ORIGINAL ARTICLE

Cyasterone accelerates fracture healing by promoting MSCs migration and osteogenesis

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ABSTRACT

Background: Mesenchymal Stem Cells (MSCs) therapy has become a new coming focus of clinical research in regenerative medicine. However, only a small number of implanted MSCs could successfully reach the injured areas. The previous studies have shown that fracture healing time is inversely proportional to concentration of MSCs in injured tissue.

Methods: The migration and osteogenesis of MSCs were assessed by transwell assay and Alizarin Red S staining. Levels of gene and protein expression were checked by qPCR and Western Blot. On the other hand, the enhanced migration ability of MSCs induced by Cyasterone was retarded by CXCR4 siRNA. In addition, the rat model of femoral fracture was established to evaluate the effect of Cyasterone on fracture healing. What's more, we also checked the effect of Cyasterone on mobilisation of MSCs in vivo.

Results: The results showed that Cyasteron increased the number of MSCs in peripheral blood. The concentrations of SDF-1α in serum at different time points were determined by ELISA assay. Micro-CT and histological analysis were used to evaluate the fractured femurs.Our results showed that Cyasterone could promote the migration and osteogenesis capacities of MSCs. The fractured femurs healed faster with treatment of Cyasterone. Meanwhile, Cyasterone could significantly increase the level of SDF-1α in rats with femur fracture.

Conclusion: Cyasterone could promote migration and osteogenesis of MSCs, and most importantly, it could accelerate bone fracture healing.

Translational Potential statement: These findings provide evidence that Cyasterone could be used as a therapeutic reagent for MSCs mobilisation and osteogenesis. What's more, it could acclerate fracture healing.

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Introduction

Fracture is the most common orthopedic disease caused by accidental injury. Although common fractures often heal quickly, stalled fracture healing happens sometimes, accounting for 10~20%, which can cause nonunion of fracture and severe disability [1]. Mesenchymal stem cells (MSCs) has became an essential new research direction of stem cell-based fracture treatment in tissue engineering and regenerative medicine [2,3]. MSCs migration has been defined as the arrest of MSCs within the vasculature of a tissue followed by transmigration across the endothelium [4]. There is powerful proof that chemokine receptor type4 (CXCR4) has been proved to play an important role in MSC migration [5]. Furthermore, the main function of periosteum in fracture repair is to release SDF-1/CXCR4 by both paracrine and autocrine mechanisms, leading to cytokine secretion and cell differentiation which could explain the promoting effect of MSCs on fracture healing [6]. In addition to self-renewal and multidirectional differentiation potential (such as osteoblasts, chondrocytes, etc.), MSCs can also secrete a variety of cytokines to regulate immunity, angiogenesis and matrix formation [6,7]. SDF-1/CXCR4 axis may be involved in the increase of stem cell-mediated ossification [8]. However, without intervention factors, only a small number of MSCs were mobilized to participate in tissue repair after fracture, which could not meet the needs of bone repair after severe fracture or bone defect [9]. Therefore, how to promote the efficient directional migration of MSCs to the fracture position is an urgent problem to be solved in the chemotaxis of MSCs.

Radix cyathulae is the dry root of *Cyathula officinalis Kuan*, which is a traditional Chinese medicine to promote blood circulation and strong bones as well as muscles. And it have analgesic, anti-inflammatory and anti-consenescence effects. It has been found that the liver and kidney tonic traditional Chinese medicines play important roles in promoting osteogenic differentiation and treating osteoporosis [10]. Cyasterone is extracted from *Radix cyathulae*. The traditional herbal extracts not only provide a new way to activate the self-regeneration of stem cells, but also reduce the use of targeted drugs which have adverse effects on the normal metabolism of human body [11]. The proliferation and differentiation of MSCs treated with herbal extracts show promising result in osteoporosis, neurodegenerative diseases and other tissue degenerative diseases [12]. Our previous studies indicate that preconditioning of MSCs with Bushen Huoxue decoction can enhance MSCs migration through activating WNT5a signaling pathway [13]. Therefore, natural chemicals may be a beneficial strategy to improve the therapeutic potential of cell transplantation.

The number of MSCs at the fracture site was positively correlated with callus volume and osteogenic capacity at the fracture site, and negatively correlated with fracture healing time [9]. MSCs have been widely used in tissue engineering, and have been proved to be able to homing to injuries portion [14]. Homing refers to the migration process of MSCs through multi-level vascular network, across vascular endothelium and extracellular matrix to the target area. Recruiting MSCs from surrounding tissues or circulation to the fracture callus is very important for bone repair and regeneration, and is a key scientific issue in promoting fracture healing [15,16]. SDF-1 and its receptor CXCR4 play an important role in MSCs mobilization, migration and homing during fracture repair. Intravenously transplanted MSCs can combine with SDF-1 through its membrane receptor CXCR4, and migrate to the bone fracture ischemia area along the concentration gradient of SDF-1 [17,18]. Studies have shown that ROCK plays an important role in MSC migration [19]. Although the importance of BMSC migration for their potential therapeutic uses, the mechanisms and signalling governing stem cell migration are still not fully elucidated. Study identified MCP-1-mediated signalling, which are important for stem cell migration. RhoGTPase family members and the Rho kinase ROCK which are important mediators of polarisation and migration in many cells types included MSCs [20]. Rho-associated coiled-coil kinase (ROCK) is a major downstream effector of RhoA small GTPase, and is thought to contribute to the cellular contraction process

and possibly regulate migration through other cellular layers [21,22]. In the present study, we investigated the effect of Cyasterone on MSCs migration and osteogenic differentiation in vitro, as well as the relevant underlying mechanism. We also explored its role in the healing of rat fractures. The finding of this study could provide a theoretical basis for the treatment of fracture, shortening the time of fracture healing, preventing delayed fracture healing and nonunion.

Materials and Methods

Experimental animal

Male Sprague-Dawley rats were used in the study (license number: SCXK (Guangdong) 2016-0041). The rats were 12 weeks old, weighing approximately 320–340g. They were randomly divided into two experimental groups and one control group (n = 10, each). Middle femoral shaft fracture was created in all rats with animal general anesthesia of chloral hydrate. The study was approved by the Guangzhou University of Traditional Chinese Medicine animal experiment ethics board, Guangzhou, Guangdong province, China (animal ethics number: 20190227050). The research was conducted in accordance with the Declaration of Helsinki and with the Guide for Care and Use of Laboratory Animals.

Cyasterone

Cyasterone (Batch NO: DST180328-039. CAS No: 17086-76-9) was purchased from Chengdu Desite Biotech Co.,Ltd (Chengdu, Sichuan, China).

Isolation and culture of MSCs

The rats were sacrificed by cervical dislocation, and the femurs were isolated aseptically. The soft tissue around the femur was removed in the ultraclean table. Then isolated femurs were put in PBS 1X Sterile containing 5% Penicillin-Streptomycin Solution (Gibco) to wash two times. The bilateral metaphysis were cut off, and the remaining femoral shalfs were placed in α-MEM (Gibco) containing 5% Penicillin-Streptomycin Solution and 10% fetal bovine serum (FBS, Gibco). The femoral marrow cavities were rinsed repeatedly using α-MEM medium by syringes, and then washing solution was collected. The collected solution was centrifuged at 1000r/min for 8 min, and the supernatant was discarded. Then the precipitate was mixed with 4 mL complete medium (containing 10% FBS) and inoculated in 25 cm^2 culture bottle. The culture bottle was placed in the incubator for cultivation (37 °C, 5% CO_2). After first 72 h, culture medium was changed firstly, and then it was changed once every 48 h until cells covered the entire culture bottles. Cells were digested with trypsin and labelled as Passage 1 (P1). Then culture medium was still changed once every 48 h for keep cells active. As long as cell confluence reached 80%, cells were passaged until P3 for experimentation. After culturing and passaging to P3, Rat Mesenchyma Stem Cell Detection Kit (Cyagen) was used to characterize MSCs by using flow cytometer (BriCyte E6, Mindray, China). MSCs in this study are consistent with those used by the research group in the early study, and need not be identified by clone formation assay and multidirectional differentiation analysis [23].

Proliferation assay

The proliferation of MSCs with treatment of Cyasterone was detected by CCK-8 (Dojindo, Japan). MSCs were cultured in 96-well culture plates at cell concentration of 5 × 10^3/100 μl. The outer rings of 96-well were filled with PBS. When MSCs reached 80% confluence, MSCs were incubated with basic medium with or without different dosages of Cyasterone (2.5, 5, 10, 20, 40, 80 μg/ml) at 37 °C and 5% CO_2 for 24~48 h. The absorbance of the 96-well plate at 450 nm (PE-1420; Bio-Kinetics Corporation, Sioux Center, IA, USA) was measured after incubation with 10

ul CCK-8/well for 1 h in the cell incubator. The proliferation of MSCs was indicated by absorbance [13]. Besides , clone formation assay that support the effect of Cyasterone on MSCs proliferation. P3 MSCS were cultured in 6-well plate, 2 ml/well, and 1000 MSCs per well were ensured, and the culture was continued for 48 h. Removed the α-MEM from 6-well plate, the fresh α-MEM containing Cyasterone of different concentrations (0, 2.5, 5, 10, 20, 40 μg/ml) was added and continued cultured for 48 h. Removed all of the liquid, fresh α-MEM was added to 6-well plate, MSCs were cultured for 10 days. Crystal violet (Bikeman , China) staining and scanner photography.

Transwell assay

The migration ability of MSCs could be detected by transwell chambers (24-well plate, 8-μm pore size, Corning , USA) as previously reported [24]. MSCs were pretreated with α-MEM containing with or without different concentrations of Cyasterone (5 and 10 μg/ml) in 24-well plates for 48 h in cell incubator. Then MSCs were digested and inoculated in the upper chambers of 24-well transwell plates. The upper chambers were loaded with total 3×10^4 MSCs in 200 μl α-MEM containing 1% FBS. The transwell plates were left in the incubator for 1 h, after which the lower chambers were loaded with 700 μl α-MEM containing 10% FBS. Then the transwell plates were incubated at 37 °C and 5% CO_2 for 12 h in the incubator. The internal bottoms of upper chambers were swabbed and washed with PBS over three times. Thereafter, the enteral bottoms of upper chambers were fixed with 4% paraformaldehyde for 20 min, washed with PBS for 2 times and then stained with 1% Crystal Violet (Bikeman , China) Staining Solution for 60 min. In the end, the whole chambers were washed twice with PBS, and MSCs were observed under an inverted photomicroscope (IX79P2F, OLYMPUS).

Induction of osteogenic differentiation

MSCs were digested and inoculated in 12-well plates (5×10^4 MSCs/well). The formula of osteogenic induction solution (50 ml volume): 50 ml ordinary low sugar medium (α-MEM) containing 10% FBS, 100 nm dexamethasone (Baomanbio, China), 50 μM ascorbic acid (Baomanbio, China) and 20 mm β-glycerophosphate (Acamec, China). After reaching to 80% confluence, MSCs were treated with osteogenic differentiation medium with different dosages of Cyasterone. The medium was changed every 48 h, cells were induced continuously for 14 days and eventually stained by Alizarin Red S (Solarbio, China). According to alizarin red staining results, the optimal concentration (10 μg/ml) was selected for subsequent experiments (RT-qPCR and Western Blot). RT-qPCR and Western Blot were used to detect the expression of marker genes.

Real-time quantitative-PCR (RT-qPCR)

MSCs were digested and inoculated in 24-well plates, and pretreated with α-MEM or osteogenic induction medium containing gradient dosages of Cyasterone (5, 10 μg/ml and 0 μg/ml) in a 24-well plate for 48 h in cell incubator. Total RNAs were extracted by RNAiso Plus (Takara, Japan). RT-qPCR was operated on an ABI Step One Real-Time PCR System (Applied Biosystems, USA) using SYBR premix Ex Taq (Takara, Japan) act up to the manufacturer's instructions. Specific primers and probes were designed with the Primer5 software, as shown in Table 1. Primers were reacted with cDNA from mRNA. The formula of amplification solution (15 μl): primer (0.004 μmol each), 6.7 μl cDNA and 7.5 μl SYBR premix Ex Taq.

Gene silencing of CXCR4

siRNA targeting CXCR4 and negative mismatched control were designed and synthesized from Genepharma (China). The sequences for siRNA targeting CXCR4 were as follows: sense: (5′-3′)

Table 1
Primer sequences were as follows.

Gene name	Sequence
CXCR4	Forward primer: 5′-CAGCGGAAGCGTCTTAAGTCCAG-3′
	Reverse primer: 5′-GGCATGGTTGGTGGAGTTCAGG-3′
ROCK2	Forward primer: 5′-GCGATGTAGAGACCTTCCCG-3′
	Reverse primer: 5′-TGTTCCAGTTCCTCCTTGGC-3′
CXCL12	Forward primer: 5′-ATCAGTGACGGTAAGCCAGTC-3′
	Reverse primer: 5′-AGGGGTCTACTGGAAAGTCCT-3′
BMP-2	Forward primer: 5′-TGCGGTCTCCTAAAGGTC-3′
	Reverse primer: 5′-ACTCAAACTCGCTGAGGACG-3′
ALP	Forward primer: 5′-GAAATTCCTCGCATCCTGCCT-3′
	Reverse primer: 5′-GGCAGTGTCAGCCGTTAATTG-3′
OPN	Forward primer: 5′- GAGACCGTCTGAAACAGCGT -3′
	Reverse primer: 5′- AACCACTGCCAGTCTCATGG -3′
F-actin	Forward primer: 5′-TTGTAACCAACTGGGACGATATGG-3′
	Reverse primer: 5′-GATCTTGATCTTCATGGTGCTAGG-3′

GACUGGUACUUUGGGAAAUTT; antisense: (5′-3′) AUUUCCCAAA-GUACCAGUCTT. The transfection was performed with lipofectamine 3000 solution (Invitrogen, USA) according to the manufacturer's protocol. After the terminal transfection for 48 h, the MSCs were collected analysed.

Western blotting analysis

MSCs were digested and inoculated in 6-well plates, and treated with osteogenic differentiation medium. After treatment, the cells were lysed and total protein was extracted and electrophoresized. The transferred membrane was blocked at room temperature for 1 h, and then incubated with primary antibody (GAPDH, OPN, ALP and BMP-2, Abcam) at 4 °C overnight. The membrane was washed with PBST for 5 min × 4 times. Then the membrane was incubated with corresponding secondary antibody at 37 °C for 1 h. The membrane was washed with PBST for 5 min × 5 times and then developed by chemiluminescence (ECL, Pierce, ThermoFisher). Image J image processing software was used to analyze the band intensity.

Fracture surgery

Male Sprague-Dawley rats (n = 30), 12 weeks old, weighing approximately 320–340g were randomly divided into two experimental groups and one control group (n = 10, each). All rats were anesthetized with 0.3% pentobarbital sodium (Sigma, USA) given intraperitoneally at 30 mg/kg body weight [25]. Making an incision along the median of the right knee joint, opening the capsule, and moving the patellar ligament aside. 18-gauge needle was used to ream the medulla from the intercondylar of the femur. Then an electric pendulum was used to saw off the middle section of femoral shaft, a 1.2 mm kirschner (JH00024, Jinhuang, China) wire was inserted into the bone marrow cavity through the femoral medullary cavity for fixation. Finally, the patellar ligament was put back to original position, sterilized, and the incision closed [26].

ELISA assay

All rats were injected with Cyasterone everyday. At day 1, 7 and 14, the serum of rats from each group was checked. Blood was drawn from the posterior venous plexus of eyeball, and serum was isolated by centrifugation and stored at − 20 °C. Total SDF-1α/CXCR4 levels of serum were measured by using a rat **total** SDF-1α/CXCR4 enzyme-linked immunosorbent assay (ELISA, Catalog NO: E-EL-R2495, elabscience, China) kit.

Measure the number of MSCs in PB (peripheral blood)

To determine the number of MSCs mobilized to PB, PB was collected by orbital blood collection before and at 6th day after Cyasterone

treatment. The cell surface markers of PBMSCs were determined by flow cytometry. Red blood cell lysis buffer (Beyotime) was used to treat the blood twice, operated according to the instructions. The cells were incubated for 30 min at room temperature with monoclonal antibodies for the stem cell markers CD73 and the haematopoietic marker CD45, of course, there are isotypes. All antibodies come from Rat Mesenchyma Stem Cell Detection Kit (Cyagen). Cells were analysed using a BriCyte E6 (Mindray) flow cytometer.

Micro-CT analysis

The animal micro-CT scanner (Skyscan1172, Bruker Micro-CT, Belgium) was used to evaluate the morphometric parameters of regenerated new bone and previous femur bone. The sample was placed in the test tube of Micro-CT system and scanned along the long axis of the specimen, and then 3D model of sample was reconstructed with NRecon Version, 1.7.0.4 and CTvox Version 3.2. The contoured regions of interest (ROI) were selected from 2D CT images covering the whole 400 slices. CT Analyser software was used to analyze ROI of 400 pictures between above and below the fracture line. The following morphometric parameters were evaluated by the built-in software: relative bone volume (BV/TV). Newly formed callus (the low-density bone) was considered at threshold (100–130) ; old cortical bone and highly mineralized callus (high-density bone) were considered at 130–255, and newly formed callus plus high-density bone (threshold at 100–255) [26]. Threshold value was defined as regenerated new bone (100–130), previous bone (130–255), and total bone (100–255).

Hematoxylin-eosin staining and Safranin O staining

After Micro-CT scanning and analyzing, the harvested samples were decalcified with 10% EDTA (pH = 7.4) and dehydrated with 70–100% ethanol. Eventually, all the femurs were embedded in paraffin and sectioned into 5 μm sections for staining. Hematoxylin and Eosin staining as well as safranin O staining were used to stain sections. Observing them in a photomicroscope (IX79P2F, OLYMPUS).

Immunohistochemistry

Sections were dehydrated, hydrated and douched. The antigens of sections were repaired by Trypsin for 30min, and then sections were washed with PBS. Endogenous peroxidase was blocked with 3% hydrogen peroxide (H_2O_2) for 20 min, and then washed with PBS. Samples were incubated with immunostaining occult solution at room temperature for 60 min, and then washed with PBS. Then they were incubated with rabbit anti-rat OPN antibody (Beijing 4A Biotech Co., Ltd) in the 4 °C refrigerator overnight, and incubated with sheep anti-rabbit secondary antibody at room temperature for 40 min, then DAB was added to develop color. They were restained with Hematoxylin for 2~3 min, and then fully rinsed with running water. They were differentiated with 1% hydrochloric acid alcohol for 3~5 s, then rinsed under running water, dehydrated and observed under optical microscope.

Statistical analysis

Data were presented as the mean ± SD. One-way ANOVA was used for multiple comparisons. A value of P within 0.05 was considered statistically significant.

Results

Identification of bone-derived MSCs in SD-rat

MSCs were obtained by whole bone marrow adherent culture, and passaged to P3. After 6 days of primary culture, spindle-shaped MSCs had attached and reached 80% confluence. P3 cells were detected using flow cytometry (Fig. 1). The results of flow cytometry identification showed that the surface antigen of MSCs including CD29, CD44 and CD73 were positive, while the expression of hematopoietic stem cell surface antigen CD11 ，CD34 and CD45 was negative.
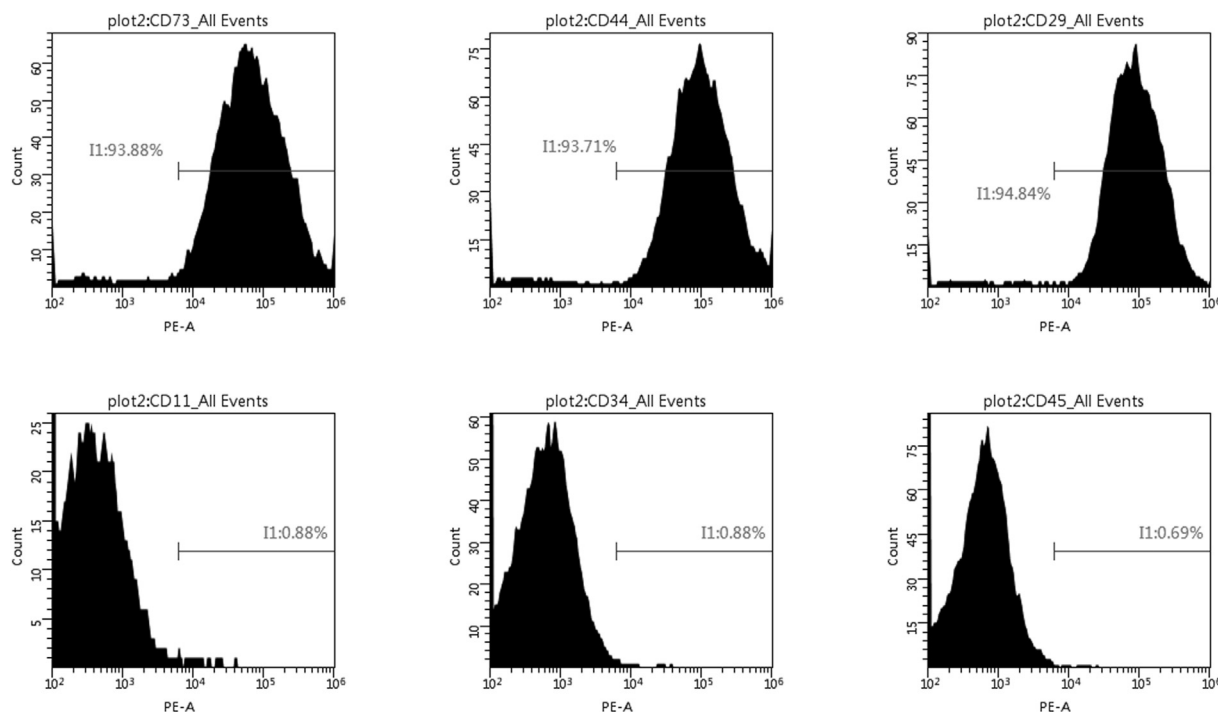

Fig. 1. Identification of mesenchymal stem cells (MSCs). MSCs at passage 3 were identified using flow cytometry. Flow cytometry was used to analyze the levels of the biomarkers in MSCs. Companied with corresponding isotype control Flow cytometry identification of MSCs surface antigen showed that the surface antigen CD29, CD44 and CD73 of cortical bone and bone marrow derived MSCs were positive, while the expression of hematopoietic stem cell surface antigen CD11 , CD34 and CD45 was negative. It was proved that the cells used in this experiment were MSCs.
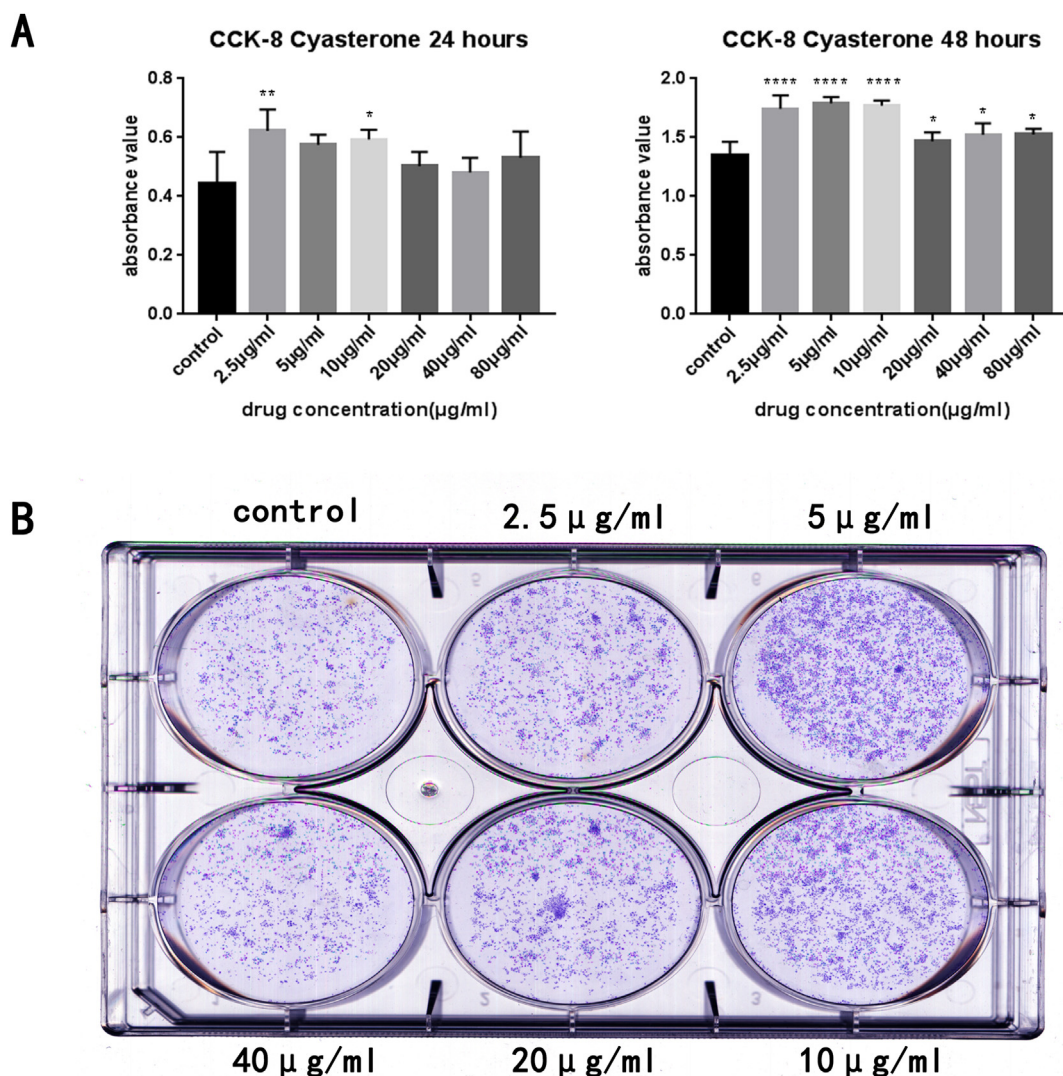
Fig. 2. Cell Counting Kit-8 assay prove the Cyasterone promoted MSCs proliferation. (A)Pretreatment with different dose Cyasterone for 24h and 48h, the figure showed Cyasterone can promoted MSCs proliferation (n = 4). *$p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ compared to control group at the same time point. The data are presented as the means ± SD of independent experiments. (B) Colony formation assay using Cyasterone (2.5, 5, 10, 20, 40μ g/ml) to culture MSCs.The colonies were fixed and stained with crystal violet.

The effect of Cyasterone on MSCs proliferation

To evaluate the effect of Cyasterone on MSCs proliferation, MSCs were incubated in basic medium with or without different concentrations of Cyasterone (2.5, 5, 10, 20, 40, 80 μg/ml) for 24~48 h. According to the results of CCK-8 assay, at 24 and 48 h, Cyasterone group could improve MSCs proliferation, particularly when cells were treated with 2.5–10 μg/ml Cyasterone (**** $p < 0.0001$) (Fig. 2A). As shown in clone formation assay, the colonies in different dosages of Cyasterone (2.5, 5, 10, 20, 40,80 μ g/ml) were much bigger and larger, which means the Cyasterone had no cytotoxicity to MSCs (Fig. 2B).

The effect of Cyasterone on MSCs migration

MSCs were treated with or without Cyasterone, and then the transwell and RT-qPCR assay were used to determine whether or not Cyasterone promoted migration. The result of tranwell demonstrated that the Cyasterone groups demonstrated that Cyasterone significantly promoted MSCs migration in comparison with the control group (Fig. 3A). We found that Cyasterone could promote the expression of migration-related mRNA, such as CXCR4, CXCL12 and ROCK2 (*$p < 0.05$) (Fig. 3B).

Silencing CXCR4 inhibits Cyasterone 's effect on migration

In front studies, we found that the expression of CXCR4 was significantly increased. We wondered whether CXCR4, as an important pathway for cell migration, was involved in MSCs migration. Therefore, we confirm its function by silencing the expression of CXCR4 by transfection siRNA (Fig. 4A). We found that the effect of Cyasterone on CXCR4 expression was significantly suppressed by siRNA-CXCR4 (Fig. 4C). And the transwell assay also demonstrated that silencing CXCR4 significantly suppressed the effect of Cyasterone on migration capacity of MSCs (Fig. 4B).

The effect of Cyasterone on MSCs osteogenic differentiation

Alizarin red staining, RT-qPCR, and WesternBlot were used to determine the effect of Cyasterone on the osteogenic differentiation of MSCs. In Alizarin red staining, MSCs were cultured in OIM with or without Cyasterone for 14 days. Cyasterone significantly simulated MSCs osteogenic differentiation on Day 14, as evidenced by Alizarin red staining, with the maximal effect observed at 10 μg/ml Cyasterone (Fig. 5A-B). We further conducted RT-qPCR and WesternBlot to detect the expression of osteogenic marker genes (ALP, OPN, and BMP-2) in
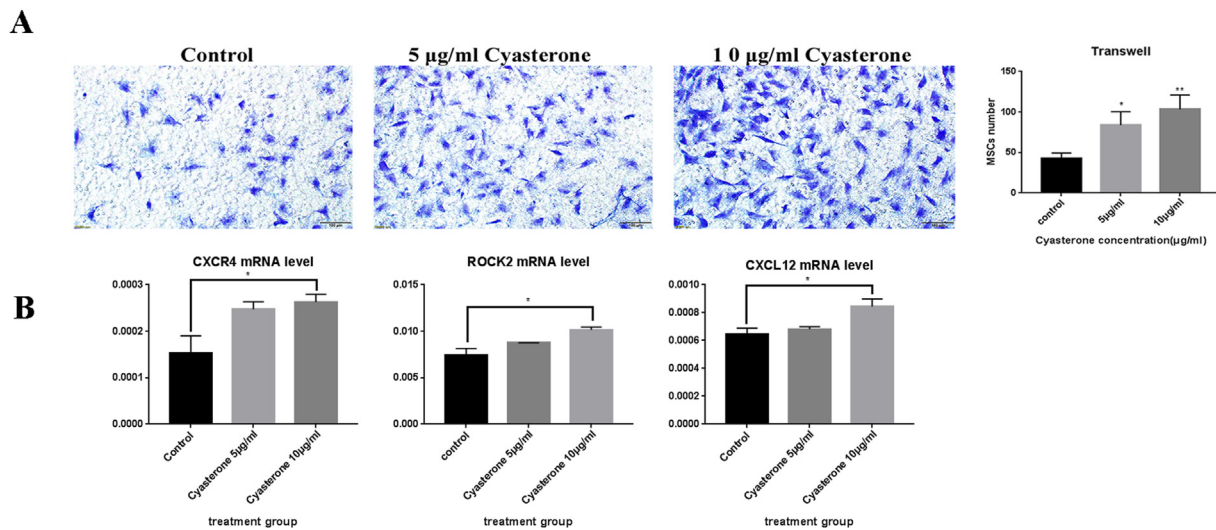
Fig. 3. The Cyasterone -treated MSCs showed a very strong migration ability in vitro. (A) Transwell assays showed that Cyasterone enhanced MSCs migration capacity (n = 3). * $p < 0.05$,** $p < 0.01$, for the Cyasterone groups vs. the control group. The data are presented as the means ± SD of independent experiments. (B) Migration-related mRNA were analyzed by RT-qPCR. Cyasterone group can promote the express of migration-related mRNA (n = 3). *$p < 0.05$, for the Cyasterone groups vs. the control group. The data are presented as the means ± SD of independent experiments.
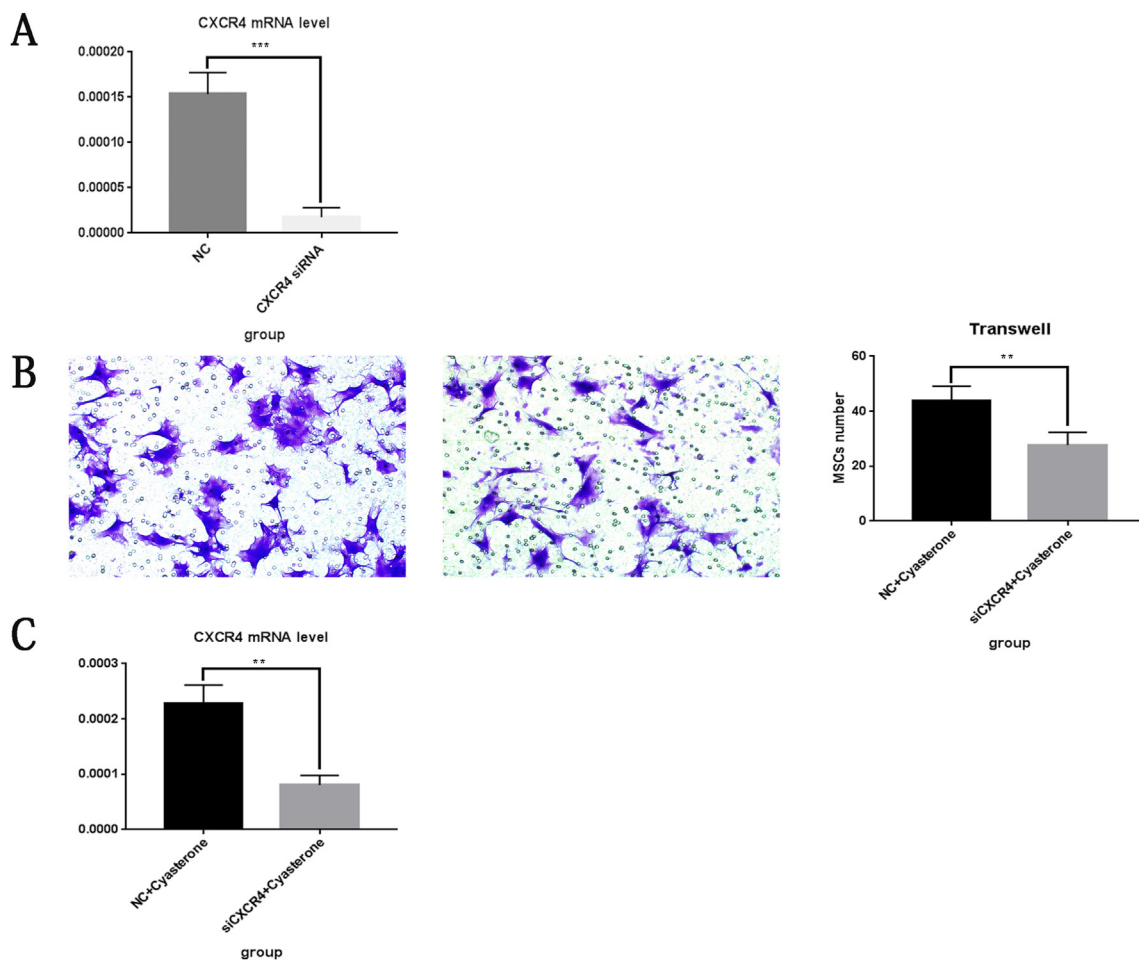

Fig. 4. Stimulatory effect of Cyasterone on MSCs migration was suppressed by silencing endogenous CXCR4. (A) The siRNA targeting CXCR4 was transfected into MSCs as mentioned in Materials and Methods. SiCXCR4 showed the best knockdown efficiency (n = 3). *** $p < 0.001$. (B) Transwell assay. The number of migrated cells were quantified by averaging five random fields per well under microscope. ** $p < 0.01$. (C) The MSCs were treated with Cyasterone or not. CXCR4 expression was significantly decreased in the siRNA+ Cyasterone group. ** $p < 0.01$.
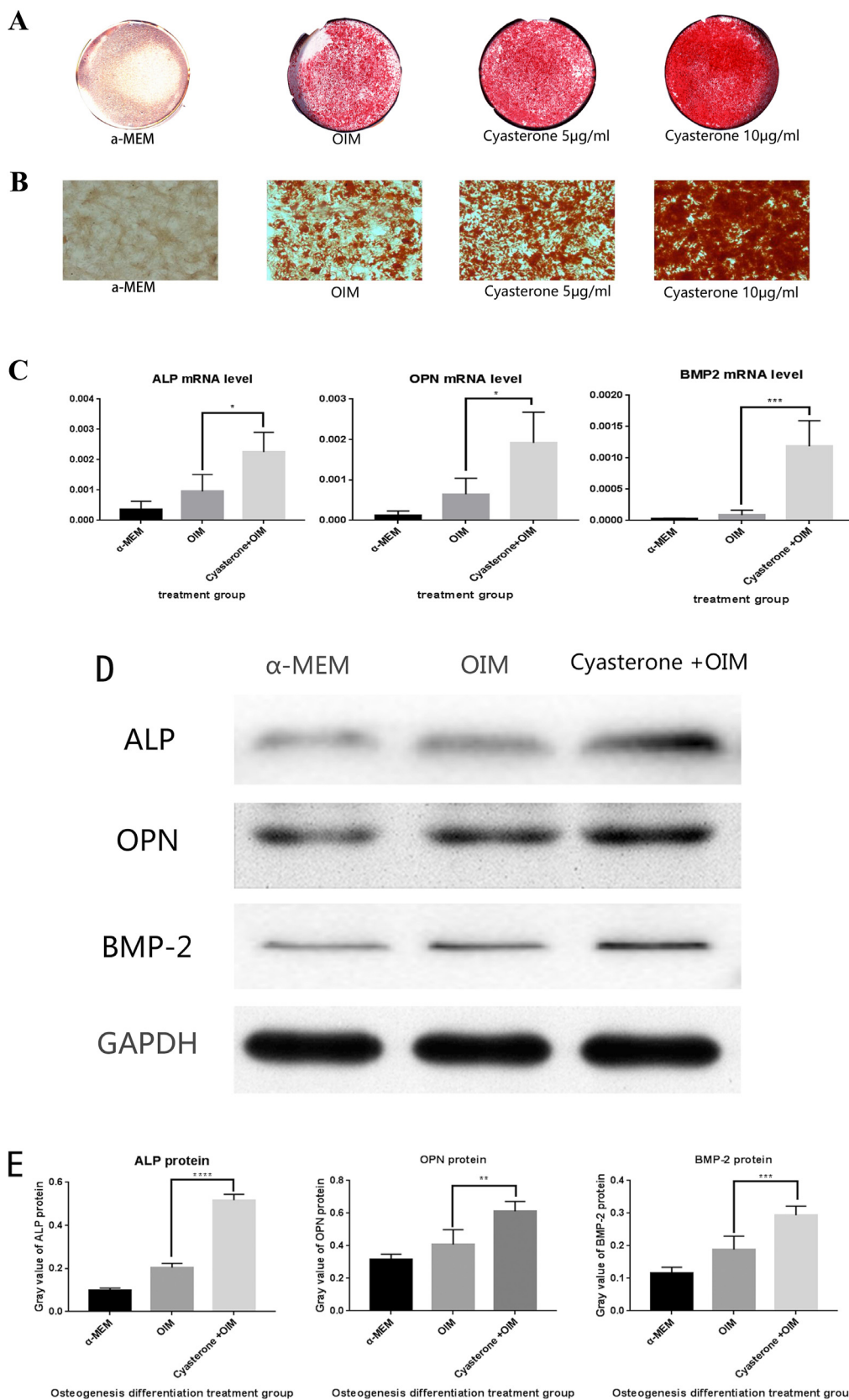
Fig. 5. The Cyasterone treated MSCs showed a very strong osteogenic ability in vitro. (A) & (B) Compare with OIM group, Cyasterone promoted the formation of calcium nodules in MSCs (alizarin red staining, 40×). (C) Osteogenesis related mRNA expression of ALP, BMP-2, OPN. The data show that Cyasterone unregulated mRNA of ALP, BMP-2, OPN (n = 3).* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Compared to OIM group at the same time point. The data are presented as the means $\pm$ SD of independent experiments. (D) Protein expression levels of ALP, BMP-2, OPN in all groups. (E) Image J was used to analyze the gray scale of the Western blotting electrophoresis strip. The data showed that Cyasterone unregulated protein of ALP, BMP-2, OPN (n = 3). * ***p*** < 0.05, ** ***p*** < 0.01, *** ***p*** < 0.001 compared to OIM group at the same time point. The data are presented as the means $\pm$ SD of independent experiments.
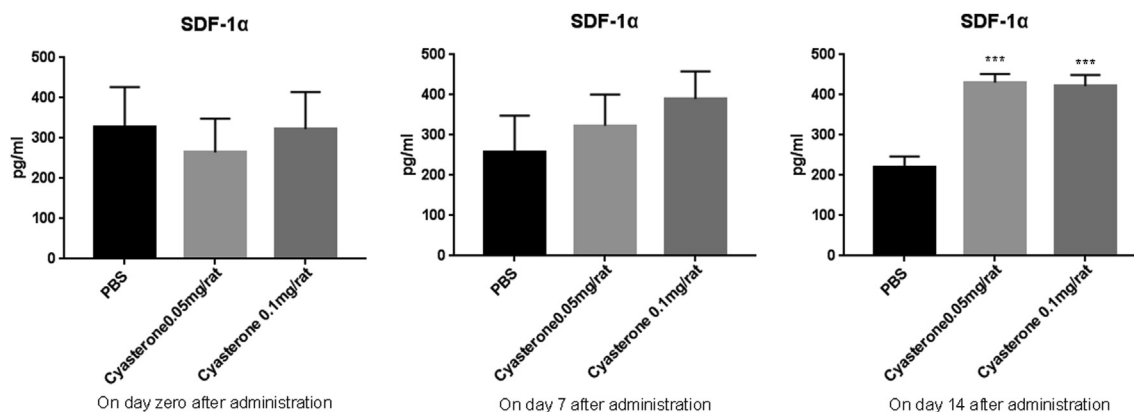
Fig. 6. SDF-1α/CXCR4 is an important factor of migration and homing of MSCs in vivo. The Elisa result shows the Cyasterone increased SDF-1α/CXCR4 concentration in SD rat fracture model. It has been shown that Cyasterone can mobilize MSCs migration in animals (n = 3). *** $p < 0.001$ compared to PBS group at the same time point. The data are presented as the means ± SD of independent experiments.

MSCs for investigating the effect of Cyasterone on osteogenesis. Compared with OIM group, OIM+Cysterone group indicated that Cyasterone significantly promoted the osteogenic differentiation of MSCs (Fig. 5C-E).

SDF-1α concentration in serum of Sprague-Dawley rats with fracture

To investigate whether or not Cyasterone simulated MSCs homing via the SDF-1/CXCR4 pathway, we conducted ELISA test to determine the expression of SDF-1α in the serum of rats with fracture. There was no significant difference between PBS and Cyasterone groups before injections ($p > 0.05$). However, Cyasterone increased the levels of SDF-1α on Day 7 and 14 in comparison with PBS groups. Moreover, the levels of SDF-1α showed an opposite trend in PBS group. After 7 days, the concentration of SDF-1α in Cyasterone group was higher than that of PBS group. After 14 days, the concentration of SDF-1α was significantly increased in the Cyasterone group in comparison with PBS group (*** $p < 0.001$) (Fig. 6).

Effect of Cyasterone on mobilization of endogenous MSCs

To make sure whether Cyasterone can mobilize endogenous MSCs in blood, flow cytometry was used to detect the quantity of PBMSCs (defined as CD45-/CD73+ mononuclear cells). Compared with the model group, the percentage of CD45-/CD73 + PBMSCs in rats treated with Cyasterone was significantly increased in both low and high dose group (Fig. 7A-D).

Micro-CT analysis for fracture femur

After treated with or without Cyasterone for 6 weeks, reconstruction and bone volume were measured within the fracture region by micro-CT analysis. The reconstructed mineralized calluses showed that different morphological characteristics among each group in 3D micro-CT images (Fig. 8A). In 6 weeks after surgery, the closure degree of fracture line in Cyasterone group was better than that of the control group. Bone volume in ROI range was calculated by software analysis. Compared with PBS group, the BV/TV value of Cyasterone group was significantly increased in low density bone (*$p < 0.05$, **$p < 0.01$) (Fig. 8B). These results showed that fracture healing was delayed in PBS group, while fracture healing was promoted and low density bone was increased in Cyasterone group.

Histological examination of fracture site

Hematoxylin-eosin (HE) staining illustrated a significant reduction of the soft ossification center (purple zone) in the fracture site in low and high dose group compared with PBS group (Fig. 9A). Safranin O staining illustrated the area of chondrocytes in the fracture end (red zone) decreased with the increase of Cyasterone concentration, the area of bone tissue at the fracture end (green zone) increase with Cyasterone concentration (Fig. 9B). Expressions of osteopontin (OPN) in rats' fracture site were evaluated by immunohistochemistry. Immunohistochemical results illustrated that the expression of OPN in the low and high dose group increased in comparison with PBS group. Besides, we used image J software to immunohistochemical quantitative analysis (Fig. 9C).

Discussion

Fracture is the most common orthopedic disease caused by accidental injury. With the rapid development of society, the incidence of severe fracture has increased significantly. The risk of bone defect is increased in chronic inflammation, diabetes, vitamin deficiency, old age, and multiple injuries, with longer treatment time, disability rates increase [27]. There are two ways of bone repair: intramembranous osteogenesis and endochondral osteogenesis, by which MSCs are directly or indirectly differentiated into osteoblasts and even bone tissue [9]. It was found that the concentration and total amount of MSCs at the fracture site are positively correlated with the quantity of callus and osteogenic ability at the fracture site, and negatively correlated with the healing time of the fracture [9]. Cyasterone was extracted from *Radix cyathulae*, which was used to promote fracture healing in traditional Chinese medicine (TCM). However, just few studies found that herbal extract of TCM effected on migration of MSCs through CXCR4 and also on osteogenic differentiation of MSCs. In this study, we demonstrated that Cyasterone could promote not only the homing and osteogenic differentiation of MSCs , but also upregulation of osteogenic-related and migration-related mRNA. There was also an important finding that Cyasterone could also promote the early healing of fracture in rats.

The promotion of the migration and osteogenic differentiation of MSCs would improve the efficiency of MSC engraftment in clinical applications, which could significantly improve MSC-based cell therapy and regenerative medicine outcomes. Herbal medicine of TCM was a promising alternative therapies, which could not only improve physical condition but also significantly reduce morbidities and alleviate symptoms [28]. Studies showed that the active ingredients of TCM prescription could promote MSC homing to the injured tissues through CXCR4 [29]. In present study, we investigate whether or not Cyasterone simulated MSCs homing via the SDF-1/CXCR4 pathway. We found that the effect of Cyasterone on CXCR4 expression was significantly suppressed by siRNA-CXCR4. And the transwell assay also demonstrated that silencing CXCR4 significantly suppressed the effect of Cyasterone on migration
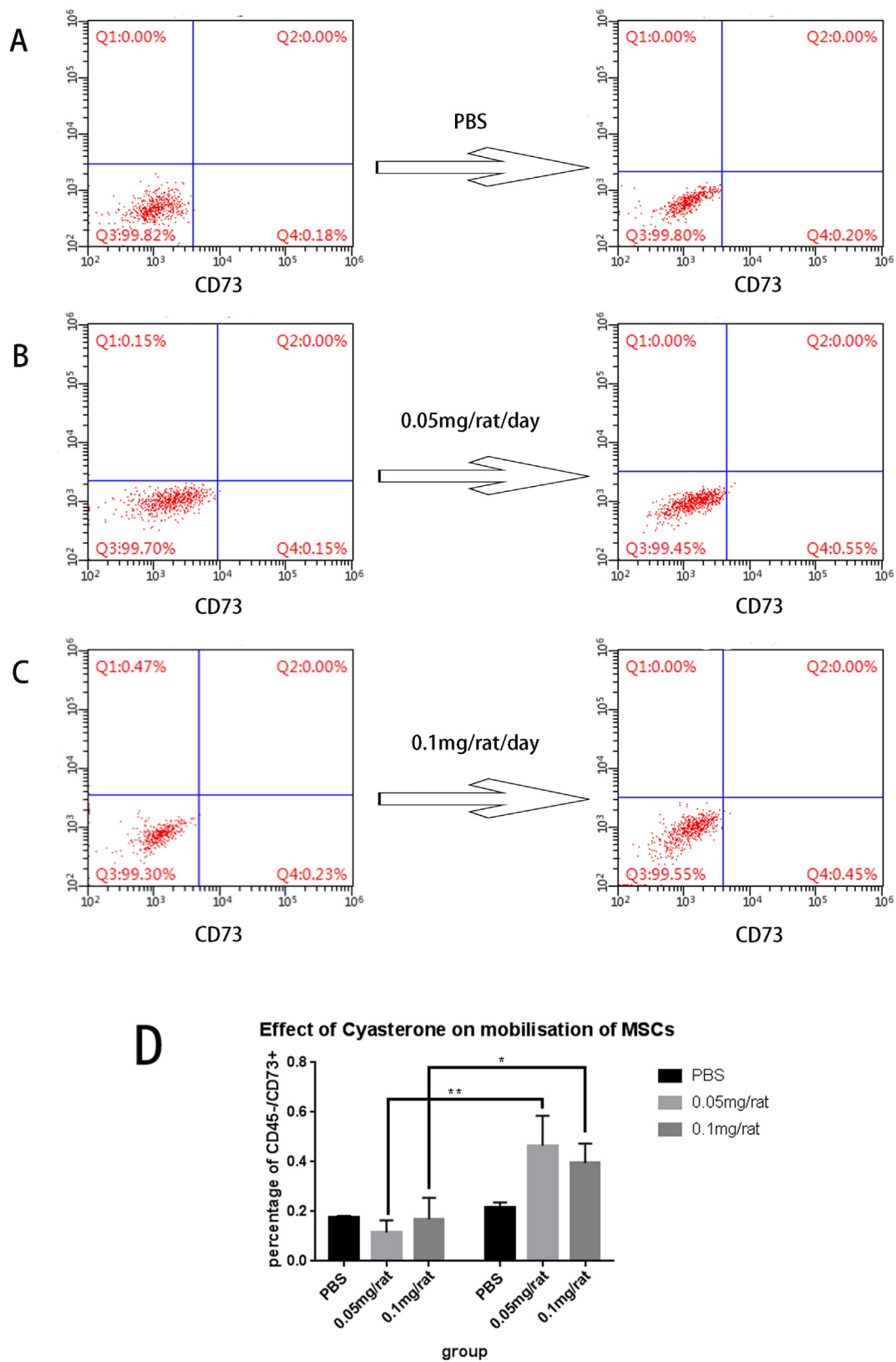
Fig. 7. Effect of Cyasterone on mobilisation of MSCs. (A–C) Representative images of flow cytometry showing the changes of circulating MSCs in rats administrated with Cyasterone or control PBS. (D) The number of MSCs in peripheral blood in Cyasterone treated group was significantly increased after treatment (*$p < 0.05$), especially in low dosage group.
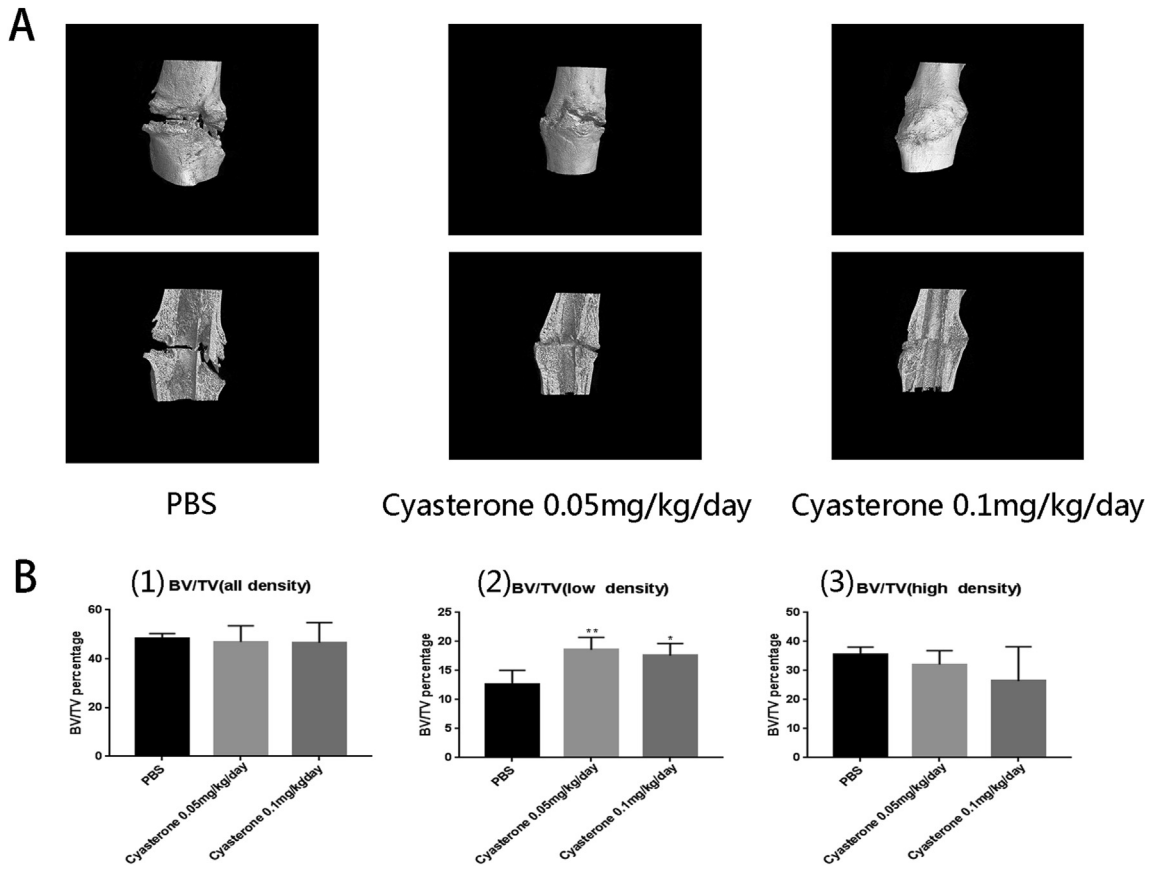
Fig. 8. 3D reconstruction of fracture site was displayed by Micro-CT. (A) Entire volume and Longitudinal cut view of Region Of Interest (ROI). Compared with the PBS group, the fracture site of Cyasterone treatment group heal better. (B) Bone volume fraction (BV/TV) in ROI range was calculated by software analysis. The data showed that bone volume fraction' result high density, low density, all density (n = 3). * $p < 0.05$, ** $p < 0.01$ compared to PBS group at the same time point. The data are presented as the means ± SD of independent experiments.
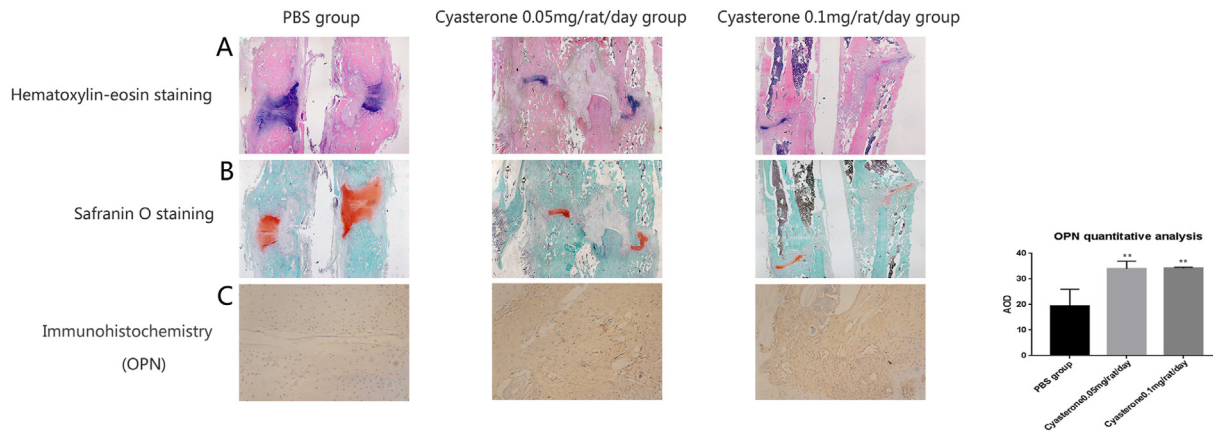

Fig. 9. Histological examination of each group. Including immunohistochemical assay, HE staining assay, saffron O staining assay. (A) The area of chondrocytes at the fracture end (red zone) decreased with the increase of Cyasterone concentration, (B) The area of bone tissue at the fracture end (green zone) increase with Cyasterone concentration. (C) Immunohistochemical staining of Cyasterone treatment group and PBS groups. (200×). This picture demonstrated the expression of OPN in callus among groups. As the Cyasterone concentration increases, the OPN' antibody expression becomes stronger. What's more image J software was used in the immunohistochemical quantitative analysis (n = 3). ** $p < 0.01$ compared to PBS group at the same time point. The data are presented as the means ± SD of independent experiments.

capacity of MSCs. With a high affinity with its ligand CXCR4, SDF-1α was the most important signal channel of chemokine-related and homing-related cytokines in MSCs. SDF-1α could be produced by paracrine effect in bone tissue injured by ischemia. MSCs transplanted intravenously could be binded with SDF-1α through CXCR4, its membrane receptor, and migrate to the ischemic fracture area along the

SDF-1α concentration gradient [17,18]. 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 [30]. In this study, the results of flow cytometry analysis shown that Cyasterone significantly increase the number of CD45-/CD73+ circulating MSCs. In this study, SDF-1α

concentration in serum of Cyasterone-treated rats was significantly increased. MSCs could be attractive candidate cells for the treatment of bone diseases due to their ability to differentiate into a variety types of functional cells in mesenchymal tissues (bone, cartilage, muscle and tendon). Micro-CT [31], immunohistochemistry and mechanical tests can be used to evaluate animal fracture experiments [32]. Osteogenic differentiation of MSC was estimated by observation of cellular morphology, accumulation of extracellular calcium, and expression of osteogenic markers, including runx2, osteopontin (OPN), and osteocalcin (OCN) [33]. OPN is a major non-collagen bone matrix protein produced by osteoblasts and osteoclasts, and is involved in bone resorption, formation and remodeling [34]. Histological and Micro-CT results showed that the degree of fracture healing in Cyasterone group was better than that of PBS group, and the OPN protein expression in the callus in Cyasterone group was significantly increased. The osteogenic differentiation experiment of MSCs also confirmed that Cyasterone group could promote the osteogenic differentiation of MSCs and improve the expression of osteogenic markers. In a word, we found that Cyasterone was a naturally extractive with potentially therapeutic effect, which could be used in clinical practice to promote fracture healing, improve the homing and osteogenic differentiation efficiency of MSCs, and significantly improve the efficacy of MSCs therapy and regenerative medicine.

Conclusion

In conclusion, Cyasterone could significantly promote the migration and osteogenic of MSCs, reducing the fracture healing time, through related genes, including OPN, ALP and BMP-2.

Ethical approval

The study was approved by the Guangzhou University of Traditional Chinese Medicine animal experiment ethics board, Guangzhou, Guangdong province, China (animal ethics number: 20190227050). All animals received humane care in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Conflict of Interest

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

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