Paeoniflorin-6'O-benzene sulfonate suppresses fibroblast-like synoviocytes proliferation and migration in rheumatoid arthritis through regulating GRK2-Gβγ interaction

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Abstract. Rheumatoid arthritis (RA) is a chronic autoimmune disease. Enhanced G protein coupled receptor kinase 2 (GRK2) translocation and prostaglandin E4 receptor (EP4) desensitization play a critical role in fibroblast-like synoviocytes (FLS) dysfunction. Paeoniflorin-6'O-benzene sulfonate (CP-25) exerts a protective effect in arthritis in the RA animal models. To demonstrate the role of Gβγ in EP4 desensitization and the mechanisms of CP-25 that protects FLS in RA, RA-FLS and adjuvant-induced arthritis (AA-FLS) were isolated from synovium of RA patients and AA rats. RA-FLS, AA-FLS and MH7A were treated with CP-25, Gβγ agonist and antagonist. The cell membrane expression of EP4, GRK2, and Gβγ were detected using western blot analysis. Co-immunoprecipitation (Co-IP) and immunofluorescence were adopted to detect the interactions of GRK2-Gβγ, GRK2-EP4, and EP4-Gβγ. Cell Counting Kit-8 and Transwell assay were used to analyze the proliferation and migration of the FLS. An increased membrane expression of GRK2 and Gβγ, enhanced GRK2-Gβγ interaction and decreased EP4 membrane expression in the RA synovial tissue were identified. *In vitro*, prostaglandin E2 (PGE_2) enhanced the proliferation and migration of FLS. CP-25 exhibited an inhibition effect similar to Gβγ inhibitor, which downregulated GRK2-EP4 interaction, blocked the translocation of GRK2, and reversed EP4 desensitization, leading to the suppression of the proliferation and migration induced by PGE_2. These results elucidated that an enhanced GRK2-Gβγ interaction was involved in the EP4 desensitization and dysfunction. CP-25 regulated EP4-GRK2-Gβγ signaling and re-sensitized EP4 by inhibiting GRK2-Gβγ interaction. The regulation of EP4-Gβγ-GRK2 signaling may be a novel potential therapeutic target in RA.

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Introduction

Rheumatoid arthritis (RA) is a chronic, autoimmune, inflammatory disease. The pathological hallmarks of synovium in RA are synovial hyperplasia, pannus formation, cartilage damage and bone erosion (1). Abnormal expansion of fibroblast-like synoviocytes (FLS) leads to synovial hyperplasia, which is the leading pathophysiology of RA. Making up two thirds of the hyperplastic synovial membrane, FLS secrete several adhesion molecules and inflammatory cytokines, and this also leads to bone erosion and cartilage damage. Migration of FLS to unaffected cartilage drives the progression of polyarthritis in RA (2). It has been reported that there is a large amount of prostaglandin E2 (PGE_2) in the synovial fluid of RA patients that triggers anomalous proliferation and migration of FLS (3).

Guanine nucleotide binding protein, also known as the G protein, is a ubiquitous signal molecule that plays an important role in transmembrane signal transduction. G protein couples with the corresponding G protein-coupled receptor (GPCR). The binding of ligand to GPCR induces the dissociation of G protein into Gα and Gβγ dimers. Gβγ subunit plays multifunctional roles, such as anchoring the G protein to the cell membrane and conducting signal transduction. GPCRs and their effector molecules bind G protein subunits before targeting to the cell surface. Interacting with various effectors, including G protein-coupled receptor kinase 2 (GRK2), adenylyl cyclase II, and PI-3 kinase (4), Gβγ is important in recruiting GRKs to activated receptors and regulating cellular functions.

GRK2 is associated with GPCR desensitization. PGE_2 binds to prostaglandin receptors (EPs). In the physiologic state, EP4 primarily couples to the Gαs subunit to mediate increases in the cAMP concentrations. In a pathological environment, EP4 perceives continuous stimulation from abnormal levels of inflammatory cytokines such as PGE_2, causing an overactive

inflammatory response. Previous studies have shown that in RA animal models, the PGE_2-EP4-GRK2 signaling pathway plays an important role in the occurrence and development of arthritis. PGE_2 binds to the EP4 receptor, inducing excessive GRK2 translocation to the cell membrane and EP4 desensitization, thereby promoting downregulation of cAMP, FLS dysfunction and synovium hyperplasia with the mechanism of decreased protective Gαs signaling (5,6).

Paeoniflorin-6'O-benzene sulfonate (CP-25; patent number in China: ZL201210030616.4) is a structurally modified compound from paeoniflorin with anti-inflammatory and immunomodulatory effects in RA animal models and other immune diseases (7-10). It was found that CP-25 ameliorated FLS abnormal proliferation and migration by inhibiting GRK2 translocation (11) and re-sensitizing EP4 (6), which was an important mechanism of CP-25 in improving FLS dysfunction.

The GPCR-Gβγ-GRK complex is crucial for the regulation of receptor function and GRK catalysis, and this has been studied in adrenergic and opioid receptors, but has not been reported in EP4 receptors. GRK2 binds both delta opioid receptor and Gβγ to form a receptor-GRK2-Gβγ complex, which is required for GRK-mediated receptor phosphorylation and desensitization (12). In heart failure animal model, GRK2 translocates to β-adrenergic receptor after binding with Gβγ to phosphorylate the agonist-occupied receptor, thus cardiac contractile function collapses as β-adrenergic receptor signaling is attenuated (13). It remains unclear whether Gβγ is involved in PGE_2-EP4-GRK2 signaling. Hence, in the present study, the role of Gβγ and the GRK2-Gβγ interaction in regulating FLS proliferation and migration was mainly explored. In addition, the specific mechanism of CP-25 in restoring dysfunction of EP4 was investigated.

Materials and methods

Sample collection. RA synovial tissue samples were obtained from six patients (4 females, 2 males; age range, 52-73 years) with RA undergoing surgical synovectomy and knee replacement. Trauma synovium samples were obtained from six patients (3 females, 3 males; age range, 28-50 years) with traumatic synovitis undergoing arthroscopic synovectomy. The sample collection period was from December 2017 to December 2018. Patients had been diagnosed with RA at least three years prior to surgery and were treated using the standard of care (non-steroidal anti-inflammatory drugs and disease-modifying anti-rheumatic drugs DMARDs). Informed consent was obtained from all patients. The study protocol was approved (approval no. 20131321) by the Biomedical Ethics Committee of Anhui Medical University (Hefei, China).

Animals. A total of 12 male Sprague Dawley rats (body weight, 180-200 g) were obtained from SPF Animal Laboratory of Anhui Medical University (Hefei, China). All rats were housed in pathogen-free animal houses at 20-26˚C under a 12-h light/dark cycle and had free access to sterile water and food. All experiments were conducted in accordance with the principles of the laboratory animal care guidelines and were approved (approval no. 20131321) by Biomedical Ethics Committee of Anhui Medical University (Hefei, China).

Drugs and reagents. PGE_2 was purchased from Cayman Chemical Company. Gallein was purchased from Santa Cruz Biotechnology, Inc. mSIRK was purchased from Sigma-Aldrich; Merck KGaA. GRK2i was purchased from Tocris Bioscience. The Cell Counting Kit-8 (CCK-8) kit was purchased from Beyotime Institute of Biotechnology. The Transwell inserts (8-μm) were purchased from Corning, Inc. CP-25 (purity >98%) was provided by the Institute of Clinical Pharmacology, Anhui Medical University (Hefei, China). Gβ1 siRNA(h) and the control siRNA(h) were purchased from Santa Cruz Biotechnology, Inc. Lipofectamine® 2000 and fetal bovine serum (FBS) were purchased from Invitrogen; Thermo Fisher Scientific, Inc. Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Hyclone; Cytiva.

Induction of adjuvant-induced arthritis (AA). There were six rats in model group, and the other six rats were in control group. The method of AA rat preparation was consistent with that described in a previous study (5). A total of 0.1 ml of complete Freund's adjuvant (CFA) emulsion was injected into the right hind metatarsal footpad. The rats were assessed every three days for the signs of arthritis by two independent observers. If tissue necrosis or ulcerated masses were present (no signs of healing within 3 days) and signs of deterioration such as inability to reach food or water for ~12 h, weight loss >20% of starting weight or any sign of suffering not relieved by pharmacologic analgesia, the rat would be euthanized. The rats in model group were sacrificed for the AA-FLS at day 30 after immunization under anesthesia using isoflurane (induction 3-4%; maintenance 1-2%). Subsequently, all the rats were euthanized via the inhalation of carbon dioxide. The displacement of the euthanasia chamber was between 30-70% of the chamber volume per min. Death would be confirmed by an appropriate method, such as ascertaining cardiac and respiratory arrest or noting fixed and dilated pupils of an animal.

Cell culture and identification. The RA-FLS and AA-FLS were obtained from the knee synovium of the RA patients and AA rats. Human rheumatoid FLS (MH7A) were purchased from Jennio Biotech Co., Ltd. All cells were cultured in DMEM containing 20% FBS, 1% penicillin and 1% streptomycin at 37˚C and 5%-CO_2 containing incubator. The RA-FLS and AA-FLS were cultured using Anti-Vimentin (cat. no. sc-373717; Santa Cruz Biotechnology, Inc.) and Anti-CD55 (cat. no. 26580-1-AP; Proteintech) at a dilution ratio of 1:1,000 for 30 min and then detected using immunofluorescence. Mycoplasma testing was carried out for the cell lines used.

Histological analysis. Synovial tissue was fixed with 4% paraformaldehyde solution at room temperature for 24 h and embedded in paraffin. The paraffin sections (4 μm) were dewaxed in xylene, stained with hematoxylin and eosin (H&E) at room temperature for 3 min and 30 sec, respectively, and were examined using standard light microscopy.

Immunofluorescence assay. The synovial tissue samples were snap-frozen in OCT medium. Cryostat sections (5 μm) were cut and mounted on adhesive glass slides. Slides of the tissue

samples and cells were fixed with 4% paraformaldehyde at 4°C for 10 min, permeabilized with 0.5% Triton X-100 for 7 min at room temperature and blocked with 5% bovine serum albumin (MilliporeSigma) for 1 h at 37°C. After incubating with primary antibodies (1:100 dilution) at 4°C overnight, the slides were incubated with anti-mouse-Alexa Fluor 594 (1:200 dilution; cat. no. ab150116; Abcam) and anti-rabbit-Alexa Fluor 488 (1:200 dilution; cat. no. ab150073; Abcam) for 1 h at 37°C. DAPI (20 μl) was used to stain the nuclei for 5 min at room temperature. The slides were then observed under a fluorescence microscope (BX53; Olympus Corporation). The primary antibodies were as follows: Gβ (cat. no. sc-166123), EP4 (1:1,000 dilution; cat. no. sc-55596; both from Santa Cruz Biotechnology, Inc.) and GRK2 (cat. no. ab228705; Abcam).

Isolation of membranes from synovial tissue. The synovial tissue was washed using PBS, cut into pieces and added with a cold homogenization buffer (0.32 M sucrose, 5 mM Tris-HCl, pH 7.5, 120 mM KCl, 1 mM EDTA and 0.2 mM PMSF). Samples were sonicated and centrifuged at 100,000 x g for 1 h at 4°C. The supernatants were pooled and centrifuged at 100,000 x g for 1 h. The pellets were resuspended in a cold extraction buffer (20 mM HEPES, pH 7.5, 10% glycerol, 2% Triton X-100, 1 mM EDTA, 1 mM EGTA and 0.2 mM PMSF) and incubated at 4°C overnight. The supernatants containing the cell membrane were collected after centrifuging at 7,800 x g for 30 min at 4°C.

Isolation of proteins from the cell cytoplasm and the membrane. RA-FLS, AA-FLS and MH7A cells were lysed with lysis buffer (cat. no. P0013; Beyotime Institute of Biotechnology) and centrifuged at 12,000 x g for 10 min at 4°C. The supernatants were centrifuged at 100,000 x g for 1 h at 4°C. The precipitate was kept as the membrane fraction, while the supernatant was collected as the cytoplasm fraction.

Cell proliferation assay. Cell proliferation was examined using a CCK-8 assay. RA-FLS, AA-FLA and MH7A cells were counted and seeded on to 96-well plates at a concentration of $5x10^6$/ml and incubated in DMEM at 37°C for 48 h. The cells were then treated with PGE_2 (2 μm) with or without different agonists or antagonists and CP-25 (10^{-7}, 10^{-6} and 10^{-5} mol/l) for 24 h. After treatment, the cells were added to a 10-μl CCK-8 solution for each well. The plate was incubated for 1 h at 37°C. The optical density values of the plate were measured at 490 nm on a Tecan Infinite M200 Microplate Reader (Tecan Group, Ltd).

Cell migration assay. Cells were seeded into the upper wells of the Transwell chambers at a concentration of $1x10^5$/ml and maintained in a medium containing 5% FBS. The lower chamber was filled with DMEM containing 20% FBS. After 24 h, the upper chamber was washed with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature and stained with 0.1% crystal violet for 15 min at room temperature. All images were captured using a light microscope. Cells were counted from at least four random microscopic fields for each sample in three independent experiments.

Small interfering RNA (siRNA). The Gβ1 siRNA (cat. no. sc-41762) and negative control siRNA (cat. no. sc-37007) were used to knockdown Gβ1 in MH7A cells. The MH7A were transfected with 50 nM siRNA at 70% confluency in 24-well plates using Lipofectamine 2000 at room temperature according to the manufacturer's protocol. After 48 h, silencing of Gβ1 was confirmed using western blot analysis.

Western blot analysis. Protein samples were collected and boiled in a loading buffer for 10 min at 100°C. The protein concentration was detected using a BCA protein assay kit (Thermo Fisher Scientific, Inc.). Equal amounts of protein (20 μg) were electrophoretically separated using 10% SDS-PAGE and transferred to a PVDF membrane (MilliporeSigma). The PVDF membrane was then incubated in blocking solution (cat. no. P0205; Beyotime Institute of Biotechnology) at room temperature for 1 h. Subsequently, the membrane was incubated with primary antibodies at 4°C overnight, followed by the horseradish peroxidase-conjugated goat anti-mouse antibody (cat. no. STAR207P;) or goat anti-rabbit antibody (cat. no. STAR208P; both 1:10,000 dilution; Bio-Rad Laboratories, Inc.) at room temperature for 2 h. The bands were visualized using chemiluminescence detection reagents (MilliporeSigma) according to the manufacturer's protocol. The following primary antibodies were used: Gβ (1:1,000; cat. no. sc-166123), Gβ1 (1:1,000; cat. no. sc-515764; both from Santa Cruz Biotechnology, Inc.), ATP1A1 (1:3,000; cat. no. 14418-1-AP; ProteinTech Group, Inc.), GRK2 (1:1,000; cat. no. sc-13143; Santa Cruz Biotechnology, Inc.), GRK2 (1:1,000; cat. no. ab228705; Abcam), EP4 (1:1,000; cat. no. sc-55596), β-arrestin2 (1:1,000; cat. no. sc-13140; both from Santa Cruz Biotechnology, Inc.), β-actin (1:5,000; cat. no. 60008-1-Ig) and GAPDH (1:5,000; cat. no. 60004-1-Ig; both from ProteinTech Group, Inc.). The protein bands were visualized using an enhanced chemiluminescence detection system and quantified using ImageJ software (version 1.46; National Institutes of Health).

Coimmunoprecipitation (Co-IP) assay. To examine the association between GRK2 and Gβ in the synovial tissue or in FLS, the tissue or cell lysate was immunoprecipitated using an anti-GRK2, anti-EP4 or anti-Gβ antibody and analyzed using western blot analysis. Briefly, the sample was precleared for 2 h at 4°C with 20 μl of protein A + G agarose beads and subsequently incubated with 4 μl primary antibody for 2 h at 4°C. Then the precleared lysates were associated with 20 μl of protein A + G agarose beads and the primary antibody and incubated overnight at 4°C on the rotation shaker. Tubes were then centrifuged at 14,000 x g for 5 sec at 4°C. The pellets were washed with lysis buffer for 10 min. The resin-bound immune complexes were boiled and the following steps were according to the western blot protocol.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from synovial tissue using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), which was then used to synthesize cDNA by RT using the PrimeScript™ RT Master Mix Kit (TaKaRa Bio, Inc.) in a reaction volume of 20 μl according to the manufacturer's protocol. Real-time qPCR assays were performed with an Applied Biosystems 7500 fast real-time PCR system with TB Green Premix Ex Taq II (TaKaRa Bio, Inc.). The conditions of amplification were as

follows: Initial denaturation at 95˚C for 30 sec, followed by 40 cycles of annealing and extension at 95˚C for 3 sec and 60˚C for 30 sec. The oligonucleotide primer sequences were as follows: GNB1 forward, 5'-TCGTCTCTGGTGCTTGTG ATGC-3' and reverse, 5'-GTCGTCTGAGCCAGTGGCAAA T-3'; and GNB2 forward, 5'-TGTTGCCGCTTCCTGGAT GACA-3' and GNB2 reverse, 5'-CACTGTGTCCAGCAA AACCCAC-3'; GAPDH forward, 5'-GGAGCGAGATCCCTC CAAAAT-3' and reverse, 5'-GGCTGTTGTCATACTTCT CATGG-3'. GAPDH was used as an internal reference and the $2^{-\Delta\Delta Cq}$ method was utilized to calculate the relative expression levels (14).

Statistical analysis. Data were analyzed using the GraphPad Prism v7 statistical software (GraphPad Software, Inc.). Values are expressed as the mean ± standard deviation. An Unpaired Student's t-test was used to analyze the data between two independent groups. A one-way analysis of variance followed by Bonferroni's post hoc test was used to analyze the data from multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

The GRK2-Gβγ interaction is overactivated in the synovium tissue of patients with RA. It was previously revealed that PGE_2 levels were increased in the RA synovium and AA rats. It was also found that after PGE_2 stimulation, the membrane expression of GRK2 was increased in AA-FLS, which was related to FLS dysfunction (5). In an attempt to identify whether Gβγ was involved in the GRK2 translocation to the membrane, the expression of Gβ and the interaction between GRK2 and Gβ in the synovial tissues were evaluated using western blot analysis, Co-IP and double-stained immunofluorescence. The pathological feature of the synovial tissue in patients with RA was first assessed using H&E staining and expression of relevant proteins was detected. Compared with the trauma patients, the RA patients had synovial hyperplasia and inflammatory cell infiltration (Fig. 1A). The RA group showed a significantly increased expression of GRK2 (Fig. S1). In the RA group, membrane expression of GRK2 and Gβ was increased, whereas EP4 was decreased (Fig. 1B). The aforementioned results indicated GRK2 and Gβ translocation to the membrane and EP4 desensitization in the RA synovium. The results of the Co-IP and immunofluorescence demonstrated that GRK2-Gβ, EP4-Gβ and GRK2-EP4 interactions were enhanced in the RA group compared with those in the trauma group (Fig. 1C-F). These results suggested that Gβγ was involved in GRK2 translocation and EP4 desensitization in RA.

CP-25 inhibits proliferation and migration of RA-FLS and AA-FLS by downregulating the GRK2-Gβγ interaction. Since FLS is the major component of hyperplastic pannus in the RA synovium, the primary synoviocytes from RA patients and AA rats were isolated and identified as cells positive for labeling with Vimentin and CD55 by immunofluorescence (Fig. S2). Before the experimental endpoint, none of the rats succumbed. After the stimulation of PGE_2 (2 μM) for 24 h, the proliferation and migration in both RA-FLS and AA-FLS was enhanced. CP-25 (10^{-6} and 10^{-5} mol/l) significantly inhibited proliferation and migration induced by PGE_2 in both primary cells. Equivalent effects were observed in the Gβγ inhibitor and the gallein (10 μM) group (Fig. 2A and B), which indicated that inhibition of both Gβγ and CP-25 may decrease the abnormal FLS proliferation migration in the RA condition. To identify whether Gβγ subunits were involved in the protection of CP-25, the membrane expression of Gβ and its interaction with GRK2 were examined. It was revealed that membrane expression of GRK2 and Gβ was increased, whereas EP4 was decreased in the PGE_2 group. CP-25 (10^{-6} mol/l) restored membrane expression of GRK2, Gβ and EP4 (Fig. 2C). The Co-IP assay showed enhanced EP4-Gβ, GRK2-Gβ, and GRK2-EP4 interactions in the PGE_2 group. CP-25 inhibited translocation of GRK2 and Gβ, and reduced the binding of GRK2 and Gβ (Fig. 2D). These results suggested that CP-25 inhibited proliferation and migration of RA-FLS and AA-FLS by downregulating GRK2-Gβγ interaction.

CP-25 inhibits EP4-GRK2-Gβγ signaling and promotes Gβγ-dependent EP4 re-sensitization in MH7A cells. In the next few experiments, drugs that interfered with Gβγ in the immortal human synovial fibroblast cell line MH7A were used to investigate whether Gβγ controlled the interaction between GRK2 and EP4 and the role of CP-25.

First, excessive proliferation and migration induced by PGE_2 was observed in MH7A cells (Fig. 3A and B). GRK2i, gallein and CP-25 reversed the abnormalities. The results were similar to that in the RA-FLS and the AA-FLS. The proliferation and migration rate were further enhanced in the mSIRK (Gβγ agonist) group compared with the PGE_2 group. PGE_2 upregulated the membrane expression of GRK2 and downregulated the membrane expression of EP4. β-arrestin2, related to GPCR desensitization, increased in the PGE_2 group. Gβγ inhibitor, GRK2i, and gallein decreased the membrane expression of GRK2 and β-arrestin2 and restored the membrane expression of EP4. Membrane GRK2 was significantly increased in the mSIRK group, whereas β-arrestin2 and EP4 remained unchanged compared with the PGE_2 group (Fig. 3C). Subsequently, it was identified that the GRK2-EP4, EP4-Gβ, GRK2-Gβ and EP4-β-arrestin2 interactions were enhanced in the PGE_2 group, while they were inhibited in the gallein group (Fig. 3E and G). Compared with the PGE_2 group, GRK2i decreased the combination of GRK2 and EP4, while mSIRK enhanced the GRK2-EP4 interaction (Fig. 3D and F). CP-25 suppressed the GRK2-Gβ interaction (Fig. 3E and G), decreased membrane expression of GRK2 and β-arrestin2 (Fig. 3C) and blocked EP4 binding with GRK2 or β-arrestin2 (Fig. 3E), and thus restored membrane EP4 expression (Fig. 3C). These results demonstrated that CP-25 inhibited PGE_2-induced EP4-GRK2-Gβγ signaling activation in MH7A. Exogenous stimulation of PGE_2 overactivated EP4-GRK2-Gβγ signaling and promoted EP4 desensitization and dysfunction. CP-25 abrogated the binding of GRK2 with Gβγ and caused Gβγ-dependent re-sensitization of EP4, thus inhibiting abnormal proliferation and migration in the RA condition. It is worthy to note that the total expression of Gβ nearly remained unchanged between the control, PGE_2, CP-25 and gallein groups (Fig. S3).

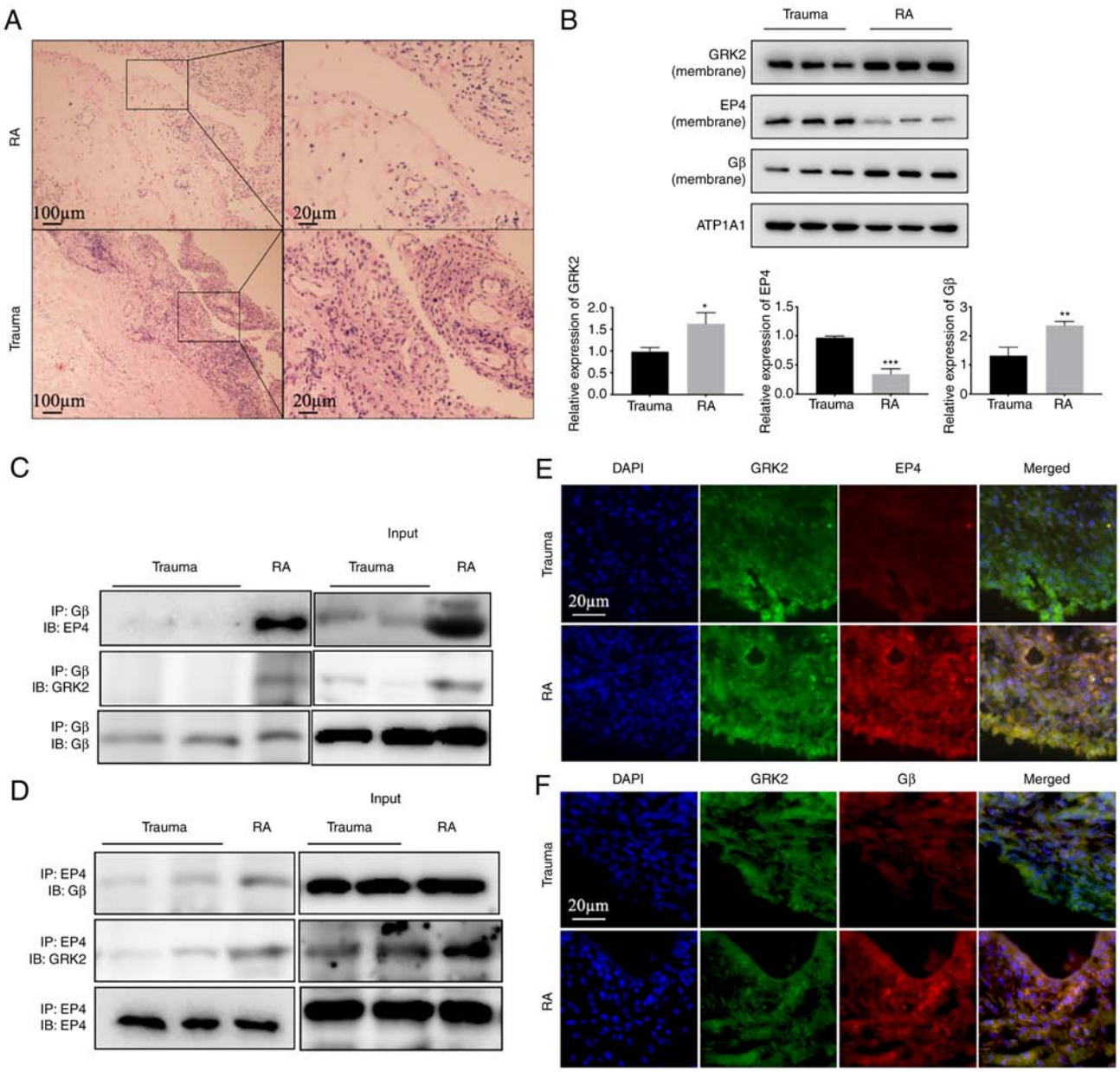

Figure 1. Enhanced GRK2-Gβγ interaction in the synovial tissue of patients with RA. (A) H&E-stained histological sections. Scale bars=100 and 20 μm. (B) Membrane protein expression of GRK2, EP4 and Gβ detected using western blotting. (C and D) Interactions of (C) GRK2-Gβ, EP4-Gβ and (D) GRK2-EP4 by Co-immunoprecipitation. (E and F) The co-localization of GRK2-EP4 and GRK2-Gβ in frozen section of synovial tissue of patients with RA was detected using immunofluorescence. Scale bars, 20 μm. *P<0.05, **P<0.01 and ***P<0.001 vs. trauma group (n=6). GRK2, G protein coupled receptor kinase 2; RA, rheumatoid arthritis; EP4, prostaglandin E4 receptor.

Gβγ knockdown inhibits the PGE_2-induced proliferation and migration by possibly interfering with the GRK2-EP4 interaction. The mRNA expression of Gβ1 and Gβ2 in synovial tissue of patients with trauma and RA was compared. It was observed that the expression of Gβ1 mRNA was higher in the synovial tissue of patients with RA (Fig. S4). To further confirm the functions of Gβγ in MH7A cells, the Gβ1 siRNA was transfected to knock down the endogenous Gβ1. It was found that the knockdown of Gβ1 significantly decreased PGE_2-induced proliferation (Fig. 4A) and migration (Fig. 4B). In untransfected cells, there was increased total expression of GRK2 (Fig. 4C), decreased membrane expression of EP4 (Fig. 4D) and enhanced GRK2-EP4 interaction after the PGE_2 induction (Fig. 4E). These results suggested that Gβγ knockdown inhibits PGE_2-induced proliferation and migration of MH7A cells, possibly by interfering with the GRK2-EP4 interaction. Collectively, Gβγ was involved in the anomalous proliferation and migration by hindering the interaction between GRK2 and EP4.

Discussion

FLS is a prominent component of the hyperplastic synovium. Inflammatory environments induce FLS to migrate, and this results in bone damage and cartilage erosion. Compared with normal synovial tissue, there are high expression levels of GRK2 in patients with RA and in RA animal models (6,7). Accordingly, GRK2 upregulation and translocation to the cell

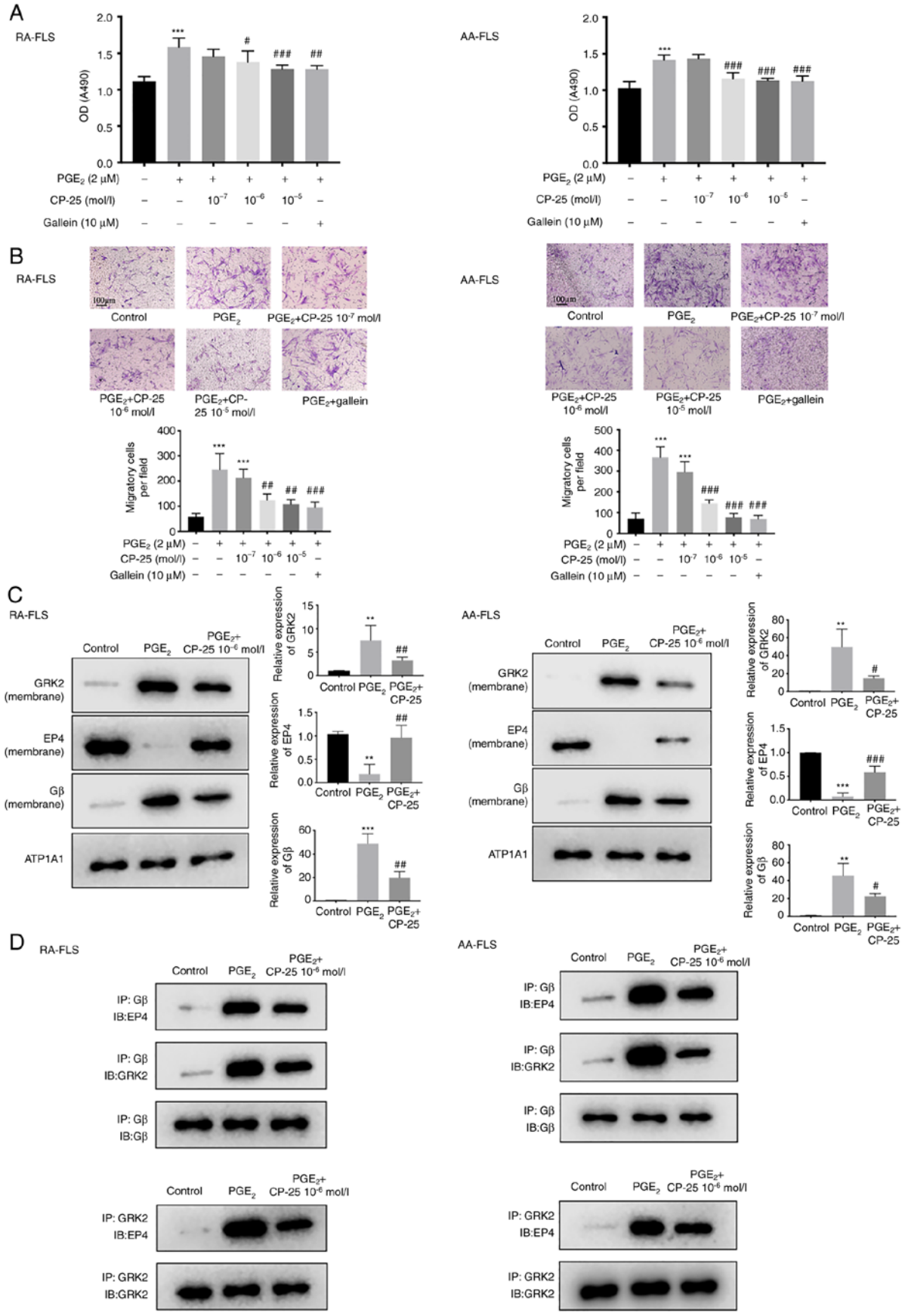

Figure 2. CP-25 inhibits proliferation and migration of RA-FLS and AA-FLS by downregulating GRK2-Gβγ interaction. (A and B) Inhibition of abnormal (A) proliferation and (B) migration of RA-FLS and AA-FLS by CP-25 and gallein was detected using Cell Counting Kit-8 and Transwell assays. (C) Membrane expression of GRK2, EP4 and Gβ in RA-FLS and AA-FLS was evaluated by western blotting. (D) Combination of EP4-Gβ, GRK2-Gβ, and GRK2-EP4 in RA-FLS and AA-FLS using Co-immunoprecipitation. Scale bars, 100 μm. **P<0.01 and ***P<0.001 vs. control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. PGE_2 group (n=6). RA, rheumatoid arthritis; AA, adjuvant-induced arthritis; FLS, fibroblast-like synoviocytes; GRK2, G protein coupled receptor kinase 2; EP4, prostaglandin E4 receptor; PGE_2, prostaglandin E2.

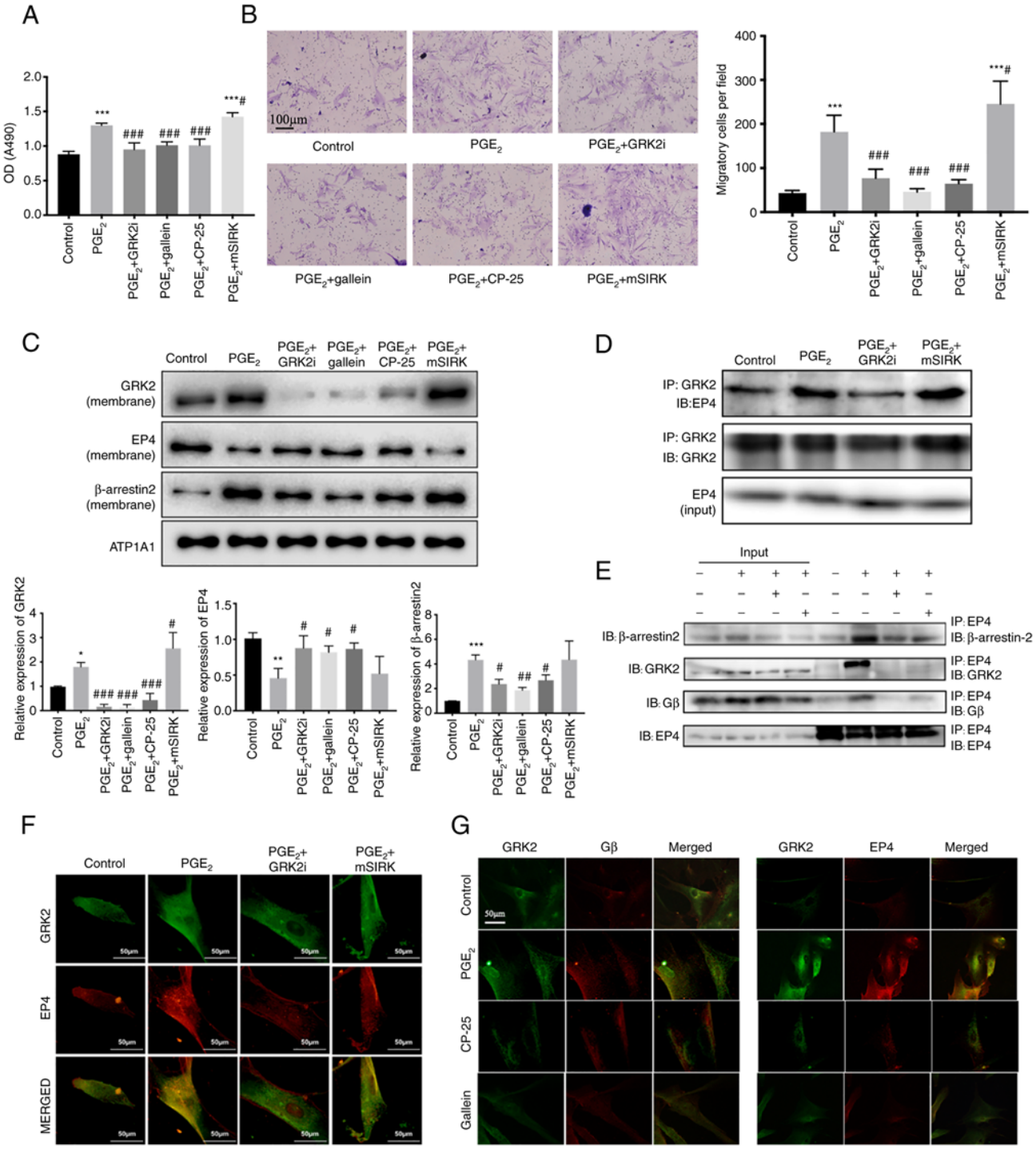

Figure 3. Gβγ on proliferation and migration of MH7A cells and the involvement of EP4-GRK2-Gβγ signaling. MH7A cells were treated with Gβγ antagonist (5 μM GRK2i and 10 μM gallein), Gβγ agonist (1 μM mSIRK), CP-25 (10^{-6} mol/l) and stimulated by PGE_2 (2 μM). (A) Cell proliferation was detected using Cell Counting Kit-8. (B) Cell migration was detected using Transwell assay. Scale bars, 100 μm. (C) Membrane expression of GKR2, EP4 and Gβ in PGE_2-stimulated MH7A cells was detected using western blotting. (D) Co-expression of GRK2 and EP4 with GRK2i and mSIRK treatment detected by Co-IP. (E) Co-expression of EP4-β-arrestin2, EP4-GRK2 and EP4-Gβ with CP-25 and gallein treatment was detected by Co-IP. (F) Co-localization of GRK2 and EP4 with GRK2i and mSIRK treatment was evaluated by immunofluorescence. Scale bars, 50 μm. (G) Co-localization of GRK2 and Gβ, EP4 and GRK2 in PGE_2 stimulated MH7A cells was evaluated by immunofluorescence. Scale bars=50 μm. *P<0.05, **P<0.01 and ***P<0.001 vs. control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. PGE_2 group (n=5). EP4, prostaglandin E4 receptor; GRK2, G protein coupled receptor kinase 2; PGE_2, prostaglandin E2; Co-IP, Co-immunoprecipitation.

membrane play a role in the mediation of the pathological progression of RA. In the present study, the protein expression of EP4-GRK2-Gβγ signaling was detected using western blot analysis and protein-protein interaction was evaluated using Co-IP and immunofluorescence. Increased GRK2 membrane expression and enhanced interactions of GRK2-Gβ, EP4-Gβ, and GRK2-EP4 were revealed in the collected synovial tissue of patients with RA.

To mimic the chronic inflammatory environment of RA, RA-FLS, AA-FLS and MH7A cells were stimulated with

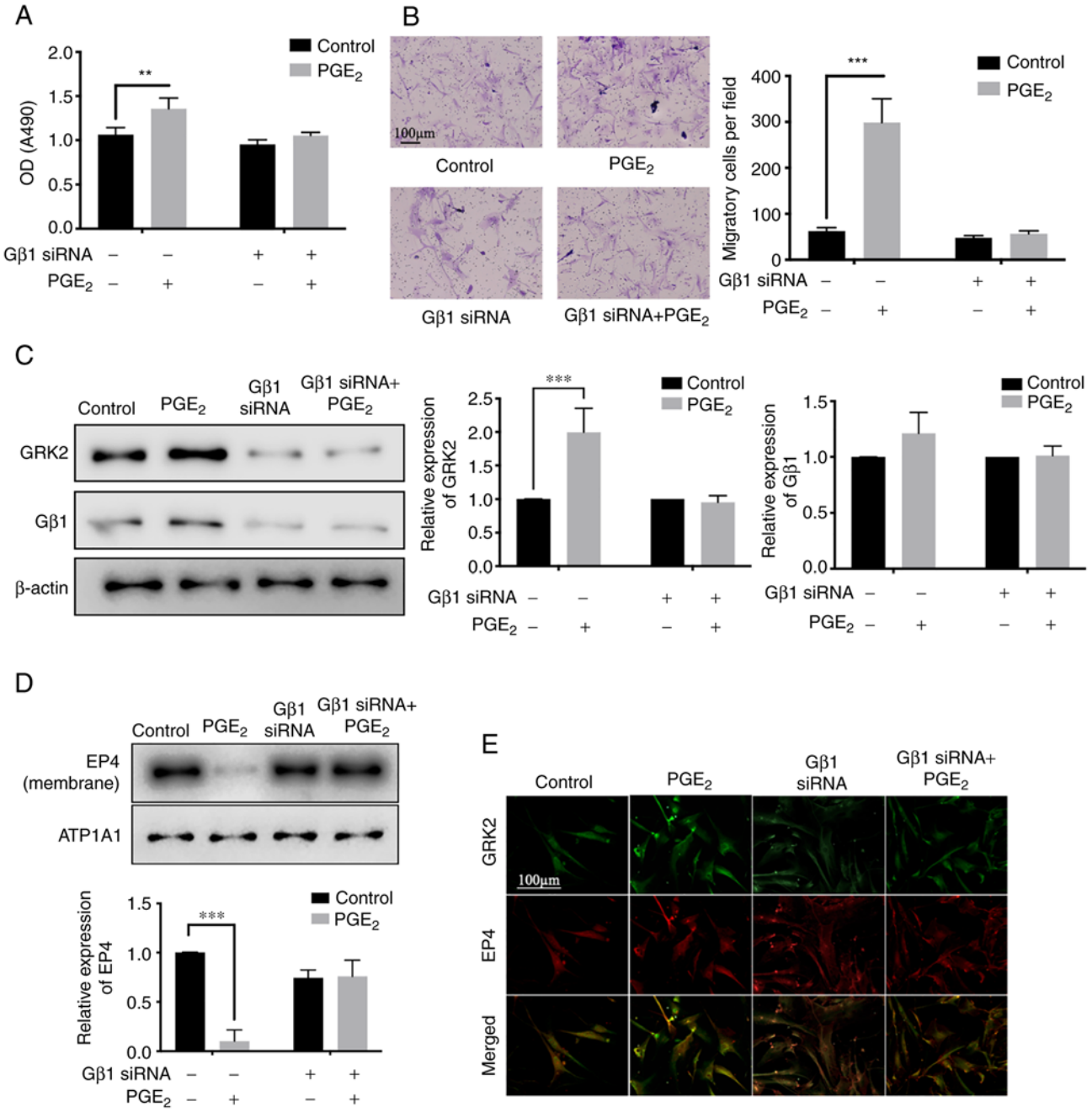

Figure 4. Gβγ knockdown inhibits the PGE_2-induced proliferation and migration of MH7A cells. (A and B) PGE_2-induced (A) proliferation and (B) migration of MH7A cells after silencing Gβ1 was detected by Cell Counting Kit-8 and Transwell assays. Scale bars=100 μm. (C) Total expression of GRK2, Gβ1 and (D) membrane expression of EP4 stimulated by PGE_2 in Gβ1 siRNA-transfected MH7A cells was determined by western blotting. (E) Co-localization of GRK2 and EP4 in Gβ1 siRNA-transfected MH7A cells. Scale bars, 100 μm. $^{**}P<0.01$ and $^{***}P<0.001$ vs. control group (n=3). PGE_2, prostaglandin E2; GRK2, G protein coupled receptor kinase 2; siRNA, small interfering RNA.

PGE_2 for 24 h. Similar results as in the synovial tissue of patients with RA were observed, suggesting that Gβγ was involved in synovial hyperplasia. The enhanced GRK2-Gβγ and the downregulated expression of EP4 in the FLS were significantly associated with synovium hypertrophy. The aforementioned mechanisms promoted the occurrence and development of RA. To further identify the role of Gβγ in RA, Gβγ interference with pharmacological and genetic methods was conducted. Gβγ agonist, mSIRK, promoted abnormalities by PGE_2. However, the membrane expression of EP4 and β-arrestin2 were unchanged compared with the PGE_2 alone group. It was hypothesized that the desensitized EP4 reached a saturated state. There may be other mechanisms independent of EP4 signaling that account for the extra elevated proliferation and migration after the mSIRK treatment. For example, the PI3K/AKT pathway was activated by Gβγ (15). Intervention abrogating GRK2-Gβγ or a Gβγ blocker and Gβγ knockdown mitigated the GRK2 translocation and resulted in a decreased membrane expression of EP4. This finally alleviated the abnormal proliferation and migration of FLS in the RA model. As revealed by the present data, the activation of Gβγ was involved in FLS proliferation and migration.

In the present study, FLS were treated with CP-25 at a concentration of 10^{-7},10^{-6} and 10^{-5} mol/l. The results showed that the 10^{-6} and 10^{-5} mol/l CP-25 significantly inhibited the proliferation and migration after the stimulation of PGE_2, suggesting that CP-25 may provide a potential novel strategy for RA treatment in terms of synovial hyperplasia. In consistency with previous studies, CP-25 suppressed proliferation (5) and migration (15) in the AA-FLS and MH7A cells. In addition, CP-25 inhibits the progress of AA rats by reducing inflammation, immunity and joint injury (16). All of the previous studies by our group proved that CP-25 may be an effective drug for RA (5-11,15,16). In the present study, the effect of CP-25 on the interaction between GRK2 and Gβγ was evaluated. A decreased GRK2-Gβγ interaction and less EP4 desensitization was observed under the CP-25 treatment. The results supported the hypotheses that the interaction of Gβγ and GRK2 contributes to the effect of CP-25 on relieving FLS dysfunction.

GRK2 is a family of protein kinases that regulates the activity of GPCR by phosphorylating the intracellular domain. The pleckstrin homology (PH) domain in the C-terminal of GRK2 is considered to interact with free Gβγ subunits (17). Phosphorylation at Ser^{685} of GRK2 by PKA facilitates the GRK2-Gβγ interaction and enhances the kinase activity of GRK2 (18). It was recently found that CP-25 downregulates phosphorylation at Ser^{685} of GRK2 and directly binds to the kinase domain of GRK2 *in vitro* and inhibits GRK2 activity by controlling the key amino acid residue of Ala^{321} of GRK2 (7,19). Other studies have revealed that when Ser^{670} in the C-terminal domain of GRK2 was phosphorylated by MAPK, the binding of Gβγ with GRK2 was impaired. Thus, GRK2 translocation to the membrane and subsequent GPCR regulation are inhibited (20,21). Manipulation of GRK2 represents a promising molecular basis for treating RA. Previous studies by our group emphasized the importance of inhibiting GRK2 in maintaining receptor function (6,7). Approaches such as the genetic deletion of *Grk2* and systemic inhibition of GRK2 showed some drawbacks or potential side effects differentiated from a pharmacological approach such as a selective GRK2 inhibitor. For instance, *Grk2*-knockout mice succumbed on day 15 of embryonic development from cardiac hypoplasia and cardiac dysfunction (21). GRK2 knockdown also caused glomerular injury and renal damage when treating heart disease (22). It is worth exploring whether there exists a safer and efficacious influence on the balanced receptor function and kinase activity with minimal side effects, but does not just completely abolish the expression of the receptor or kinase.

At present, small molecule targeting GPCR-Gβγ-GRK2 signaling is an area of increased research attention in pathologic conditions such as heart failure and kidney dysfunction (23,24). In this pathway, one approach is the pharmacological inhibition of GRK2, such as paroxetine. The other strategy is to interfere with GRK2-Gβγ or inhibit Gβγ. Preclinical evaluations have revealed a cardioprotective effect for GRK2 genetic deletion or pharmacological inhibition in cardiovascular diseases by binding to and stabilizing GRK2, thus inhibiting downstream β-adrenergic receptor (β-AR) signaling (25,26). Furthermore, M119 and gallein hindered Gβγ binding to GRK2, leading to decreased translocation to the membrane of GRK2 and thus preserving the normal function of the β-adrenaline receptor so as to mitigate heart failure progression and cardiac hypertrophy (27). In the present study, gallein and CP-25 suppressed the abnormal proliferation and migration in PGE_2-stimulated FLS by interfering with the binding of Gβγ with GRK2. The present results are in a manner analogous to those observed of the β-AR.

There are five different Gβ and 12 Gγ in humans. An investigation of the binding preferences revealed that GRK2 binds preferentially to Gβ1 and Gβ2 (28). It was revealed that the expression of Gβ1 mRNA was higher than Gβ2 in the synovial tissue of patients with RA; hence, Gβ1 was silenced in MH7A cells. It has become clear that Gβγ is a multifunctional protein complex that interacts not only with the Gα subunit and GPCRs but also with intracellular proteins such as PI3K, AC and GIRK (29). Targeting Gβγ signaling appears to be a growing potential pharmacological intervention for treating cancer cell migration (30). It has been reported that the EP4 antagonist suppresses cancer cell invasion and migration in prostate cancer (31). However, a balance should be maintained in suppressing cancer cell growth and motility without destroying the normal functions of cells. FLS displays cancerous properties of proliferation and migration in RA synovial tissue that resemble cancer-associated fibroblasts in tumors (32). FLS has its own physiological function to maintain the homeostasis of joints (33). Excessive inhibition of the normal function of FLS in rheumatoid or non-rheumatoid joints would cause detrimental outcomes. This notion may be related to the soft regulation of the inflammatory immune response (SRIIR) proposed by our group. This emphasized that the normal function as well as gene and protein expression or activity of the cell should not be completely disabled by drugs. The most appropriate drug is characterized by regulating and restoring abnormal activity to achieve physiological levels with minimal adverse reactions. In the present study, CP-25 likely is a SRIIR drug due to its ability to re-sensitize EP4 and reduce the excessive proliferation and migration in PGE_2-stimulated FLS.

The long-term activation of GPCRs after ligand exposure has been revealed to desensitize receptors and downregulate downstream signals. GPCR desensitization is generally explained by paradigms proposed in studies on the β2-AR (34,35). Once the activated receptor is phosphorylated by GRK2/3, its affinity for β-arrestin is enhanced. β-arrestin2 binds GPCRs that are targeted for endocytosis and receptor internalization (35). Agonist-induced GRK2-Gβγ interaction is a prerequisite for GRK2-mediated receptor desensitization and downregulation. The PH domain of GRK2 competitively binds to Gβγ and sequesters it from the Gα subunit. Evidence has demonstrated that the Gβγ-mediated recruitment of PI3K in complex with GRK2 is directly implicated in β-AR desensitization (36). In the present study, in response to PGE_2, GRK2 was passively pulled to the cell membrane by the binding of Gβγ subunits, and then both simultaneously translocated to the cell membrane. GRK2 was phosphorylated on the membrane. β-arrestin2 was recruited to the membrane simultaneously, inducing excessive desensitization and internalization of EP4. Blocking Gβγ signaling, either with the small molecule Gβγ selective inhibitor, gallein and GRK2i, or with Gβ1 siRNA or CP-25, led to the decreased translocation of GRK2 and EP4

re-sensitization. In this regard, EP4 desensitization via the GRK2-Gβγ interaction could be a novel therapeutic target to treat RA. Although the present study was focused primarily on the interaction between GRK2 and Gβγ, it was found that GRK2, Gβγ and EP4 interacted with each other. It is a possibility that these three proteins bind directly to form a ternary complex. Further research is required to demonstrate the complex formed by EP4, GRK2 and Gβγ in a sequential order, as well as the association and disassociation of it. Several issues, including the details of Gβγ releases from Gα-GDP, the binding site of Gβγ with GRK2, and the Gβγ downstream signal pathways, remain to be further confirmed.

The limitation of the present study is that Gβ antibody was selected instead of Gβγ. Although distinct Gβ and Gγ antibodies may reveal in an improved way the activity of Gβγ, in the present study, main focus was addressed on the interaction between Gβγ and GRK2; it is considered that one kind of Gβ antibodies may not be optimal, but should be sufficient to draw the conclusion of the present study. It is considered that future work is needed to explore changes of different subunits of Gβ and Gγ.

Collectively, it was revealed that GRK2-Gβγ interaction was enhanced in RA synovial tissue and FLS. PGE_2-induced abnormal proliferation and migration in FLS were associated with strengthened GRK2-Gβγ interaction and EP4 desensitization. Regulating EP4-GRK2-Gβγ signaling may be one of the mechanisms that underlie the salutary effect of CP-25. Additional *in vivo* studies are required to confirm the therapeutic potential of the interfering GRK2-Gβγ interaction in RA.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ participated in the design of the study, performed most of the experiments and wrote the manuscript. XY, CH and DW performed experiments. YM and WW conceived the study and revised the manuscript. YZ and DW confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved (approval no. 20131321) by the Biomedical Ethics Committee of Anhui Medical University (Hefei, China). Informed consent was obtained from all patients for the use of their samples in scientific research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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