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Glucocorticoids Inhibit BMSC Homing by Upregulating PPARγ through Genomic Pathways

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Background: In the context of glucocorticoid-induced femoral head necrosis, the overexpression of peroxisome proliferator-activated receptor γ (PPARγ), induced by glucocorticoid (GC), inhibits adipogenesis and promotes osteogenesis. Some studies have found that the stromal cell-derived factor-1 (SDF-1)/CXC chemokine receptor 4 (CXCR4) pathway mediates the homing of bone marrow mesenchymal stem cells (BMSCs). However, the mechanism of BMSCs homing regulated by GC is not fully understood. In this study, we aim to investigate the mechanism of how GC regulates BMSCs homing through PPARγ from the glucocorticoid receptors (GR) downstream, based on the regulatory signal ahead of the CXCR4.

Methods: BMSCs were cultured, dexamethasone (DXMS) was applied to intervene the BMSCs, and the expression sites of GRα, PPARγ and CXCR4 were detected by immunofluorescence. The expression changes of cytokines GRα, PPARγ and CXCR4 after cell intervention were detected by quantitative real time polymerase chain reaction (qRT-PCR) and Western blot (WB) to explore cellular chemokines and their signaling pathways.

Results: DXMS enhanced the expression of PPARγ and inhibited the expression of SDF-1 and CXCR4. The results revealed that the G protein-coupled receptor was involved in DXMS-regulated expression of SDF-1 and CXCR4, but did not play a major role, and this effect was GR dependent. The Western blot results showed that the downstream SDF-1/CXCR4 was significantly enhanced after GR silencing, suggesting that GR was involved in the BMSCs homing ($p < 0.05$). After DXMS treatment, the expression of SDF-1 and CXCR4 in BMSCs was inhibited, while PPARγ expression was significantly increased ($p < 0.05$). The fluorescence quantitation and the Western blot results showed a significant increase in the expression of SDF-1 and CXCR4 after PPARγ silencing ($p < 0.05$), suggesting the involvement of PPARγ in the expression of BMSCs. No significant change was observed in GR expression when PPARγ was inhibited, suggesting that GR is located in upstream of PPARγ. Additionally, the expression of SDF-1 and CXCR4 decreased significantly upon the addition of DXMS ($p < 0.05$). The inhibition of SDF-1 and CXCR4 by PPARγ is not complete, indicating the existence of other regulatory mechanisms. The *PPARγ* gene plays a crucial regulatory role in the DXMS-regulated expression of SDF-1 and CXCR4.

Conclusions: DXMS inhibited the homing of BMSCs by upregulating PPARγ through genomic pathways. PPARγ plays a significant regulatory role in the downregulated expression of SDF-1 and CXCR4.

Keywords: glucocorticoid osteonecrosis of femoral head; bone marrow mesenchymal stem cells (BMSCs); peroxisome proliferator-activated receptor γ (PPARγ); homing; genomic effect

Introduction

An independent study has found that ischemia, hypoxia, and injury following femoral head necrosis lead to the directional migration of stem cells to the affected areas [1].

In the case of femoral head necrosis caused by glucocorticoids (GC), the expression of peroxisome proliferator-activated receptor γ (PPARγ), mainly PPARγ2, is promoted by GC [2–4]. Inhibition of the proliferation of bone marrow mesenchymal stem cells (BMSCs) results in a significant decrease in the number of BMSCs in glucocorticoid-induced femoral head necrosis (GNFH) [5], and the osteogenic differentiation ability of the remaining BMSCs declines noticeably [6].

In our previous study, we successfully constructed a PPARγ-siRNA lentiviral vector and transfected rat BMSCs with the lentivirus. The BMSCs function of adhesion, proliferation, and apoptosis showed no significant changes, but the BMSCs function of the adipogenic differentiation ability was weakened, and the osteogenic differentiation ability was enhanced ($p < 0.01$) [7]. Therefore, it is of greater significance to study the homing mechanism of bone marrow mesenchymal stem cells and enhance their osteogenic ability.

At present, SDF-1 is the most effective chemokine mediating BMSCs migration [8]. Inflammatory factors in femoral head necrosis induce the local secretion of a large amount of SDF-1 [1,9]. There is a large number of stromal cell-derived factor-1 (SDF-1) receptor CXC chemokine 4

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(CXCR4) on the surface of BMSCs, and the coupling of the two mediates BMSCs migration to the femoral head necrosis area. Further studies also found that the SDF-1/CXCR4 axis can inhibit the apoptosis of BMSCs, increase the survival rate, proliferation activity, and migration ability of BMSCs, and improve the homing efficiency of BMSCs in many aspects [10]. However, glucocorticoids inhibit the expression of CXCR4 and inhibit SDF-1-mediated BMSCs migration [11,12].

Glucocorticoid dependence on the genomic regulatory mechanism involves specific association with cell membrane proteins or nonspecific binding to lipids. This leads to changes in the physical and chemical properties of the membranes or certain cell membrane protein structures, resulting in the secretion, outward capacity adjustment, change of membrane potential and ion flow. These changes activate the intracellular second messenger system, causing a rapid response to external stimuli. A recent study demonstrated the existence of high-affinity G-protein-coupled receptors for glucocorticoids in cell membranes [13]. Glucocorticoids binding to G-protein-coupled receptors regulate multiple intracellular signal transduction pathways, including the cAMP-PKA pathway, PKC pathway, and Ca^{2+} pathway [14]. This rapid effect of glucocorticoids cannot be blocked by the classical genomic steroid hormone receptor antagonist RU486 (roussel.uclaf 38486, also known as mifepristone).

Glucocorticoids have a classical genomic pathway in which they bind to intracellular glucocorticoid receptor (GR). The GC-GR is known to change gene expression in two ways. First, glucocorticoids can bind to intracytoplasmic ligands and form complexes. These complexes then enter the nucleus and directly bind to the positive glucocorticoid regulatory element (pGRE) or negative glucocorticoid regulatory element (nGRE) on DNA. They act as ligand-dependent transcription factors, either promoting or inhibiting gene expression and regulating protein synthesis [15]. This effect can be blocked by the glucocorticoid classical receptor antagonist RU486 [12].

The second mechanism involves the interaction with nuclear transcription factors, such as PPARγ, NF-κB, AP-1, etc., leading to the inhibition of their transcriptional activity [16,17]. During the onset of glucocorticoid-induced necrosis of the femoral head, PPARγ is predominantly expressed. *PPAR*γ regulates the transcription of downstream related genes, influences the production of proteins, and blocks cell adhesion and migration through ligand-dependent transcriptional activation mechanisms in the nucleus [18]. Following upregulation of PPARγ expression by glucocorticoids, CXCR4 acts downstream to PPARγ, leading to inhibition, and a decrease in the expression of CXCR4 receptor on BMSCs [18,19]. Furthermore, it attenuates the interaction between VEGF-1 and the homing factor of SDF-1, thereby inhibiting the role of the VEGF-1/CXCR4 regulatory chain in the passage of cells [20,21]. It also impairs the VEGF-SDF-1/CXCR4 signaling pathway. The upregulation of VEGF-A expression by SDF-1 and the upregulation of CXCR4 expression by VEGF-1 are inhibited. This, in turn, blocks the VEGF-mediated migration of BMSCs in the vascular endothelial space [22], ultimately hindering the homing of BMSCs to the necrotic area. Additionally, the PPARγ agonist rosiglitazone downregulates CXCR4 expression, while the PPARγ antagonist GW9662 downregulates agonist-induced CXCR4 downregulation, thus supporting the involvement of PPARγ [23].

The specific mechanism by which glucocorticoids and PPARγ affect BMSCs homing remains unclear. A study [11] suggested that the CXCR4 promoter region might contain positive and negative regulatory elements of GR (pGRE or nGRE) and binding elements of PPARγ (PPRE-l and PPRE-2). We hypothesized that glucocorticoids activate GRE activity and increase the binding of the nuclear transcription factor GR to nGRE, affecting PPRE1 activity and binding of nuclear transcription factor PPARγ to PPRE, thereby downregulating the transcription and expression of CXCR4 and inhibiting the homing of BMSCs.

In light of the above hypothesis, we cultured experiments with BMSCs and employed molecular biology techniques to address the following questions. First, we aimed to determine whether glucocorticoids regulate PPARγ in a GR-dependent manner. Second, we sought to investigate whether glucocorticoids regulate the downstream SDF-1/CXCR4 signaling pathway through PPARγ. Lastly, we aimed to clarify whether glucocorticoids regulate the homing of BMSCs in a PPARγ-dependent manner. The potential implications of this study include shedding light on the roles of GR and PPARγ in BMSCs homing and elucidating their functions in glucocorticoid-activated genomic pathways. The findings from this study are expected to establish a new experimental foundation for BMSCs homing in the early treatment of GNFH.

Materials and Methods

Reagents and Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotics, and trypsin were obtained from Hyclone (Logan, UT, USA). Antibodies against PPARγ (bs-4590R, WB: 1:500, IF: 1:100), SDF-1 (bs-0783R, WB: 1:500, IF: 1:100), CXCR4 (bs-20317R, WB: 1:500, IF: 1:100), and GR (bs-13385R, WB: 1:500, IF: 1:100) were obtained from Bioss (Beijing, China). The GPCR antagonist suramin from MedMol (S80257, Milan, Italy) was used to pretreat cells for 30 min at 4 μM. β-actin antibody was obtained from Beyotime (abs132001, Shanghai, China). The GR antagonist RU486 (4 μM) (84371-65-3, Princeton, NJ, USA) and dexamethasone (DXMS) were from MCE. Polyformaldehyde was purchased from Beyotime Biotechnology (P0099, Shanghai, China). DAPI dye was obtained from Solarbio Life Sciences (C0060, Beijing,

China). The siRNA was synthesized by Sangon Biotech (Shanghai, China). The sequences for the siRNAs used are as follows: siNC, TTCTCCGAACGTGTCACGT; siGR1, GGAGATCAGACCTGTTGATAG; siGR2, GGAGATGACAACTTGACTTCT; siGR3, GCAGTACTCCTGGATGTTTCT; siPPARγ1, GCAAGGAACTCTGAAAGTGTG; siPPARγ2, GGAAAGAACATCTTGGGAAGA; siPPARγ3, GGAGCAAACGACACCAGATTT.

Cell Culture and Transfection

BMSCs were purchased from Saiye Biotechnology Company (HUXMA-01001, Guangzhou, China). The BMSCs used in this study were tested for mycoplasma and showed no mycoplasma contamination. All cell lines have been authenticated by STR analysis to confirm their identity. The cells were cultured in αMEM (Gibco, 12571-063, Waltham, MA, USA) supplemented with 20% FBS (GE Healthcare Life Sciences, SH30071.03, Chicago, IL, USA), and 1% Penicillin-Streptomycin (P/S) (Gibco, 15140-122, Waltham, MA, USA) at 37 °C in a 5% CO_2 atmosphere. BMSCs were seeded into a 24-well plate at a density of 1 $\times$ 10^4 cells/well. Then, 1 µg of siRNA was dissolved in 150 µL of serum-free αMEM, and 9 µL of Lipofectamine™ 2000 (16680191, Thermo, Waltham, MA, USA) was dissolved in 150 µL serum-free αMEM. After 10 min at room temperature, the two mixtures were gently mixed and incubated for 20 min at room temperature. Next, 50 µL of the mixture was added into each well containing 500 µL of serum-free αMEM. The medium was changed after 6 h at 37 °C.

Immunofluorescence Staining

BMSCs were washed twice with pre-cooled phosphate-buffered saline (PBS), then fixed with 4% paraformaldehyde for 20 min at room temperature. Subsequently, the cells were washed with PBS and permeabilized with 0.3% Triton X-100 for 20 min. After another PBS wash, the cells were blocked with 5% bovine serum albumin (BSA) at 37 °C for 1 h. The primary antibodies were then added and incubated overnight at 4 °C. The following morning, the cells were washed with PBS three times and incubated with a fluorescent secondary antibody for 2 h at 37 °C. The cells were then stained with DAPI dye in the dark for 10 min. The images were captured and collected using a fluorescence microscope. The protein localization was detected using a laser confocal microscope.

Real-Time PCR

Total RNA was isolated from harvested cells (1 $\times$ 10^5 cells per condition) using Trizol following the manufacturer's protocol. Subsequently, 1 µg of total RNA was treated with RQ1 DNase and reverse transcribed using 1 µM MLV Reverse Transcriptase. After a 5-fold dilution, the cDNAs were used as the template for quantitative real time polymerase chain reaction (qRT-PCR). Briefly, 20 µL PCR reactions, containing 2 µL cDNA, 5 pmol of corresponding primer sets, and 10 µL 2X SYBR Green Master mix, were analyzed using a Rotor-gene 6000 real-time PCR system. PCR was performed for 10 s at 95 °C, followed by 20 s at 58 °C and 10 s at 72 °C for 40 cycles, followed by the thermal denaturation protocol. The expression of each mRNA relative to β-actin mRNA was determined using the $2^{-\Delta\Delta Ct}$ method. The primers used are listed in Table 1.

Table 1. List of primers used in quantitative real time polymerase chain reaction (qRT-PCR).

Primer name	Primer sequence (5′-3′)
GR-F	CTGCGTCTTCACCCTCAC
GR-R	GTGAAACTGCTTTGGACA
CXCR4-F	TGGTCTATGTTGGCGTCTGG
CXCR4-R	GTCATTGGGGTAGAAGCGGT
SDF-1-F	GACAAGTGTGCATTGACCCG
SDF-1-R	GCCCTTCCCTAACACTGGTT
PPARγ-F	CGTGGCCGCAGAAATGAC
PPARγ-R	AGATGCAGGCTCCACTTTGA
β-actin-F	ACCAACTGGGACGACAT
β-actin-R	TCTGGGTCATCTTCTCG

Western Blotting

Proteins were isolated from cells by using RIPA buffer and separated by 10% SDS-PAGE gels, then transferred onto PVDF membranes. The membranes were blocked with 3% BSA in 0.05% Tris-buffered saline-Tween 20 (TBS-T) for 2 h at room temperature and then incubated with primary antibody. After being washed with TBS-T three times, the membranes were incubated with a peroxidase-conjugated anti-rabbit secondary antibody. Following a 2-hour incubation, the membranes were washed with TBS-T three times. The signal was detected with an ECL reagent. GelPro software (Gel-Pro analyzer 4.0, Media Cybernetics, Rockville, MD, USA) was used to detect the gray value of Western blot results.

Regulation of PPARγ, SDF-1, and CXCR4 by GR

BMSCs were plated into 6-well plates, and four different treatments were administered after the cells reached 60%–80% confluency. Six specific treatments were applied as follows: (1) pretreatment with DMSO for 30 min; (2) pretreatment with the GR antagonist RU486 (4 µM) for 30 min; (3) stimulation of the cells with DXMS for 30 min; (4) pretreatment of the cells with GR siRNA for 48 h, followed by stimulation with DXMS for 30 min; (5) incubation of the cells with GR siRNA for 48 h; (6) pretreatment of the cells with NC siRNA for 48 h. The expression of PPARγ, SDF-1, and CXCR4 was detected by extracting cell proteins, using the same detailed experimental procedures as for the Western blot, and the differences between the treatment groups were analyzed.

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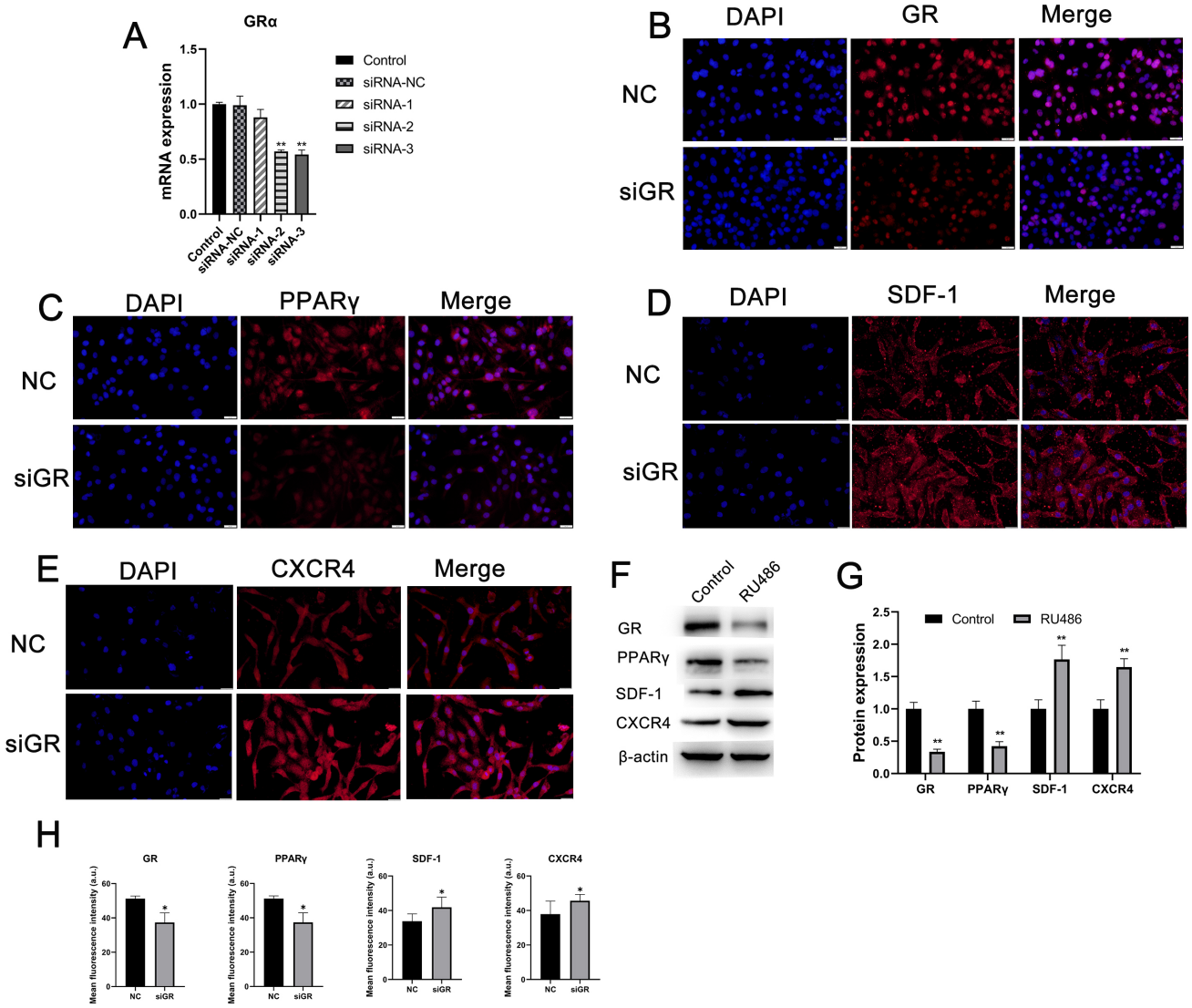

Fig. 1. Effect of the GR silencing on the localization of GR, PPARγ, SDF-1, and CXCR4. (A) The knockdown efficiency was detected by RT-PCR. Compared with siRNA-NC, $^{**}p < 0.01$. (B–E) The signals of GR, PPARγ, SDF-1, and CXCR4 were detected by immunofluorescence staining (Scale bar: 20 μm). (F,G) The effect of RU486 on the protein levels of GR, PPARγ, SDF-1, and CXCR4 was analyzed by Western blotting. Compared with Control, $^{**}p < 0.01$ (Total magnification: 400×) (1920 pixels × 1200 pixels). (H) Statistics of immunofluorescence in the NC and siGR groups. $^{*}p < 0.05$. GR, glucocorticoid receptors; PPARγ, peroxisome proliferator-activated receptor γ; SDF-1, stromal cell-derived factor-1; CXCR4, CXC chemokine receptor 4.

Regulation of PPARγ, SDF-1, and CXCR4 by G Protein-Coupled Receptors

BMSCs were plated into 6-well plates and subjected to four different treatments after reaching 60%–80% confluency. The treatments were as follows: (1) pretreatment with DMSO for 30 min; (2) pretreatment with the GPCR antagonist Suramin (4 μM) for 30 min; (3) pretreatment with Suramin (4 μM), a GPCR antagonist, for 30 min, followed by stimulation with DXMS for 30 min; (4) stimulation with DXMS for 30 min. Subsequently, cell proteins were extracted after the 30-minute DXMS stimulation to detect the expression of PPARγ, SDF-1, and CXCR4, using the same detailed experimental procedures as Western blot. The differences between the treatment groups were analyzed. There was no significant difference in protein levels between treatments 1 and 2, and between treatments 3 and 4, indicating that DXMS did not regulate PPARγ, SDF-1, and CXCR4 *via* GPCR. However, the differences between treatments 1 and 2, and between treatments 3 and 4, suggested that DXMS may require membrane G-protein coupled receptors to regulate PPARγ, SDF-1, and CXCR4.

Regulation of SDF-1 and CXCR4 by DXMS

BMSCs were subjected to the optimal concentration of DXMS and the optimal treatment duration. Subsequently, the expression levels of SDF-1 and CXCR4 were assessed

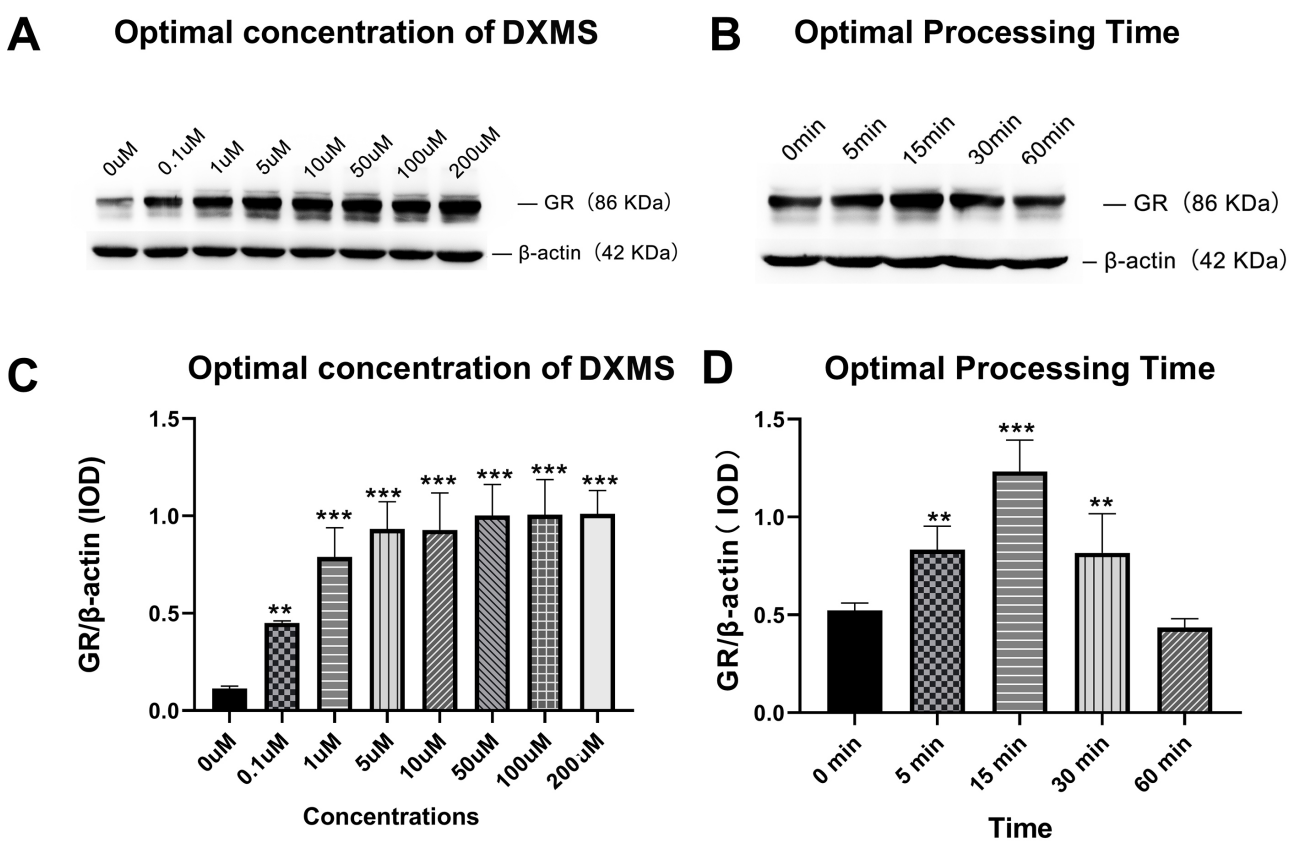

Fig. 2. Dexamethasone (DXMS) upregulates the expression of GR. (A,C) The effect of DXMS at various concentrations on GR expression. Compared with the 0 μM group, $^{**}p < 0.01$, $^{***}p < 0.001$. (B,D) The effect of DXMS incubated at various time on GR expression. Compared with the 0 min group, $^{**}p < 0.01$, $^{***}p < 0.001$.

at both mRNA and protein levels. The detailed experimental procedures for this analysis were identical to those described above for RT-PCR and Western blotting.

Regulation of SDF-1 and CXCR4 by PPARγ

BMSC cells were plated into 6-well plates, and six different treatments were performed after the cells reached 60%–80% confluency. The six different treatments were as follows: (1) pretreatment with DMSO (Control) for 30 min; (2) pretreatment with the PPARγ antagonist GW9662 (4 μM) for 30 min; (3) stimulation with DXMS for 30 min; (4) pretreatment with PPARγ siRNA for 48 h, followed by stimulation with DXMS for 30 min; (5) incubation with PPARγ siRNA for 48 h; (6) pretreatment with NC siRNA for 48 h. Subsequently, cell proteins were extracted to detect SDF-1 and CXCR4, following the same detailed experimental procedures as for Western blot. The differences between the groups were then analyzed.

Statistical Analysis

All the assays were performed a minimum of three times, and the data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) and post-hoc tests were used to compare multiple groups. A Student's *t*-test (GraphPad Prism; GraphPad Software Inc, San Diego, CA, USA) was performed where appropriate. The differences were considered significant when $p < 0.05$.

Results

The Silencing of GR does not Affect the Localization of PPARγ, SDF-1, and CXCR4

To assess the knockdown efficiency, three siRNAs targeting GR were used for transfecting BMSCs. As shown in Fig. 1A, the transfection of siRNA-2 and siRNA-3 significantly inhibited the expression of GR compared to siRNA-NC. Therefore, siRNA-3 was selected for further research. Immunofluorescence results showed that GR was mainly located in the nucleus of BMSCs (Fig. 1B). The silencing of GR significantly reduced the signal. PPARγ protein was mainly located in the nucleus with a small portion in the cytoplasm. The knockdown of GR blocked the localization of PPARγ in both the nucleus and cytoplasm (Fig. 1C). SDF-1 was mainly in the cytoplasm with a small portion outside cells, while CXCR4 was localized in both the cytoplasm and nucleus. The blockage of GR significantly increased the signals of SDF-1 and CXCR4 (Fig. 1D,E).

We further used GR antagonist RU486 to incubate the cells and examined the effect on the protein levels of

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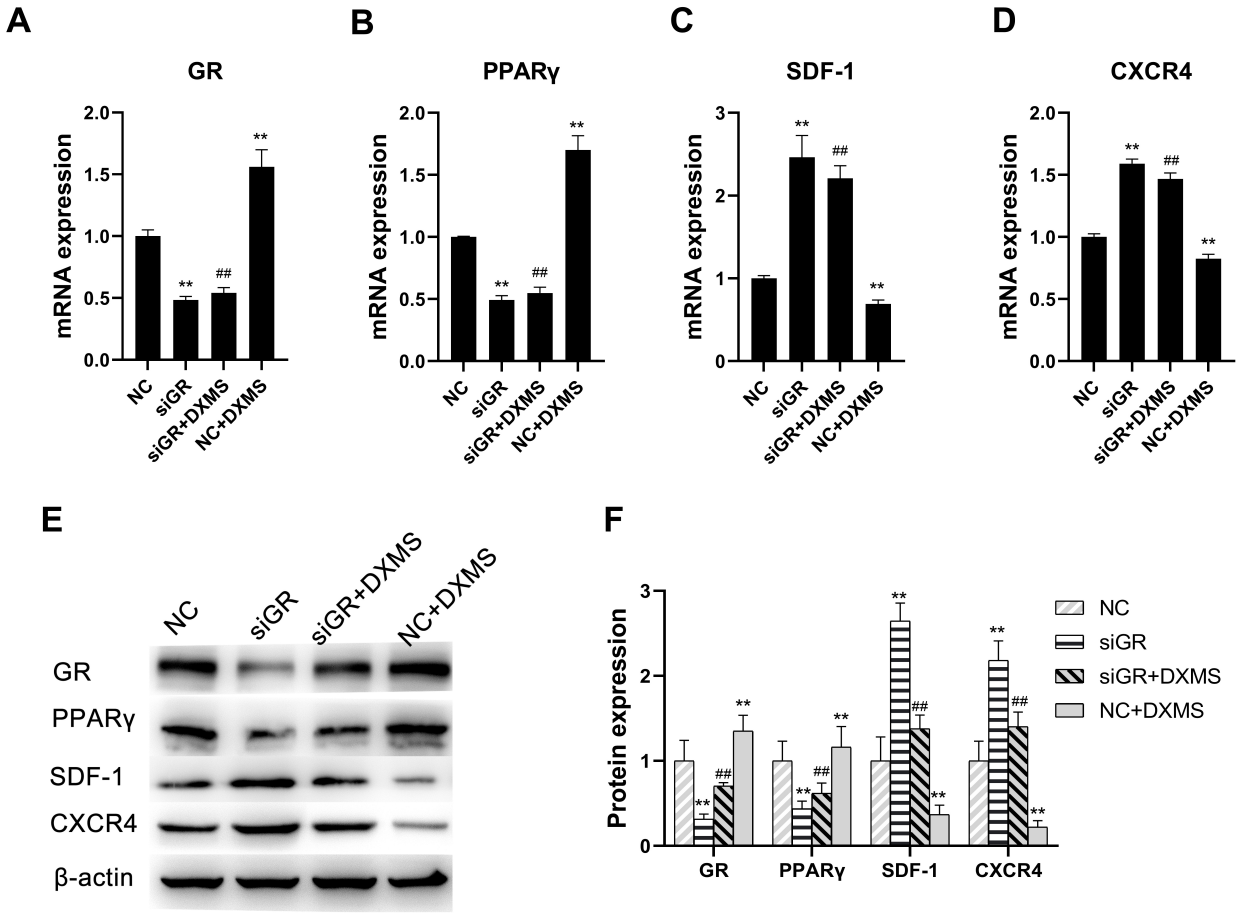

Fig. 3. DXMS regulates the expression of PPARγ, SDF-1, and CXCR4 *via* GR. (A–D) The effect of GR silencing on the expression of GR, PPARγ, SDF-1, and CXCR4 analyzed by RT-qPCR. (E,F) The effect of GR knockdown on the protein levels of GR, PPARγ, SDF-1, and CXCR4 analyzed by Western blot. Compared with the NC group, $^{**}p < 0.01$; compared with the NC+DXMS group, $^{\#\#}p < 0.01$.

PPARγ, SDF-1, and CXCR4. In Fig. 1F,G, the addition of RU486 significantly blocked the protein levels of GR and PPARγ but improved the protein levels of SDF-1 and CXCR4. The data showed that GR regulated the expression of PPARγ, SDF-1, and CXCR4, but did not affect the location of these proteins. Statistical analysis showed that the fluorescence signals of the two groups were significantly different, indicating a high siGR transfection efficiency (Fig. 1H).

DXMS Regulates the Expression of GR in Dosage- and Time-Dependent Manners

To investigate the impact of DXMS on GR, we treated cells with DXMS at various concentrations ranging from 0 to 200 μM for different durations. In Fig. 2A,C, we observed that DXMS increased the protein levels of GR at 0.1 μM in a dosage-dependent manner. Similarly, DXMS enhanced the protein levels of GR starting from 5 min, with the peak at 15 min (Fig. 2B,D). These findings demonstrate that DXMS enhances the expression of GR at the protein level in dosage- and time-dependent manners.

DXMS Regulates the Expression of PPARγ, SDF-1, and CXCR4 via GR

In Fig. 3A, compared with the NC group, the transfection of siGR significantly inhibited the expression of GR, and the addition of DXMS markedly induced the expression of GR. Similarly, DXMS increased the mRNA levels of PPARγ, and the silencing of *GR* blocked the upregulation of GR by DXMS (Fig. 3B). As shown in Fig. 3C,D, the addition of DXMS significantly inhibited the expression of SDF-1 and CXCR4. However, siRNA targeting *GR* transfection partly relieved the downregulation of SDF-1 and CXCR4 induced by DXMS. We further analyzed the protein levels of GR, PPARγ, SDF-1, and CXCR4. A similar trend was observed in Fig. 3E,F. These data indicated that DXMS modulated the mRNA and protein levels of PPARγ, SDF-1, and CXCR4 *via* GR.

PPARγ is a Downstream Target of GR

To further examine the relationship between PPARγ and GR, we knocked down PPARγ and assessed the expression of GR, SDF-1, and CXCR4. In Fig. 4A, we evalu-

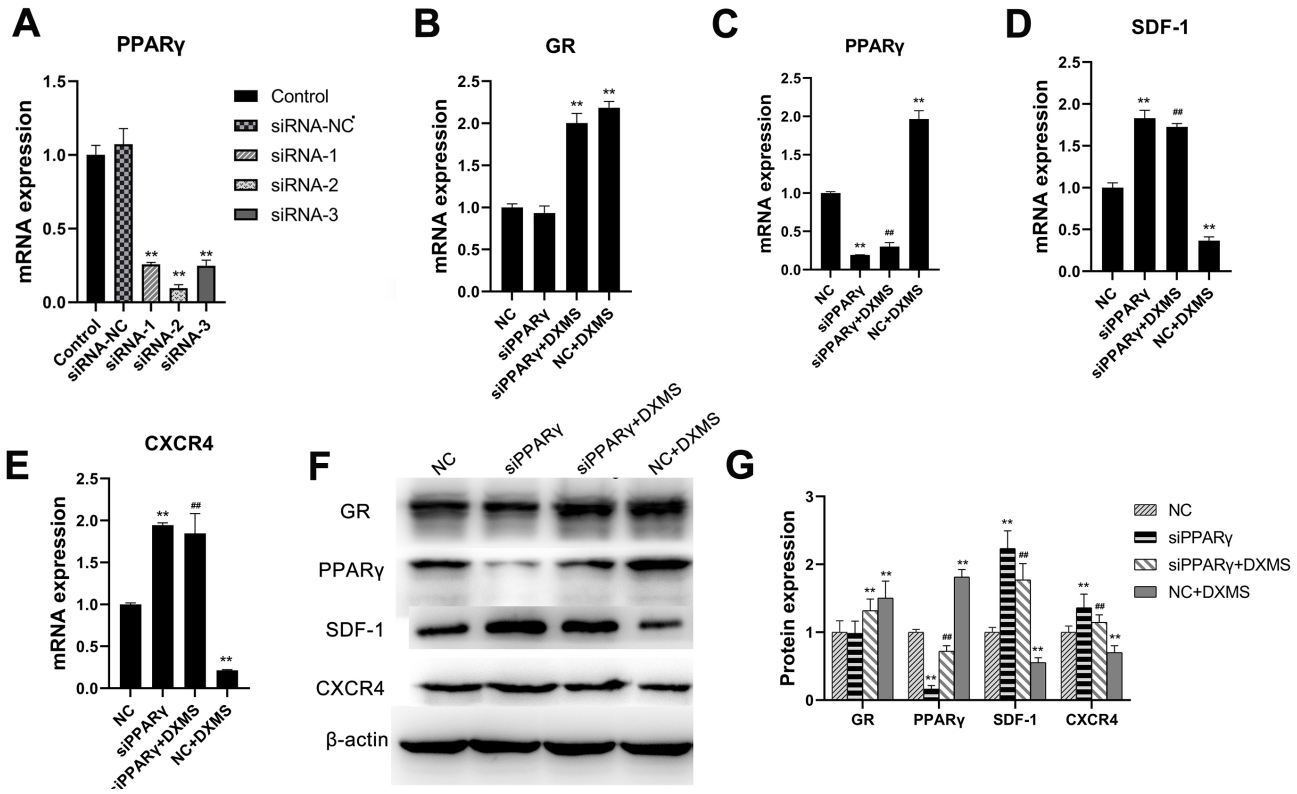

Fig. 4. PPARγ is downstream of GR. (A) The knockdown efficiency of PPARγ was detected by RT-qPCR. Compared with siRNA-NC, $^{**}p < 0.01$. (B–E) The effect of PPARγ silencing on the expression of GR, PPARγ, SDF-1, and CXCR4 analyzed by RT-qPCR. (F,G) The effect of PPARγ knockdown on the protein levels of GR, PPARγ, SDF-1, and CXCR4 was analyzed by Western blot. Compared with the NC group, $^{**}p < 0.01$; compared with the NC+DXMS group, $^{\#\#}p < 0.01$.

ated the knockdown efficiency of PPARγ siRNAs and observed that all siRNAs significantly inhibited the expression of PPARγ. In Fig. 4B,C, the silencing of PPARγ had no significant effect on the expression of GR but blocked the mRNA levels of PPARγ. As shown in Fig. 4D,E, the blockage of PPARγ significantly increased the expression of SDF-1 and CXCR4 under DXMS incubation. We further performed Western blotting to examine the protein changes. In Fig. 4F,G, the silencing of *PPARγ* decreased the protein levels of PPARγ and upregulated the levels of SDF-1 and CXCR4. DXMS addition upregulated GR and PPARγ proteins but blocked SDF-1 and CXCR4 proteins. However, the knockdown of PPARγ partially reversed the downregulation of SDF-1 and CXCR4 induced by DXMS. These findings indicate that PPARγ is downstream of GR.

DXMS Regulates PPARγ, SDF-1, and CXCR4 Partially through G Protein-Coupled Receptors

As shown in Fig. 5A, RT-qPCR showed that in the DXMS+Suramin group, there was a significant increase in the relative expression levels of PPARγ mRNA compared to the DXMS group ($p < 0.05$). Additionally, in Fig. 5B,C, the relative expression levels of SDF-1 and CXCR4 mRNA were significantly decreased in the same group ($p < 0.05$). As shown in Fig. 5D,E, the relative levels of PPARγ protein were significantly decreased in the DXMS+Suramin group compared to the DXMS group ($p < 0.05$), and the levels were higher in the DXMS+Suramin group than in the Suramin group ($p > 0.05$). Furthermore, the relative expression levels of SDF-1 and CXCR4 protein were significantly increased in the Suramin group compared to the DXMS group ($p < 0.05$) in Fig. 5D,E. These findings suggest that the G-protein-coupled receptor-tyrosine kinase family may be involved in the homing behavior of DXMS-regulated cells, but it is not the primary regulatory factor.

Discussion

When glucocorticoid-induced femoral head necrosis occurs, glucocorticoid-induced *PPARγ* gene expression is promoted, mainly PPARγ2 [2–4]. In this study, it was confirmed that the expressions of SDF-1 and CXCR4 mRNA and protein were downregulated with the increase of PPARγ expression, consistent with previous studies indicating that glucocorticoids increased PPARγ expression and decreased SDF-1 and CXCR4 expression on BMSCs [18,19].

IF results showed that, after siGR, downregulation of GR blocked PPARγ in the nucleus and cytoplasm, suggesting that *GC* regulates PPARγ in a GR-dependent manner.

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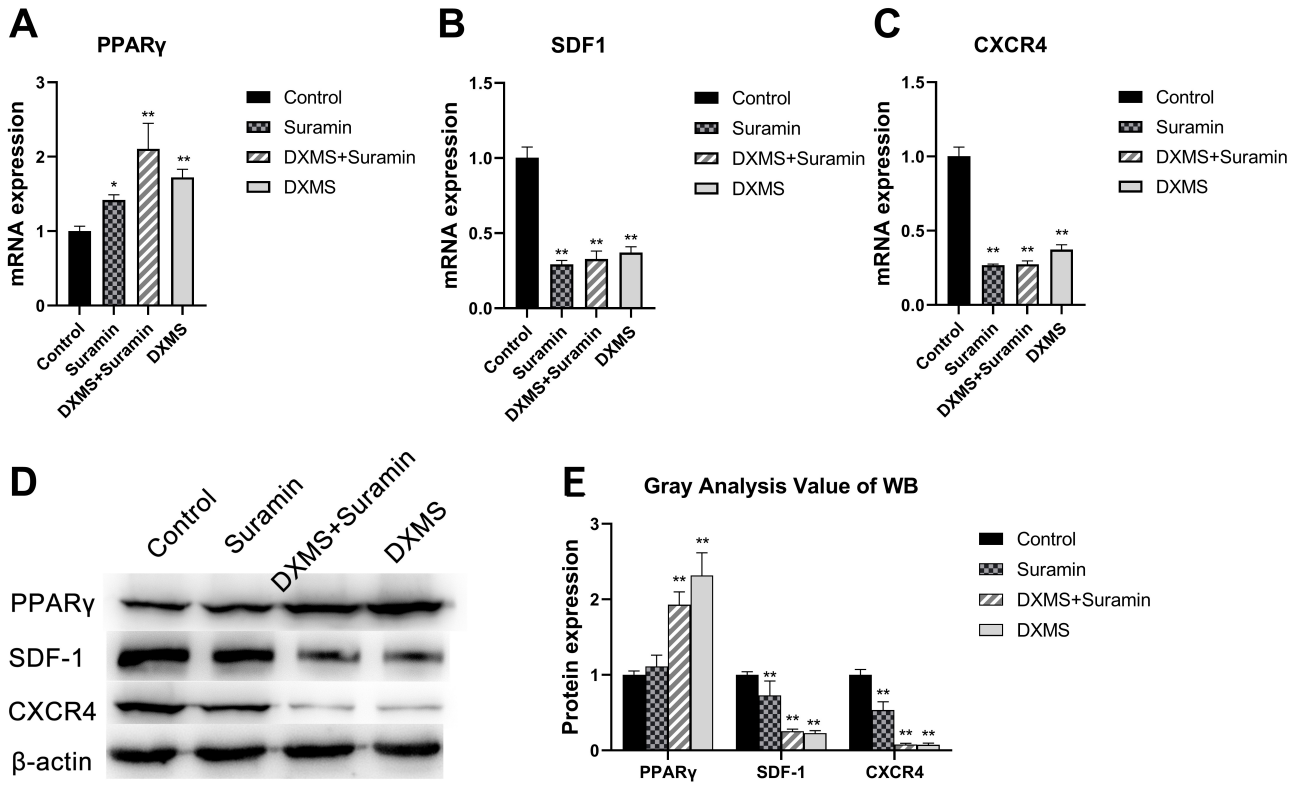

Fig. 5. DXMS regulates PPARγ, SDF-1, and CXCR4 partially through G protein-coupled receptors. (A–C) The effect of Suramin on the expression of PPARγ, SDF-1, and CXCR4 was analyzed by RT-qPCR. (D,E) The effect of Suramin on the expression of PPARγ, SDF-1, and CXCR4 was analyzed by Western blotting. Compared with the NC group, $*p < 0.05$, $**p < 0.01$.

Additionally, siGR increased the protein levels of SDF-1 and CXCR4. Protein localization showed that siGR did not significantly affect the localization of these molecules, indicating that GR may not be activated by cytokine receptors, secretory and exocrine pathways of cytokines, or subcellular structural changes. It is suggested that GR may affect the expression and localization of PPARγ through transcriptional regulation.

A previous study has demonstrated that glucocorticoids bind to glucocorticoid receptors in the nucleus and, by binding to nGRE, a negative regulatory element on DNA sequences [15]. Subsequently, after binding DNA, they initiate the recruitment of CBP/P300 and methyltransferase, which modify histones with their co-activating proteins, and recruit other regulatory proteins to bind to the promoter [24]. This process is driven by the ATPase-dependent core subunit BRGl. *In vitro* modifications to the histone tail include acetylation, methylation, ubiquitination, and other covalent modifications regulated by glucocorticoids [25]. The glucocorticoids then enter the nucleus to regulate *PPARγ* transcription and affect protein production. This study confirmed that the regulation of PPARγ by DXMS was blocked by the glucocorticoid classical receptor antagonist RU486, consistent with previous studies [12]. These results indicated that DXMS regulates the expression of PPARγ through GR.

However, it has also been demonstrated that GC plays a role by interacting with the nuclear transcription factor PPARγ and inhibiting its transcriptional activity [16, 17]. The *PPARγ* regulates the transcription of downstream related genes, affects the production of proteins, and blocks cell adhesion and migration mainly through a ligand-dependent transcriptional activation mechanism in the nucleus [18].

A recent study demonstrated the existence of high-affinity glucocorticoid G-protein-coupled receptors in cell membranes [13]. Glucocorticoids bind to these receptors to regulate various intracellular signal transduction pathways, including the cAMP-PKA pathway, PKC pathway, and Ca^{2+} pathway [14]. In this study, we confirmed that the inhibitory effect of glucocorticoids on SDF-1 and CXCR4 was only partially blocked by the G-protein-coupled receptor antagonist suramin, suggesting the involvement of the G-protein-coupled receptor family in the regulation of CXCR4 by DXMS, but the membrane receptor pathway was not the main regulatory mechanism of BMSCs homing.

In general, G protein-coupled receptor blockers have an inhibitory effect on PPARγ rather than upregulating it. After blocking this pathway, the expression and activity of PPARγ may return to normal levels or slightly increase. In this study, the increase of PPARγ in the DXMS+Suramin

group was observed compared with that in the DXMS group, but the specific mechanism was not clear. There may be unintended cross-reactivity, and G-protein-coupled receptor blockers may have non-specific effects on other intracellular pathways that affect the expression and activity of PPARγ. Additionally, there may be feedback mechanisms, where G-protein-coupled receptor blockers may trigger an instantaneous regulatory feedback loop that results in an adaptive cell response to PPARγ expression and activity, leading to temporary upregulation of PPARγ.

After the administration of G protein-coupled receptor blockers, the PPARγ protein of DXMS was higher than that of the DXMS+Suramin group. G protein-coupled receptor blockers can affect mRNA translation by regulating the activity of translation initiation factors. Specifically, they can interfere with the phosphorylation of eukaryotic initiation factor 2 alpha (eIF2α), thereby influencing the formation of transcription initiation complexes and mRNA translation. In addition, G protein-coupled receptor blockers may further regulate mRNA translation by affecting the mammalian target of the rapamycin signaling pathway and RNA-binding proteins. These mechanisms can affect protein synthesis within the cell, consequently affecting the cell's physiological function.

The mechanism by which GC-activated PPARγ prevents BMSCs from migration remains unclear. This study showed that after GC intervention in BMSCs, the expression of PPARγ was significantly increased, while the expression of SDF-1 and CXCR4 was significantly decreased. Silencing PPARγ led to a significant change in the downstream SDF-1/CXCR4, suggesting that PPARγ is involved in the homing behavior of cells. Additionally, the administration of DXMS reduced the change of SDF-1/CXCR4, indicating that the inhibition of PPARγ was not complete and still had a regulatory effect. Based on the regulation of SDF-1 and CXCR4 expression by DXMS in this study, it was inferred that *PPAR*γ plays a major direct role in regulating SDF-1 and CXCR4 expression in BMSCs cells.

After PPARγ expression was up-regulated by glucocorticoid, the downstream CXCR4 mRNA expression was inhibited and the expression of CXCR4 receptor on BMSCs was decreased [18,19]. It was deduced that GC binds to intracellular GRα to form a ligand-receptor complex, which then enters the nucleus, activates ligand-dependent PPARγ transcriptional activation mechanism, initiates gene transcription, and affects the production of SDF-1 and CXCR4 proteins. Similar to tumor cell metastasis, *PPAR*γ regulates the transcriptional activity of SDF-1 and CXCR4 and blocks the adhesion, proliferation, and migration of tumor cells [18]. Furthermore, it weakens the interaction between VEGF-A and homing factor SDF-1, inhibiting the effect of VEGF-A on the regulatory chain of SDF-1/CXCR4 during cell penetration [20,21], and hinders VEGF-mediated BMSCs migration in the vascular endothelial space [22].

In the early research on the treatment of glucocorticoid-induced avascular necrosis with BMSCs, the focus has been primarily on the proliferation of BMSCs and the enhancement of osteogenetic differentiation. There has been relatively less research on the homing of BMSCs to femoral head necrosis lesions, especially regarding the relationship between PPARγ and the homing factors SDF-1 and CXCR4, as well as the molecular mechanism of transcription regulation. This study is based on the background that glucocorticoids regulate the expression of SDF-1 and CXCR4 in BMSCs. We investigated the molecular mechanism of glucocorticoid regulation of SDF-1 and CXCR4 expression through GRα or PPARγ, focusing on the formation of protein complexes and transcriptional regulation. We also revealed the functional mechanism of the SDF-1/CXCR4 axis in BMSCs homing from a new perspective.

The migration assay is a common method used to study cell migration and homing ability. However, it has a shortcoming in detecting BMSC migration. The results of this study indicate that both BMSCs can express SDF-1 and CXCR4, and the migration behavior differs between *in vitro* and *in vivo* conditions. Therefore, the migration experiment may require a more complex experimental design to effectively demonstrate the migration effect, which will be the focus of our next investigation.

Conclusions

In conclusion, we found that (1) the glucocorticoid-dependent GR regulates PPARγ, (2) glucocorticoid-mediated SDF-1/CXCR4 signaling pathway through PPARγ, and (3) glucocorticoid-mediated homing of BMSCs is PPARγ-dependent. This study explored the regulation of GR and PPARγ on BMSCs homing and found that PPARγ affects BMSC migration through a genomic pathway. The results of this study will provide a new experimental basis for the early treatment of GNFH by BMSCs homing.

Availability of Data and Materials

All the data had been included in the main documents, and the original files could be obtained from the corresponding author upon reasonable request.

Author Contributions

LPW, TL and ZML designed and performed the research. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

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Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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