5'-CTAACAGTCCGTGGGTGGTTC A-3', ROCK2-reverse: 5'-TCCACCTGGCATGTACTCCATC-3'.

Transfection of MSCs with ROCK2 siRNA

ROCK2 were depleted using commercially available ROCK2 siRNA (sc-108088) provided by Santa Cruz Biotechnologies (Santa Cruz, CA, USA), which is a pool of 3

target-specific 19-25 nt siRNAs designed to knock down gene expression [31]. The control siRNA used for these experiments were also provided by Santa Cruz Biotechnologies (Santa Cruz, CA, USA). MSCs were transfected with control siRNA or 50 nmol·L⁻¹ ROCK2 siRNA construct using Lipofectamine TM Stem Transfection Reagent (STEM 0001, Invitrogen, USA) and stimulated in the absence or presence of volatile oil of *C. tinctorius* for 24 h following the manufacturer's siRNA Transfection protocol. The sequences for ROCK2 siRNA are 5'-GCAGCAAUGGUAAGCGUAA-3', 5'-GCAACUGGCU CGUUCAAUU-3', 5'-GUAGAAACC UCCCCAAUUC-3', and 5'-GCAAAUCUGUAAUACUC G-3'. ROCK2 expression in stably transfected cell lines was examined by western blotting.

Table 1 Chemical composition of *C. tinctorius* volatile oil

No.	Compound name	t_R /min	Area %
1	Caryophyllene	12.39	0.75%
2	Caryophyllene oxide	16.92	3.88%
3	(1 <i>R</i> , 7 <i>S</i> , <i>E</i>)-7-Isopropyl-4, 10-dimethylenecyclodec-5-enol	18.07	0.62%
4	10, 10-Dimethyl-2, 6-dimethylenebicyclo[7.2.0]undecan-5.beta.-ol	18.29	1.96%
5	Methyl 4, 7, 10, 13-hexadecatetraenoate	18.85	0.39%
6	(<i>Z</i> , <i>Z</i> , <i>Z</i>)-1, 8, 11, 14-Heptadecatetraene	19.14	0.52%
7	Methyl 4, 7, 10, 13-hexadecatetraenoate	19.22	1.04%
8	2, 5-Octadecadiynoic acid, methyl ester	19.30	0.67%
9	Cyclohexane, 1, 3-butadienylidene-	20.68	1.71%
10	Deca-4, 6-diyn-1-yl 3-methylbutanoate	23.05	43.16%
11	(<i>Z</i>)-Deca-8-en-4, 6-diyn-1-yl 3-methylbutanoate	23.36	20.83%
12	2-Pentadecanone, 6, 10, 14-trimethyl-	23.73	4.18%
13	(<i>E</i>)-Deca-8-en-4, 6-diyn-1-yl 3-methylbutanoate	24.42	1.21%
14	(2 <i>E</i> , 8 <i>Z</i>)-Deca-2, 8-dien-4, 6-diyn-1-yl 3-methylbutanoate	24.76	0.62%
15	Methyl palmitate	25.71	1.93%
16	11, 14-Octadecadienoic acid, methyl ester	29.54	0.90%
17	9, 12, 15-Octadecatrienoic acid, methyl ester, (<i>Z</i> , <i>Z</i> , <i>Z</i>)-	29.68	1.19%
18	Heneicosane	34.04	1.42%
19	Henicosane-6, 8-dione	35.47	4.98%
Total			91.95%

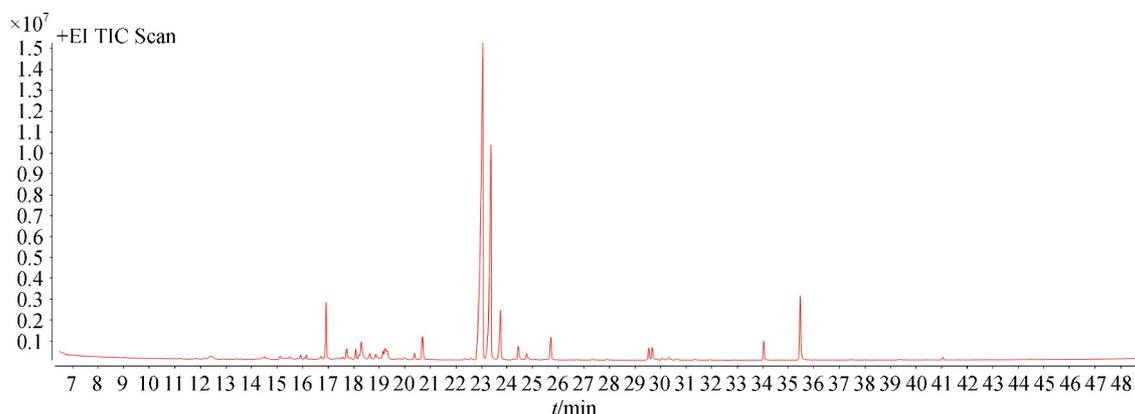


Fig. 1 GC-MS analysis of *C. tinctorius* volatile oil. The identification of volatile oil extracted from *C. tinctorius* flowers was analysed by GC-MS

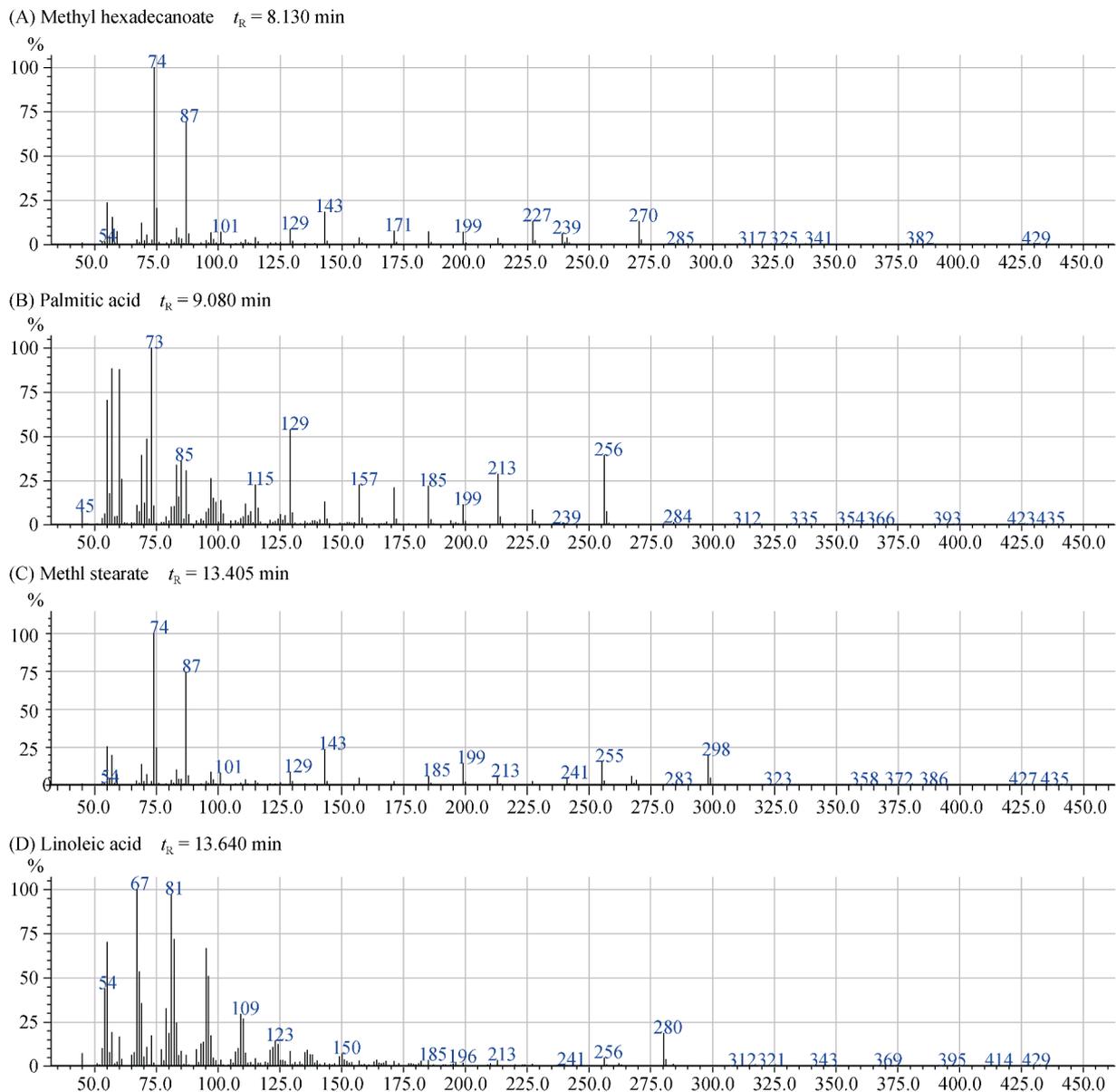


Fig. 2 GC-MS analysis of standard reference material. (A) Methyl hexadecanoate; (B) Palmitic acid; (C) Methyl stearate; (D) linoleic acid; RT: retention time

Statistical analysis

All data are presented as mean \pm standard deviation (SD) and were analyzed using GraphPad Software (GraphPad Software, Inc., La Jolla, CA, USA). Statistical analyses between two groups were performed by unpaired Student's t -test. Differences among groups were tested by one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. The $P < 0.05$ was considered statistically significant.

Results

Volatile oil analysis

Hydro-distillation of *C. tinctorius* flowers gave floral odor and light yellowish volatile oil with a yield of 0.15% (V/W), and the chemical composition of the volatile oil ana-

lyzed by GC-MS is shown in Table 1, which shows the compound names, retention time (t_R) and the percentage content of the individual components. The 19 major components of the oil were preliminarily identified by those stored in the spectrometer database of libraries, which represented about 91.91 % of the total detected constituents. In Fig. 1, the mass spectrum of the oily extract exhibited the individual peaks at which 19 components from *C. tinctorius* volatile oil were found. Furthermore, by referring to standards, we can confirm that *C. tinctorius* volatile oil contains methyl hexadecanoate, palmitic acid, methyl stearate and linoleic acid (Fig. 2).

Lineage differentiation of cultured rat MSCs

Cultured rBMSCs were adherent to the dish and showed

an elongated, spindle-shaped and fibroblast-like morphology (Fig. 3A). Osteogenic induction assay showed obvious calcium deposits in the cell culture (Fig. 3B). Adipogenic induction assay indicated that a number of isolated cells were positively stained by Oil Red O (Fig. 3C).

CTVO enhances MSCs migration in vitro

To examine the effects of CTVO on MSCs motility, cells were treated with different concentrations of CTVO (1, 10, 25, 50 and 100 $\mu\text{g}\cdot\text{mL}^{-1}$) for 24 h, and migration assay of cultured MSCs was performed using Boyden chamber assay. CTVO with the concentrations of 1, 10, 25, 50 $\mu\text{g}\cdot\text{mL}^{-1}$ could

increase the MSCs migratory capacity compared to the control group, especially in the 10 $\mu\text{g}\cdot\text{mL}^{-1}$ CTVO group (Figs. 4A and 4B).

Effects of C. tinctorius volatile oil on the viability of MSCs.

To observe whether cell proliferation affected the migration, MSCs were treated with CTVO at different concentration (1, 10, 25, 50 and 100 $\mu\text{g}\cdot\text{mL}^{-1}$) for 24 h, followed by CCK-8 assay. The results showed that CTVO ranging from 1 to 50 $\mu\text{g}\cdot\text{mL}^{-1}$ did not cause any significant changes of the cell proliferation, while 100 $\mu\text{g}\cdot\text{mL}^{-1}$ CTVO slightly increased the cell proliferation (Fig. 4C).

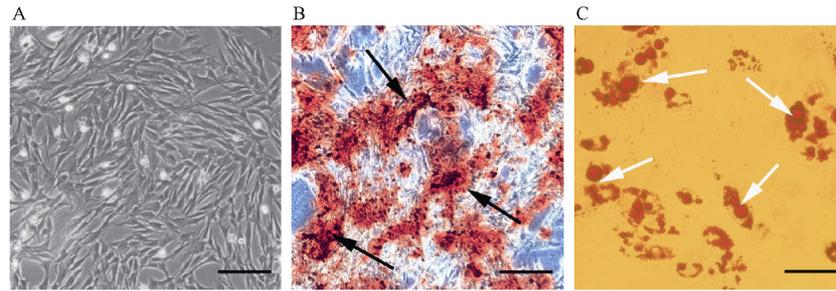


Fig. 3 MSCs characterization. (A) The isolated cells from BM of SD rats show an elongated, spindle-shaped and fibroblast-like morphology. (B) Representative fields of Alizarin Red S-stained cells isolated from BM of SD rats. The black arrows show the abundant calcium nodi in the matrix. (C) Representative fields of Oil Red O-stained cells isolated from BM of SD rats. The white arrows show the obvious small red lipids in adipose differentiated cells. Scale bar = 100 μm

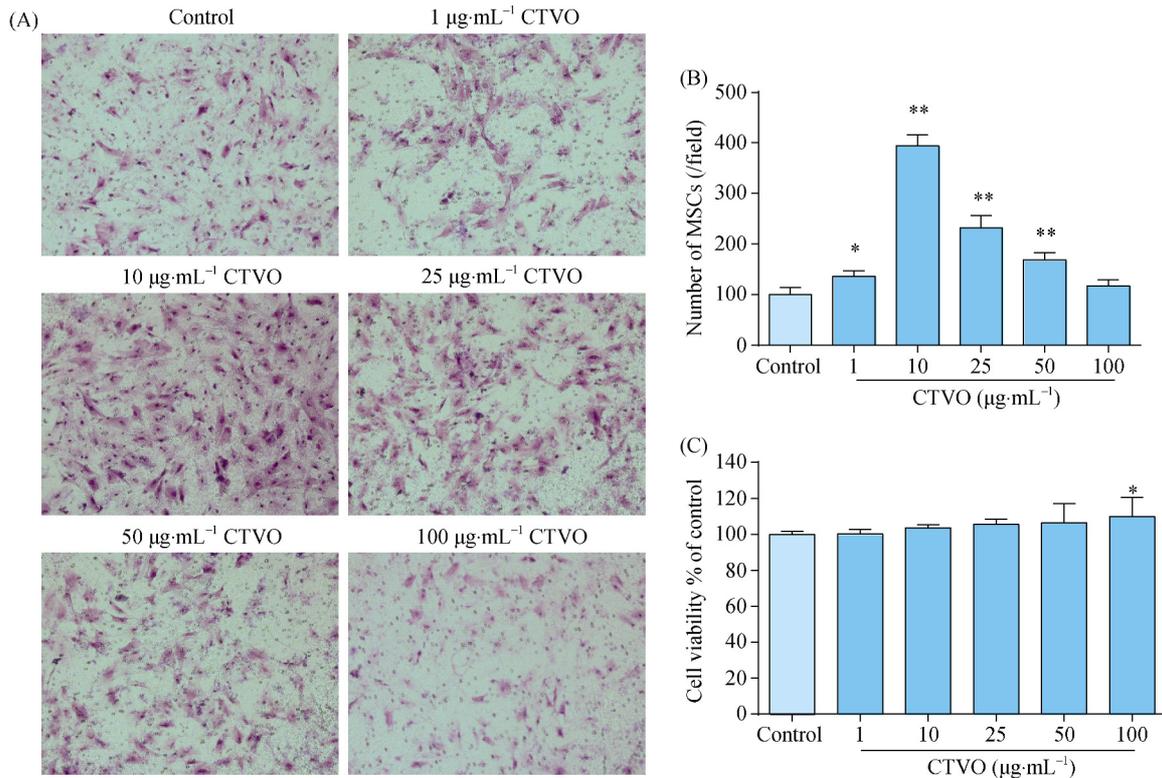


Fig. 4 Effects of *C. tinctorius* volatile oil (CTVO) on migration *in vitro* and cell viability. (A) Representative images of migrated MSCs in the transwell assay (magnification $\times 200$). The migration ability was enhanced when treated with CTVO (0–100 $\mu\text{g}\cdot\text{mL}^{-1}$) for 24 h. (B) The average number of migrated cells in five random fields were quantified. (C) MSCs were treated with various concentrations (0–100 $\mu\text{g}\cdot\text{mL}^{-1}$) of CTVO for 24 h. Cell viability was determined using a CCK-8 assay. Data are presented as the mean \pm SD from three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs control group

CTVO enhances ROCK2 expression in MSCs.

It has been reported that ROCK2 signaling pathway play an important role in promoting cells migration ability. We therefore investigated whether CTVO could enhance MSCs migration *via* ROCK2 expression by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blot. The results showed that a treatment of CTVO significantly increases the levels of ROCK2 mRNA and protein expressions (Figs. 5A and 5B).

The ROCK2/Myosin light chain signaling pathways involved in MSCs migration

To identify the mechanism underlying MSCs migration, we conducted kinase inhibition assays with the ROCK inhibitor Y27632. As the CTVO in concentration $10 \mu\text{g}\cdot\text{mL}^{-1}$ had significant effects on MSCs migration ability with transwell chamber, we therefore chose the dose for the subsequent

experiments. In transwell migration assay, the cell migration ability was significantly suppressed by the combined treatment with Y27632 ($10 \mu\text{mol}\cdot\text{L}^{-1}$) for 1 h (Figs. 6A and 6B), which did not cause any significant toxicity to the cell proliferation followed by CCK-8 assay (Fig. 6C). Considering that MLC phosphorylation mediated actomyosin contraction was considered to be essential for the force generation necessary for cell migration, we assessed MLC phosphorylation in CTVO treated cells by Western blotting. The results showed that MLC phosphorylation of $10 \mu\text{g}\cdot\text{mL}^{-1}$ CTVO treated cells was significantly higher than in non-treated control cells. However, pretreatment with Y27632 ($10 \mu\text{mol}\cdot\text{L}^{-1}$), the expression level of p-MLC was inhibited (Fig. 6D). In addition, the data also indicates that CTVO enhanced the cellular migration ability *via* ROCK-mediated MLC phosphorylation.

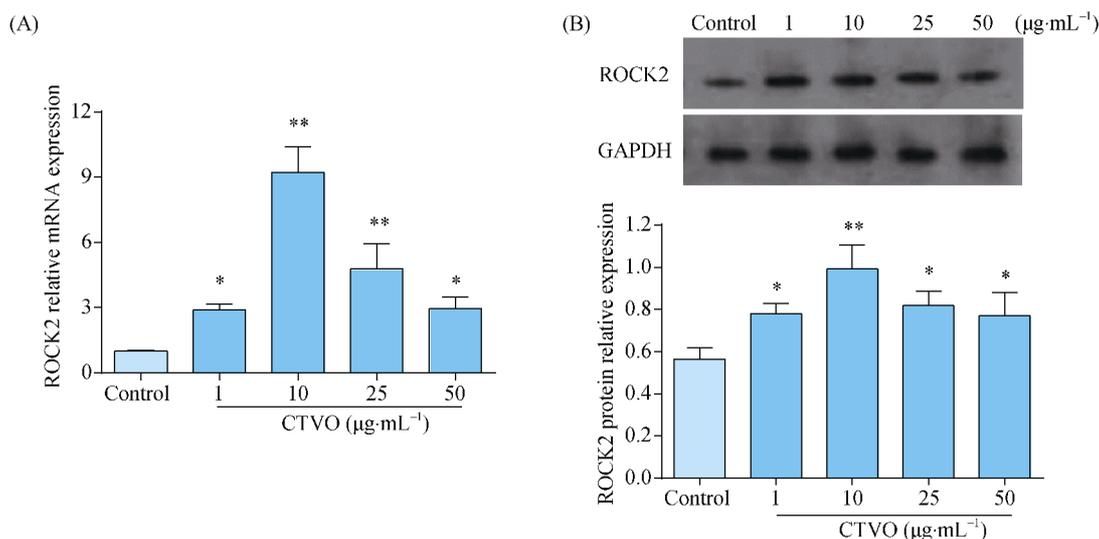


Fig. 5 CTVO enhanced the levels of ROCK2 mRNA and protein expressions *in vitro*. CTVO could increase the protein expression and mRNA levels in MSC, treated with CTVO (1, 10, 25, and $50 \mu\text{g}\cdot\text{mL}^{-1}$) for 12 h by quantitative real-time PCR (qPCR) assay and western blotting. The values represented the mean \pm SD for three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs control group

Interference with ROCK2 expression in MSCs significantly inhibited its transendothelial migration.

To gain further insight into the role of ROCK2 in MSC migration, an adenoviral vector containing ROCK2 siRNA was constructed. After MSCs were treated with control or ROCK2 siRNA for 24 h, the ROCK2 protein level and MLC phosphorylation were significantly reduced in ROCK2 siRNA infected cells evaluated by Western blotting (Figs. 7A and 7B). MSCs were transfected with control siRNA and $50 \text{nmol}\cdot\text{L}^{-1}$ ROCK2 siRNA for 24 h, and then the cells were treated with CTVO for 24 h, and the migration of MSCs were then detected using the scratch wound healing and transwell migration assays. Cells in control siRNA group increased the rate of wound closure of MSCs to 58.3%, compared to ROCK2 siRNA group with 27.8% (Fig. 7C). The number of cells that migrated to the lower chamber was overtly higher in control

siRNA infected MSCs (380/field) treated with CTVO than in ROCK2 siRNA group (122/field, Fig. 7D).

Discussion

This study reports that *C. tinctorius* volatile oil (CTVO) could promote MSC migration, and the effect is mediated by ROCK/MLC signaling. The promotion of the MSCs migration would improve the efficiency of MSC engraftment in clinical applications, which could significantly improve MSC-based cell therapy and regenerative medicine outcomes.

Traditional herbal medicines are a promising alternative, offering substantial improvement of patient conditions and significantly decreased disease and symptoms [32]. In many different studies, herbal extracts have shown much promise in the proliferation and differentiation of MSCs [33]. Recently, researchers found that the beneficial effects of *S. miltiorrhiza*

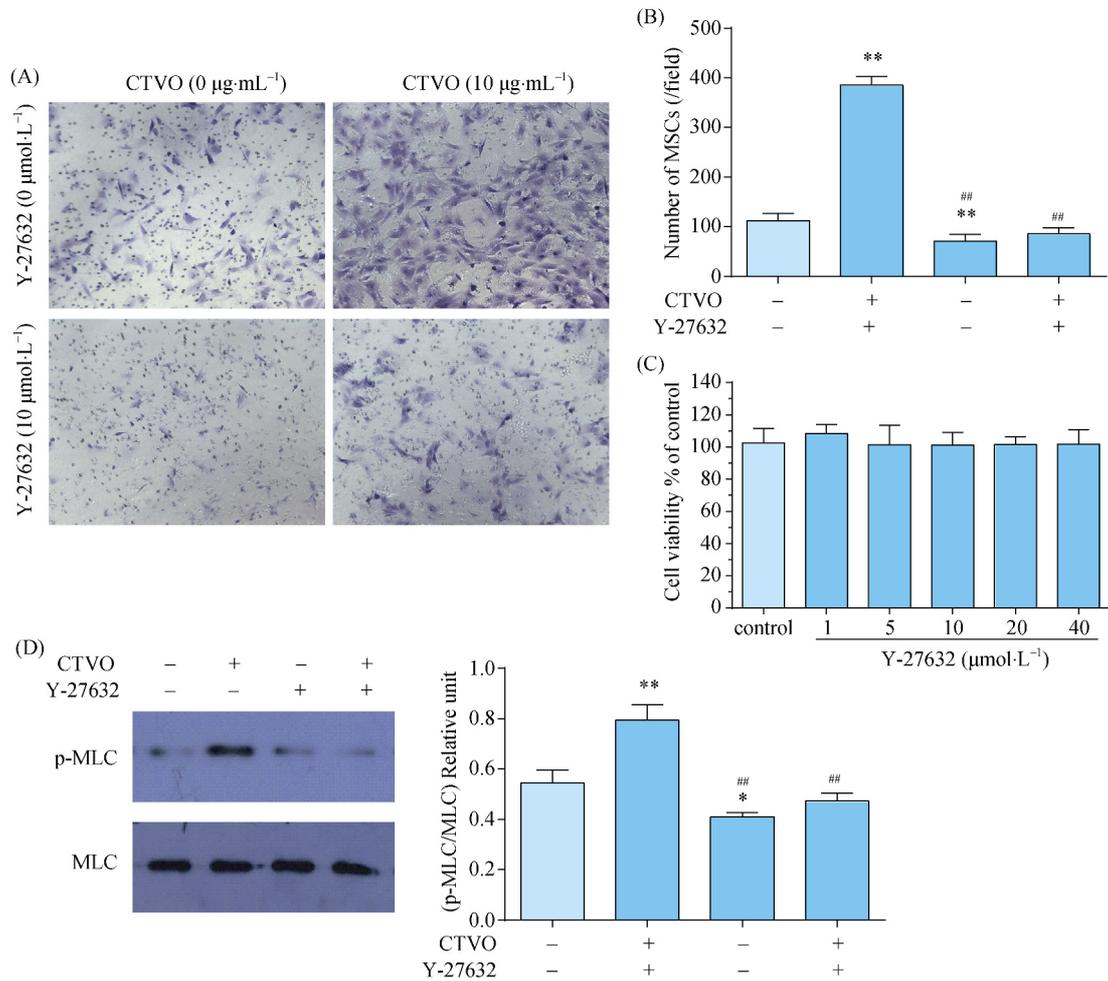
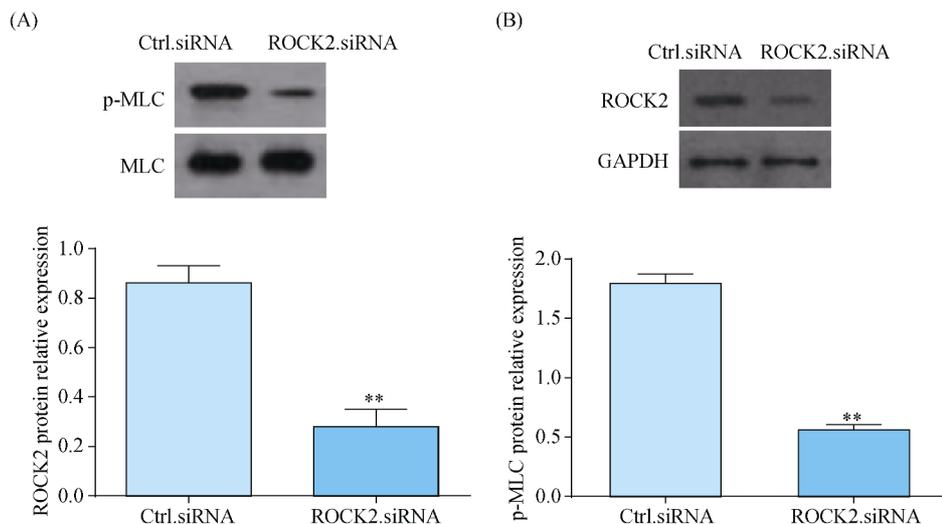


Fig. 6 Involvement of ROCK2 in CTVO-enhanced MSC transendothelial migration. (A) The cells were treated with or without volatile oil of *C. tinctorius* (10 $\mu\text{g}\cdot\text{mL}^{-1}$) in the lower chambers and/or 10 $\mu\text{mol}\cdot\text{L}^{-1}$ Y27632. After 24 h incubation, the migrated MSCs were fixed and stained with 0.5% crystal violet. Representative images of migrated MSCs in transwell migration assay (magnification $\times 200$). (B) The average number of migrated cells in five random fields was quantified. (C) MSCs were treated with Y27632 (10 $\mu\text{mol}\cdot\text{L}^{-1}$) for 1 h. Cell viability was determined using a CCK-8 assay, $n = 6$ in each group. (D) MSCs were then harvested to evaluate p-MLC phosphorylation and MLC by Western blotting. The blots were quantified by densitometry, and the results were expressed as relative unit (p-MLC/MLC). The values represented the mean \pm SD for three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs control group; ## $P < 0.01$ vs the CTVO-treated group



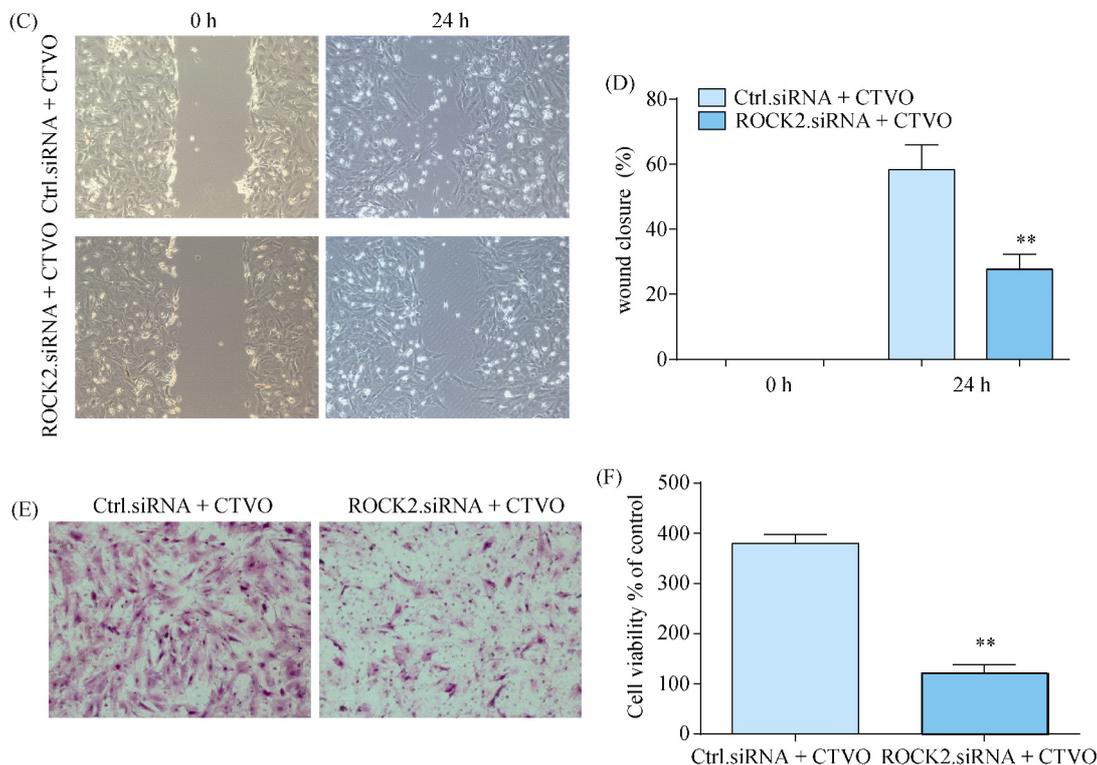


Fig. 7 The influence of interference with ROCK2 expression on MSCs transendothelial migration. MSCs were infected with commercially available ROCK2 siRNA at 24 h after transfection. (A) The interference efficiency was determined by Western blot. (B) MLC, a downstream protein of ROCK2 was analyzed by Western blotting. The blots were quantified by densitometry, and the results were expressed as a ratio relative to the values of p-MLC/MLC. (C) At 24h after transfection of ROCK2 siRNA or negative control siRNA, a scratch wound was then created, and the cells were treated with CTVO for 24 h. the wounds were then photographed (magnification $\times 200$). (D) Quantitative analysis of the rate of wound closure. (E) MSCs with ROCK2 knock-down were cultured in transwell inserts and incubated CTVO for 24 h. Representative images of transmigrated MSCs in the transwell assay (magnification $\times 200$). Quantitative analysis of the average number of migrated cells in five fields examined. Data are presented as mean \pm SD from three independent experiments. ** $P < 0.01$ vs control siRNA group

Bunge, *A. membranaceus* and Rhizoma Chuanxiong among Chinese herbals on MSC therapy are related to the promotion of MSC migration, basing on the theory of Qi and blood in traditional Chinese medicine. *C. tinctorius* has the similar medicinal properties of those herbals, which could promote blood circulation to remove blood stasis in Chinese traditional medicine [16-17]. To the best of our knowledge, the current study is the first to investigate the pro-migratory effect of CTVO on MSCs *in vitro*.

Mesenchymal stem cells have emerged as an appealing candidate in treating numerous bone diseases, owing to their capable of differentiating into functional cell types found in various mesenchymatous tissues (bone, cartilage, muscle and tendon). MSCs transplantation has been explored as a new clinical approach in bone repair and the most important aspect of MSC therapy is that systemically administered MSCs migrate to sites of injury [9]. However, It is generally acknowledged that the low migration ability of transplanted MSCs toward the ischemic region limits the efficacy of this approach and new approaches are needed to improve the migration ability of MSCs for the successful transplantation [11, 34].

Combinations of chemokines and genetic modification, have been proposed to promote the effectiveness of transplanted MSCs to migrate toward damaged injury [14-15]. However, their high cost and adverse side effects might be not feasible for widespread clinical application. In the present study, we found that CTVO significantly enhanced MSCs migration *in vitro* (Figs. 4A and 4B).

Cell migration is a complex process that orchestrates signal transduction, cytoskeleton rearrangement, and morphogenesis. The ROCK signaling has been reported to be a command central hub for the regulation of migration, and ROCK was shown to play an important role in MLC phosphorylation, which exacerbates cell migration [35]. Hence, we studied whether CTVO enhanced MSCs migration *via* the ROCK/MLC pathway. Our results suggested that CTVO could enhance ROCK2 expression in MSCs (Figs. 5A and 5B). Considering that the $10 \mu\text{g}\cdot\text{mL}^{-1}$ CTVO has a significantly effects on migration, we therefore chose the dose for subsequent experiments. MLC phosphorylation induces actomyosin contraction, which is believed to be essential for the force generation necessary for cell migration. As shown in Fig. 6C,

CTVO obviously increased phosphorylated MLC, which was significantly suppressed by the combined treatment with Y27632 ($10 \mu\text{mol}\cdot\text{L}^{-1}$). We also found that the MSCs migration capacity was hampered in the scratch wound healing and transwell migration assays, when intervention with ROCK inhibitor (Figs. 6A and 6B). Those results enabled us to conform that ROCK could facilitate MLC phosphorylation and eventually induce MSCs migration.

Y27632 is a ROCK-specific inhibitor for both ROCK1 and ROCK2. To further demonstrate whether the ROCK2 contributes to the MSCs migration, the effectiveness of suppression of ROCK2 protein expression by RNA interference (RNAi) should be considered. In this study, we showed that ROCK2 protein levels were blocked by RNAi knockdown of ROCK expression. Meanwhile, phosphorylated MLC also markedly inhibited. For interference with ROCK2 expression by siRNA in MSCs, it significantly reduced the effect CTVO on MSCs migration ability (Fig. 7).

Herbal extracts may show promising results *in vitro* studies but to apply these natural products in stem cell therapy will require more quality researches to provide a better understanding into the active constituents and the molecular mechanisms. Pharmacological preconditioning with herbal remedies as stimulant of migration in stem cell therapy may result in cost-effective, highly available, non-toxic alternatives for therapeutic application as there is vast number of herbal extracts used in traditional Chinese medicine. In our study, we found *C. tinctorius* volatile oil is a feasible pharmacology-targeted therapy to improve the efficiency of MSCs engraftment in clinical applications, which could significantly improve MSC-based cell therapy and regenerative medicine outcomes.

Conclusions

Taken together, this study indicates that *C. tinctorius* volatile oil enhances MSCs migration *via* ROCK2/MLC signaling *in vitro*. *C. tinctorius* volatile oil-targeted therapy could be a beneficial strategy to improve the therapeutic potential of cell transplantation for bone diseases in regenerative medicine.

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