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Original Article

Erythropoietin enhances meniscal regeneration and prevents osteoarthritis formation in mice

Xiao-Ning Fu[1*], Hui-Wu Li[2*], Na Du[3], Xu Liang[1], Shi-Hao He[1], Kuang-Jin Guo[1], Tian-Fang Li[1]

[1]Department of Rheumatology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China; [2]Department of Orthopaedics, Shanghai 9th Hospital, Shanghai Jiaotong University, Shanghai, China; [3]Department of Nephrology, Central Hospital of Kai-Feng City, Kai Feng, China. [*]Equal contributors.

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Abstract: Osteoarthritis (OA) is a leading cause of pain and disability, and knee is the most commonly afflicted joint. Meniscal tear due to injury or degeneration is an established factor for OA pathogenesis. Previous studies have demonstrated that meniscectomy does not reduce the OA incidence. We hypothesized that enhancing meniscal regeneration may prevent OA formation and progression. We first investigated the developmental pattern of mouse meniscus. Knee joint samples were collected at embryonic stages as well as after birth for histological and immunohistochemical studies. The results showed that meniscal cells underwent active proliferation and apoptosis at embryonic day 19.5 and Day 1 after birth. Collagen I (Col-1) is a major type of matrix protein in matured meniscus. Meniscal cells isolated from 3-month-old mice were used to examine the effect of selected factors on the molecules related to cell proliferation, angiogenesis, inflammation, extracellular matrix proteins and matrix degradation enzymes. Overall assessment indicated that EPO had optimal effect on meniscal regeneration. An organ culture system of mouse meniscus was established to test the effect of EPO on *in vitro* cultured menisci. EPO upregulated the expression of Col-1, Col-2 and VEGF-A, and downregulated the expression of MMP-13. Finally, we established a mouse model of meniscus injury induced OA (MIO), and mice were subjected to PBS or EPO treatments. The results demonstrated that EPO enhanced meniscal repair and prevented OA formation. EPO may become an effective Disease Modifying Osteoarthritis Drug and may be used for early treatment for meniscal injury to prevent OA progression.

Keywords: Erythropoietin, meniscus, osteoarthritis, regeneration

Introduction

Osteoarthritis (OA) is the leading cause of pain and disability. Current treatments are palliative and no effective disease modifying osteoarthritis drugs (DMOADs) are available [1, 2]. Because of structural and biomechanical complexities, knee is the most commonly afflicted joint [3, 4]. Meniscal tear caused by either injury or degeneration, is an established causative factor for the pathogenesis of OA [5-8]. Previous studies show that patients with OA have a prevalence of meniscal lesions of 68-90% [5, 9].

Human menisci can be roughly divided into three zones: the outer zone (red-red) with blood and nerve supply, the inner zone (white-white) without blood supply, and the middle zone (red-white). The major cells in the outer zone are fibrochondrocytes while articular chondrocyte-like cells are predominant in the inner zone. The superficial zone is covered by fibroblast-like cells. Extracellular matrix proteins are composed of both collagenous and non-collagenous molecules. Collagen type I (Col-1) is most abundant in the outer and middle zones, while Col-2 is only detectable in the inner zone. Trace amount of Col-3, Col-4, Col-5, possibly other collagens, may also present [9-12].

Low vascularity, hypocellularity, mechanical stress and joint inflammation impair regeneration of damaged meniscus. While the tears in the outer zone may regenerate, the inner zone damage are conventionally treated with either partial or total meniscectomy. However, meniscectomy may disrupt joint biomechanics and increase shear stress at the bone-cartilage

interface [13, 14]. A long-term follow-up study shows that meniscectomy in adolescents causes more than 130-fold increase in the incidence of OA [15], precluding surgery as an option for younger patients. In adults, partial meniscectomy for meniscal degeneration does not lower the incidence of OA [16]. In addition, partial meniscectomy combined with physical therapy does not affect the treatment outcome. Further, arthroscopic menisectomy to treat meniscal tear and OA increases the future risk for total knee arthroplasty [17].

Molecular events during tissue regeneration often recapitulates embryonic developmental processes. Thus, in-depth studies of embryonic development of meniscus may help identify novel targets able to promote meniscal repair. On the site of future knee joints, an interzone is formed and the cells in this zone form meniscus as well as other joint components [18]. TGF-β superfamily members such as BMP-7, TGF-β3, and GDF-5 are critical for joint formation. In addition, Wnt (especially, Wnt14) and IGF-1 may also play a role [19, 20]. Other molecules that may participate in joint formation include TGF-β pathway associated molecules such as TGFBI and Wnt pathways associated molecules such as Wnt inhibitor SFRP2 and the activator RSPO2 [20].

Erythropoietin (EPO) is a hematopoietically active factor regulated by transcription factor hypoxia inducible factor alpha (HIF-1α) [21, 22]. Previous studies have shown that EPO receptor (EPOR) is expressed in fracture callus of different stages and EPO enhances skeletal repair in fracture and bone defects by promoting chondrocyte proliferation, matrix synthesis, callus formation and angiogenesis. In a porcine model of bone defect, EPO combined with a collagen carrier augments healing. EPO is a survival factor for developing cartilage and EPO combined with bone marrow cells enhances cartilage repair [23-29]. Critical factors for meniscal regeneration include the increases in cell proliferation, neoangiogenesis and synthesis of extracellular matrix proteins, and the decrease in joint inflammation [30]. From these perspectives, EPO seems an optimal candidate.

Our current study aimed to investigate developmental pattern of mouse meniscus, to examine the effect of the relevant growth factors as well as EPO on meniscal cells with the focus on joint formation, chondrogenesis, angiogenesis, inflammation and matrix turnover, to establish an organ culture system of mouse menisci to test the effect of selected growth factors, and to unveil the effect of EPO on mice with meniscus injury induce OA (MIO). Our results show that organ culture system of mouse menisci is a useful tool for screening molecules able to enhance meniscal regeneration. In addition, EPO inhibits inflammatory response in vitro and prevents OA pathogenesis in vivo by promoting regeneration of meniscus.

Materials and methods

All the animal studies were conducted with the approval from the Committee of Laboratory Animal Use in Zhengzhou University.

Reagents and equipments

Reagents: Fetal bovine serum (FBS, Invitrogen, Carlsbad, CA); Pronase (Roche, San Francisco, CA); Collagenase D (Roche); Wortmannin (PI3K-AKT pathway inhibitor, Sigma Aldrich, St. Louis, MO); AZD1480 (JAK2-STAT pathway inhibitor, Santa Cruz Technologies, Santa Cruz, CA); Trizol reagent (Life Technologies, Grand Island, NY); SuperScript First Strand Synthesis System (Invitrogen). Kits: BrdU Cell Proliferation Assay Kit (Cell Signaling Technologies, Denvers, MA); DAB kit (Vector Laboratories, Burlingame, CA); Dual endogenous enzyme blocking kit (DAKO, Carpinteria, CA); Secondary antibodies and Fast Red kit (Abcam, Cambridge, MA). Mouse recombinant proteins: EPO, GDF-5, BMP-7 and IGF-1 (R&D System, Minneapolis, MN); TGF-β3 (Sigma); IL-1β (Invitrogen). Antibodies: from Cell Signaling Technologies: active caspase 3 (#9664, 1:200) and MMP-13 (#69926, 1:100); from Abcam: PCNA (ab19166, 1:200), Col-1 (ab233080, 1:500), Col-2 (ab34712, 1:200) and VEGF-A (ab51745, 1:100).

Knee sample collections

Sample harvesting started as early as embryonic day 13.5 (E13.5). However, the pilot study showed that at this time point, knee joint structure was difficult to identify. So samples were collected from E15.5 to 6 months after birth. These samples were subjected to H/E and Alcian blue/Orange G staining by following the protocols from University of Rochester. IHC st-

aining was performed with antibodies against PCNA (a marker for proliferation), active caspase 3 (a marker for apoptosis), and Col-1.

Isolation and culture of primary mouse meniscal cells

Meniscal samples were isolated from the knee joints of 3-month-old C57BL/6J wild type (WT) mice. Soft tissue was removed by a digestion with pronase (Roche, San Francisco, CA) at 37°C shaker for 45 min and collagenase D (Roche) for 60 min. After thorough wash, the samples were further digested with collagenase D for 5 hours. The meniscal cells were cultured on DMEM containing 10% FBS and then treated with the following factors: Vehicle (Veh), GDF-5, EPO, BMP-7, IGF-1, TGF-β3. The RNA and protein samples were collected 12 and 24 hrs after treatment for RT-PCR and western blotting, respectively.

In vitro organ culture of mouse menisci

Meniscal samples were collected *en bloc* from 3-month-old C57B6J WT mice under dissection microscope and cultured in Petri dishes with DMEM containing 10% FBS. One day later after harvesting, cultured menisci were treated with EPO (10 ng/ml) for 3 days. The tissue sections were used for histological and immunohistochemical (IHC) examination, RNA isolation for RT-PCR, protein extraction for western blotting.

After a brief wash and fixation, the samples were incubated in 30% sucrose for 24 hours. Then menisci were embedded in OCT and stored at -80°C for frozen section.

The RNA sample collection: the meniscal samples were minced into small pieces and homogenized by ultrasound in Trizol reagent. RNA was extracted by following the instructions. Protein sample collections: the meniscal samples were minced to small pieces and homogenized by ultrasound in RIPA buffer containing protease/phosphatase inhibitor cocktail. The protein samples were stored at -80°C. BrdU labelling: BrdU reagent was added to cultured menisci 4 hours before processing. BrdU labelling of cells in frozen tissue sections was detected with a kit by following the protocol.

Real-time PCR (RT-PCR)

RT-PCR was performed as previously described [31]. Briefly, RNA was extracted from meniscal cells with a kit from Qiagen and reversely transcribed into cDNA with SuperScript First Strand Synthesis System. RT-PCR was performed with the relevant primers and β-actin was used as an internal reference gene. The expression of each gene was normalized with β-actin and the results were represented as the mean and standard deviation (SD).

Western blotting

Western blotting was done as previously described [32]. The following rabbit anti mouse antibodies were used: active caspase 3, PCNA, Col-1, Col-2, and VEGF-A.

Immunohistochemistry (IHC)

Frozen sections: After antigen retrieval and blockage of endogenous peroxidase activity, the sections were incubated in primary antibodies overnight at 4°C. For IHC with the ABC (avidin-biotin-peroxidase complex) method, the sections were next incubated in biotinylated secondary antibody and AB Complex. Color was developed using a DAB kit from Vector Laboratories (Burlingame, CA). For IHC with AP (alkaline phosphatase) method, the endogenous AP activity was quenched by incubation with dual endogenous enzyme block for 30 min. The AP-conjugated secondary antibody was used and followed by an incubation from a Fast Red kit. For double staining, both endogenous HRP and AP were quenched by a dual enzyme block. The first antibody used was rabbit antibody for Col-1, followed by incubation in donkey anti-rabbit IgG conjugated with AP for 30 min. Color was developed using Fast Red solution. The #2 primary antibody was mouse antibody for Col-2 and the following steps were the same as in the regular IHC using the ABC method.

Mouse model of meniscus injury induced OA (MIO) and EPO treatment

MIO was established in 3-month-old C57B6J WT mice by excising the anterior horn of medial meniscus. The OA-like pathology can be detected 3 months after surgery. Sham surgery was done by exposing joint cavity with immediate

closure. Both MIO and Sham mice were divided to two groups: treatment group receiving subcutaneous injection of mouse recombinant EPO (1000 units/kg) consecutively for 14 days, and vehicle control group receiving PBS injection. Three time points of EPO treatment were tested: early (week 1 after surgery), intermediate (week 5 after surgery), and late intervention (week 9 after surgery). Mouse hemoglobin concentration of different treatment groups was measured with Element HT5 machine (Heska, Loveland, CO). Knee joint samples were collected 12 weeks after surgery for different examinations.

Semi-quantitative analysis was conducted with the Image J v1.8.0. For tissue sections, the threshold was set automatically or manually based on the gray-scale values to calculate the percentage of the positive stained areas in total areas. For western blotting, the band was circled manually and the gray-scale values of the target protein bands were measured. The data were presented after normalization with the values of β-actin bands. Student's T test was used for statistical analyses.

Results

Development of mouse meniscus

At E15.5, cell condensation was completed and meniscal morphology may be observed. The blue-stained cartilaginous component was detected in cartilage template, but not in menisci. After birth, the blue color appeared both in articular cartilage and menisci, suggesting the existence of Col-2 and aggrecan. At D21, the red-stained substances containing Col-1 and other types of collagens were found in menisci and the red area was gradually increased until skeletally mature at 3-month-old (**Figure 1A**).

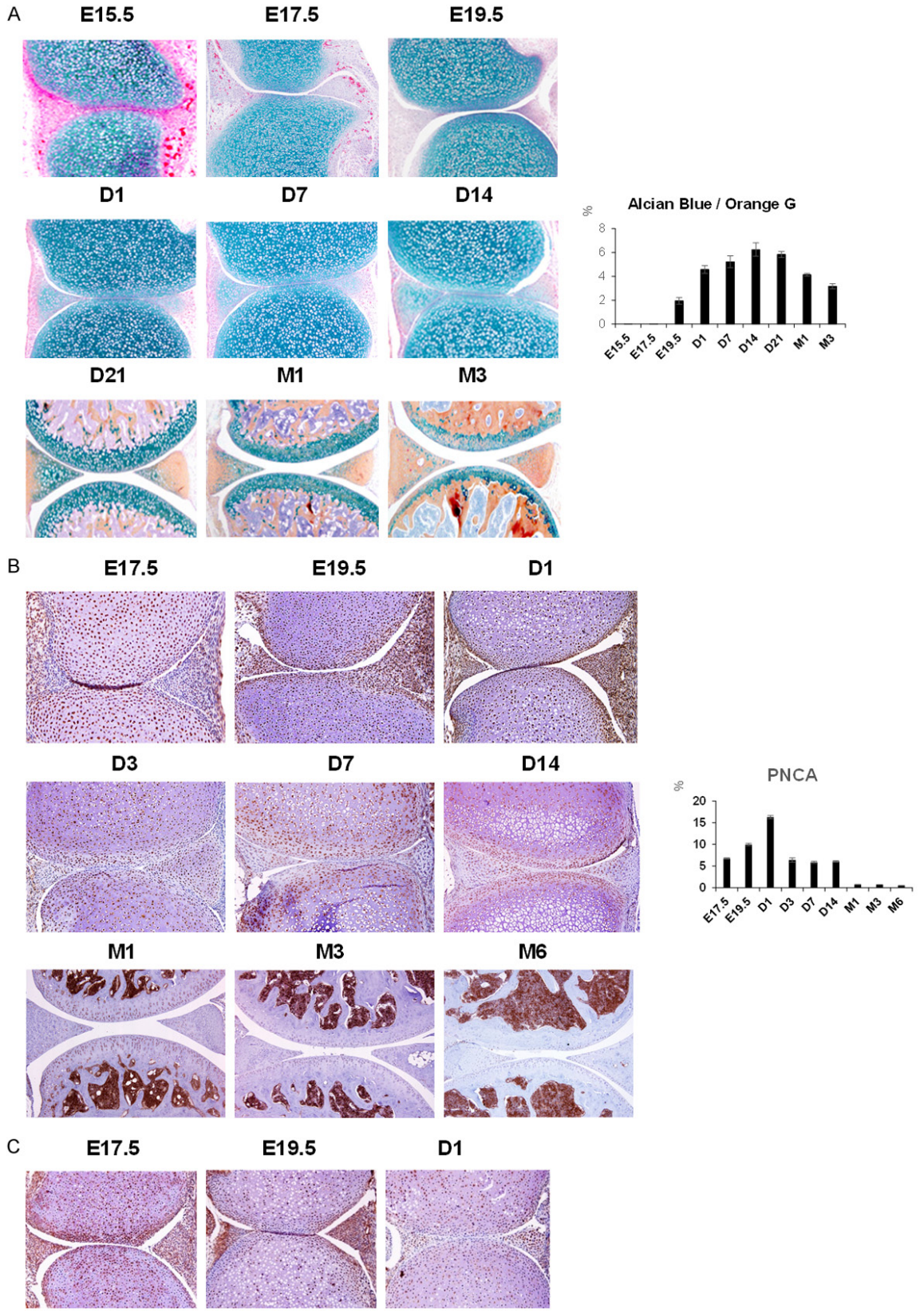
In order to evaluate the proliferation status of meniscal cells, IHC staining was performed with an antibody against mouse PCNA. At E19.5 and D1, meniscal cell proliferation was evident in menisci and superfacial layers of articular cartilage. The number of the PCNA positive cells in menisci started to decrease at D7, while cell proliferation became gradually increased in articular chondrocytes. One month after birth, cell proliferation in menisci and articular cartilage was not evident (**Figure 1B**). Cell apoptosis in knee joints was assessed with an antibody against mouse active caspase 3 because it is a critical enzyme in apoptic pathway. At E17.5, cell apoptosis was prominent in both menisci and the superfacial layer of articular cartilage, and at E19.5, apoptosis was still evident in menisci but not in articular cartilage. After birth, cell apoptosis was gradually decreased. One month after birth and thereafter, cell apoptosis in menisci was hardly detectible (**Figure 1C**). To evaluate the expression pattern of Col-1, an antibody against mouse Col-1 was used for IHC staining. At E17.5 and E19.5, Col-1 was detected in menisci, but not articular cartilage. After birth, Col-1 was detected predominently in outer areas of menisci (**Figure 1D**). Collectively, these data were put into a flow chart (**Figure 1E**).

In vitro results with primary mouse meniscal cells

We first tested the effect of the relevant factors on the expression of major extracellular matrix proteins and key degrading enzymes with RT-PCR. The results showed that EPO upregulated the expression of *Col-1*, *Col-2*, *Col-3*, *Col-5* and *aggrecan*. Interestingly, it also upregulated the expression of *Timp-1*, an inhibitor molecule for MMPs. IGF-1 and EPO were the most potent inhibitors for *Mmp-13*, and GDF-5 and BMP-7 downregulated the expression of *Adamts 4* and *Adamts 5*, respectively (**Figure 2A**).

We then studied the effect of these factors on the expression of the genes related to joint formation. Both EPO and BMP-7 significantly upregulated *Sox-6* expression, and also all growth factors increased *Sox-9* expression. Unexpectedly, these factors, except IGF-1, enhanced the expression of *Runx2*, a critical transcription factor for chondrocyte differentiation. EPO significantly upregulated *Gdf-5*, *Ki67* and *Vegf-A* expression. Regarding the genes involved in joint formation, EPO, BMP-7 and IGF-1 increased the expression of *Tgfbi* and *Rspo2*. In addition, BMP-7 was the most potent inducer for *Sprf2* expression (**Figure 2B**).

Based on these findings, EPO was selected for the following studies (**Figure 3**). To test the effect of EPO on the expression of the most important proinflammatory cytokine *Tnf-α*, IL-1β was used to increase the basal *Tnf-α* level


A
E15.5
E17.5
E19.5
D1
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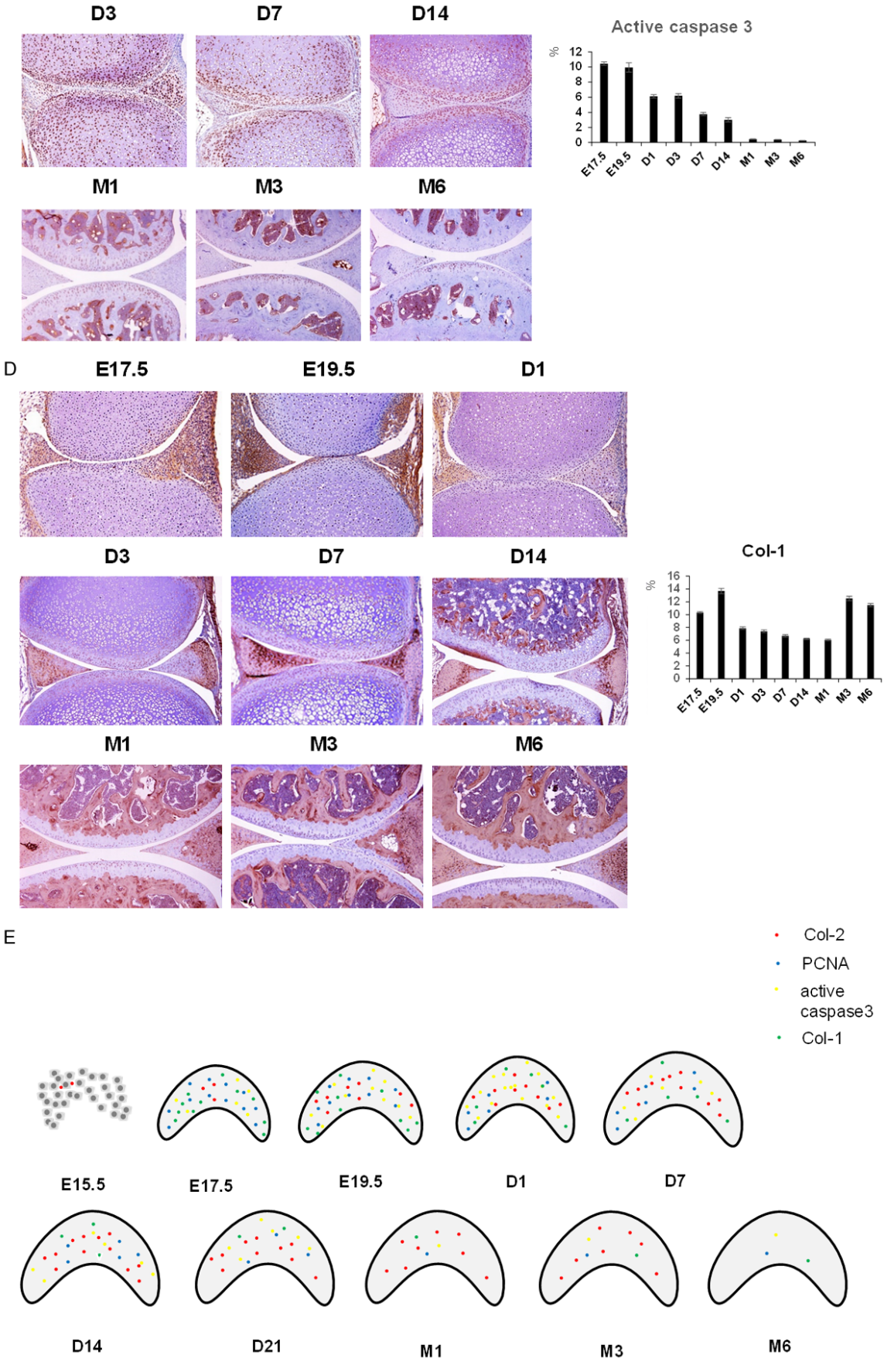
D3
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16
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E
Col-2
PCNA
active caspase3
Col-1
E15.5
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E19.5
D1
D7
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D21
M1
M3
M6

Figure 1. Development of mouse meniscus. A. Histological examinations. Meniscal samples were subjected to Alcian blue/Orange G staining. Blue staining: Col-2 containing matrix. At the embryonic day 15.5 (E15.5), cell condensation appeared in the location of future menisci. At E17.5 and E19.5, lateral and medial menisci may be clearly seen although cartilaginous components were rarely detected. After birth, Col-2 was detected at Day 1 through 14 (D1, 7, 14). Starting from D21, the red-staining area indicated the existence of other types of collagens in menisci, especially, Col-1 (original magnification: ×40). B. Proliferation of meniscal cells. An antibody to PCNA was used for IHC staining to detect proliferative cells. At E19.5 and D1, meniscal cell proliferation was robust. The number of the PCNA positive cells was decreased at D7. One month after birth, cell proliferation in menisci was hardly detectible (original magnification: ×40). C. Apoptosis of meniscal cells. An antibody to active caspase 3 was used for IHC to detect apoptotic cells. At E17.5 and E19.5, apoptosis was evident in menisci. After birth, cell apoptosis was gradually decreased. One month after birth, cell apoptosis in menisci was not prominent (original magnification: ×40). D. The expression of Col-1. An antibody to mouse Col-1 was used for IHC to detect the distribution pattern of Col-1. At E17.5 and 19.5, Col-1 was detected in menisci, but not in cartilage template. After birth, Col-1 positive area shrank, while it became more extensive from month 1 through month 6 (original magnification: ×40). E. A flow chart of meniscal development. E: embryonic days, D: days after birth, M: months after birth.

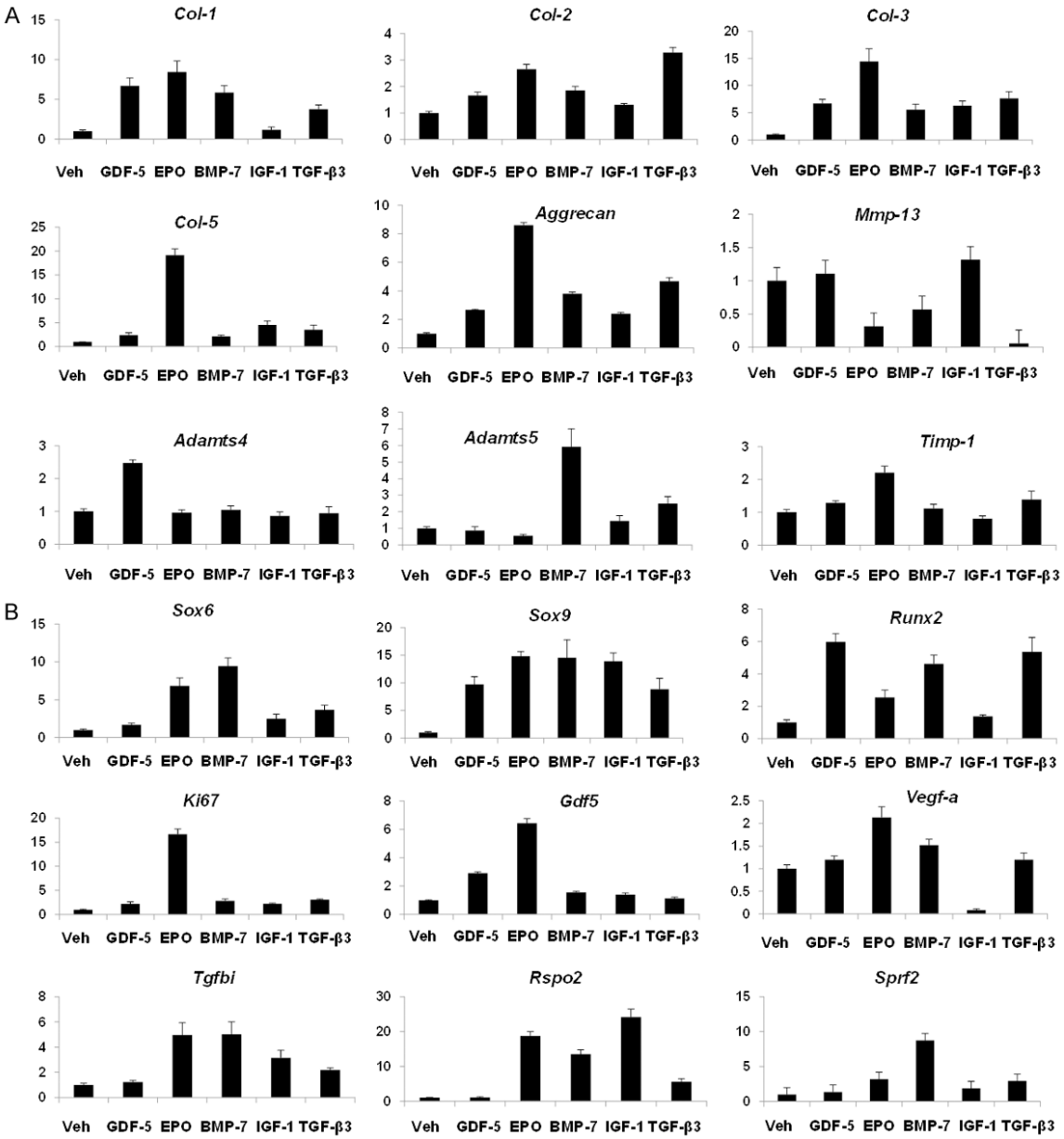

Figure 2. The effect of growth factors on mouse meniscal cells. A. The expression of extracellular matrix proteins and matrix degrading enzymes. RT-PCR analysis of gene expression in mouse meniscal cells after the following treatments: (1) Veh, (2) GDF-5, (3) EPO, (4) BMP-7, (5) IGF-1, (6) TGF-β3. EPO upregulated the expression of *Col-1, Col-2, Col-3, Col-5, Aggrecan, and Timp-1*, while it downregulated the expression of *Mmp-13*. GDF-5 also increased the expression of these genes, albeit to a lesser extent. In addition, GDF-5 upregulated the expression of *Adamts4* and BMP-7 significantly increased the expression of *Adamts5*, respectively. B. The expression of the relevant molecules. All selected factors upregulated *Sox-9* expression. However, only EPO and BMP-7 increased *Sox-6* expression. BMP-7 and TGF-β3 were the most potent inducers for *Runx-2*. EPO upregulated the expression of *Gfd-5, Ki67* and *Vegf-a*. EPO, IGF-1 and BMP-7 upregulated *Tgfbi* expression. Similar effects were noticed in *Rspo2* expression. BMP-7 increased *Sprf2* expression.

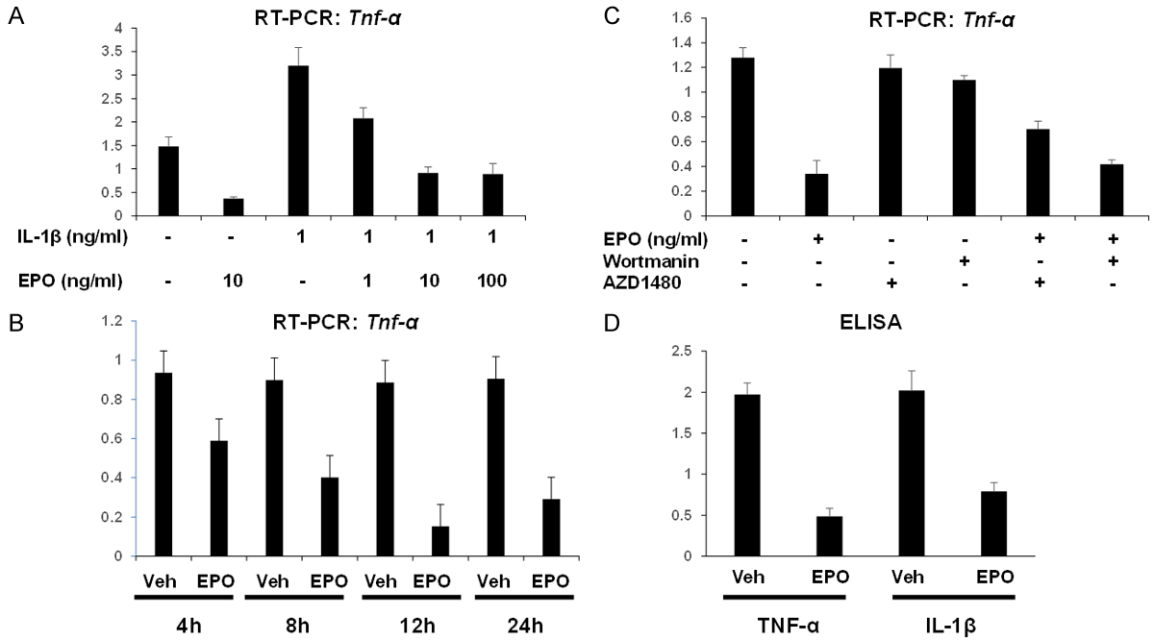

Figure 3. A. EPO dose-dependently inhibited *Tnf*-α expression induced by IL-1β. Mouse primary meniscal cells were treated with the following: (1) Veh, (2) EPO-10 ng, (3) IL-1β, (4) IL-β + EPO-1 ng, (5) IL-1β + EPO-10 ng, (6) IL-1β + EPO-100 ng. A dose of 10 ng EPO/ml largely blocked upregulation of *Tnf*-α expression induced by IL-1β, thus, this dose was chosen for the following experiments. B. A time course study: the inhibitory effect of EPO on *Tnf*-α expression peaked at 12 hours. C. The pathway through which EPO took effect on *Tnf*-α expression. (1) Veh, (2) EPO, (3) AZD1480, (4) Wortmannin, (5) EPO + AZD1480, (6) EPO + Wortmannin. Blocking JAK2-STAT pathway significantly abrogated the inhibitory effect of EPO on *Tnf*-α expression. D. The effects of EPO on protein expression of TNF-α and IL-1β. ELISA showed that EPO decreased the protein expression of both TNF-α and IL-1β.

in primary mouse meniscal cells. IL-1β significantly upregulated the expression of Tnf-α and inhibitory effect of EPO was evident at a dose of 10 ng/ml. Increasing the dose to 100 ng/ml did not increase such effect (3A). A time-course study showed that the effect of EPO peaked at 12 hours after EPO stimulation (3B). To investigate the EPO downstream signaling molecules, we utilized the inhibitors for JAK2-STAT (Wortmannin, 10 nM) and PI3K-AKT (AZD1480, 100 nM) signaling pathways, respectively. While both pathways may transduce EPO signaling, JAK2-STAT pathway seemed to play a more important role in the inhibitory effect of EPO on *Tnf-α* expression (3C). Consistent with RNA expression, ELISA results showed that EPO decreased the production of TNF-α and IL-1β proteins, with more evident effect on TNF-α (3D).

Examination of meniscal organ culture

H/E staining showed that this organ culture model largely preserved the C-shapes of the menisci and their insertion sites to tibial plateaus. EPO increased the production of extracellular matrix proteins as evidenced by increased areas of Alcian blue staining. BrdU labelling was conducted to evaluate the rate of cell proliferation, and the results showed that EPO significantly increased cell proliferation. IHC staining demonstrated EPO repressed MMP-13 expression (**Figure 4A**).

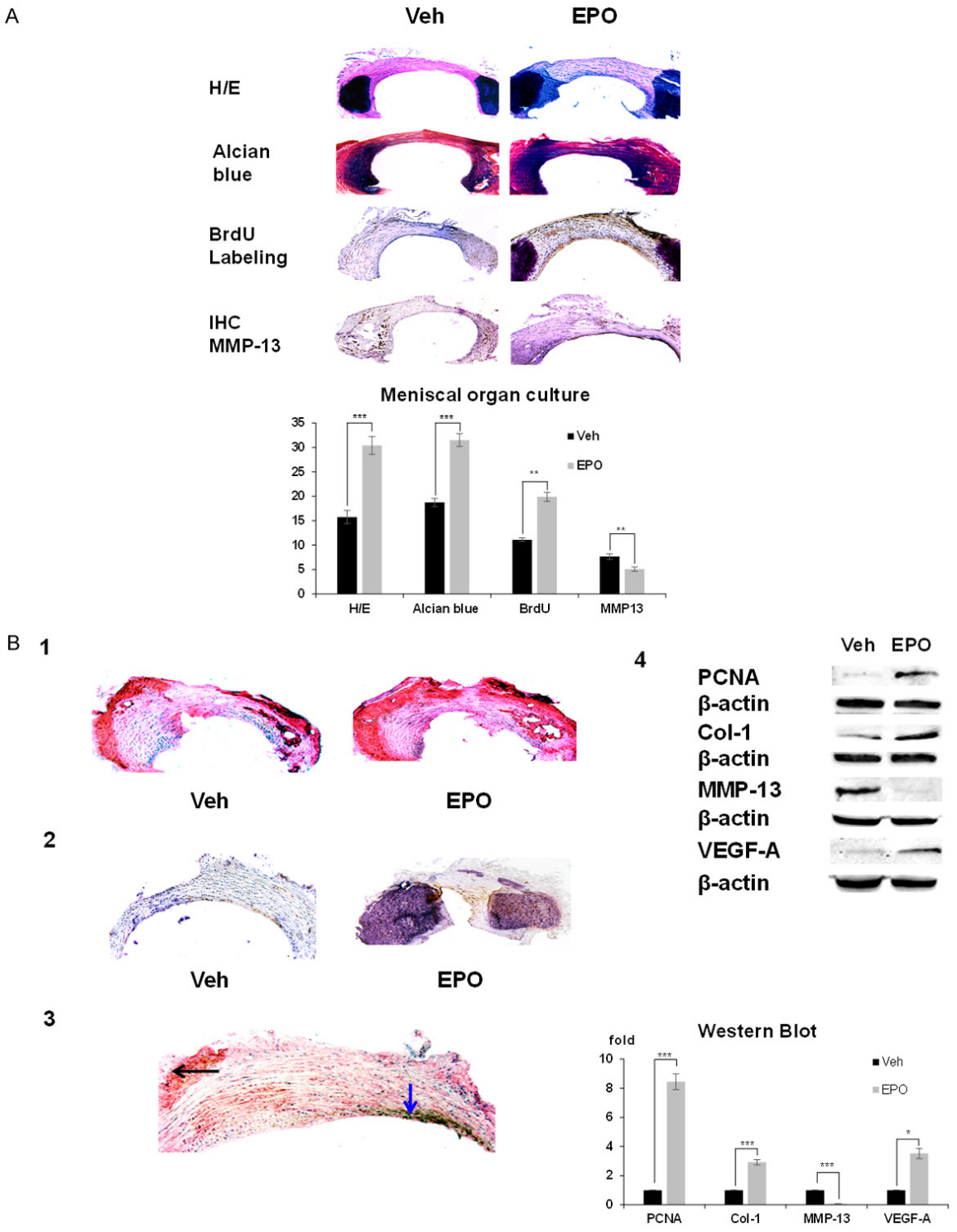

Figure 4. An organ culture system of mouse menisci. A. Histological and immunohistological examinations. H/E staining: the in vitro cultured menisci largely preserved their morphology. Alcian Blue staining: EPO increased Col-2 production. BrdU and IHC: EPO enhanced cell proliferation. IHC: EPO downregulated MMP-13 expression (original magnification: ×40). B. Double IHC staining: IHC with the AP method (red): EPO increased Col-1 production (1). IHC with the HRP method (yellow): EPO increased Col-2 expression (2). Double IHC staining with Col-1 and Col-2 antibodies using AP and HRP methods: Col-1 (black arrow) mainly located in the outer zone although some Col-1 may also exist in middle zone. In contrast, Col-2 was only detected in restricted area in inner zone (3, blue arrow) (original magnification: ×40). Western blotting: EPO upregulated the expression of PCNA, Col-1 and VEGF-A, and downregulated the expression of MMP-13 (4).

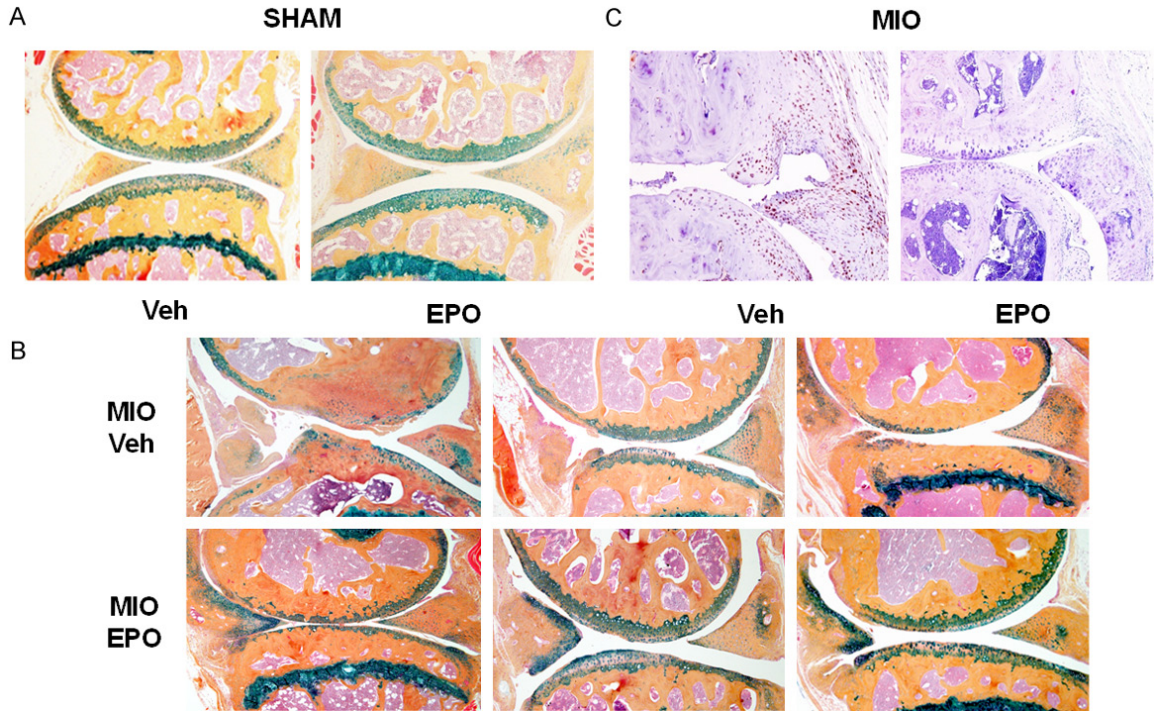

Figure 5. EPO effect on the severity of MIO: Mouse knee joint samples were subjected to Alcian blue/Orange G staining. Blue staining: Col-2 containing matrix, orange staining: Col-1 containing tissue. Mice underwent Sham surgery treated with either EPO or PBS (Veh) (A. Original magnification: ×40). Mice underwent MIO were treated with either EPO or PBS. EPO enhanced meniscal regeneration and ameliorated the severity of MIO (B. Original magnification: ×40). IHC staining: EPO suppressed MMP-13 expression in menisci and articular cartilage (C. Original magnification: ×80).

An antibody against mouse Col-1 was used for IHC with alkaline phosphatase (AP, red staining) method (**Figure 4B**). The results showed that EPO increased Col-1 expression (1). Similarly, IHC was performed using an antibody against mouse Col-2 with the horseradish peroxidase (HRP, yellow staining) method, and the results showed that EPO also enhanced Col-2 expression (2). Double IHC with both Col-1 and Col-2 antibodies disclosed that Col-2 was only detected in certain areas in the inner zone of menisci (3). Western blotting of cell lysates from meniscal culture was done with different antibodies. The results showed that EPO increased the expression of PCNA, Col-1 and VEGF-A, while it decreased the expression of MMP-13 (4).

The effect of EPO on the severity of MIO

Pilot study was done to compare the efficacy of early, intermediate and later interventions. Slight and transient increase of hemoglobin level was noticed after EPO treatment, and its level returned to normal after cessation of the treatment. With regard to the regenerative potential, early use of EPO exhibited a maximal effect. Thus, early treatment was utilized in the following studies. After continuous administration of EPO for 2 weeks, knee joint samples were subjected to Alcian Blue/Orange G staining. In Sham group mice with intact meniscal structure, EPO effect was not detectible (**Figure 5A**). In MIO mice, EPO significantly reduced the OA severity compared to mice receiving PBS injection. Of note, EPO treatment resulted in enhanced regeneration of injured menisci (**Figure 5B**). To investigate the major mechanism responsible for the protective effect of EPO on OA, we conducted an IHC study in mice underwent MIO treated with either PBS or EPO with an antibody to MMP-13, and the result showed that EPO suppressed MMP-13 expression both in meniscus and articular cartilage (**Figure 5C**).

Discussion

Meniscal regeneration after tear or degeneration, especially those in the inner zone, poses a tremendous challenge to orthopaedic society.

Due to the lack of blood and nerve supplies in the white zone and the quiescent nature of meniscal cells, the chance is low for spontaneous healing, or even after suturing [14, 33, 34]. Partial meniscectomy is palliative for pain due to meniscal tears, and does not decrease the incidence of OA [16, 17, 35, 36]. It is reasonable to postulate that the agents able to regenerate meniscal injury may prevent OA pathogenesis and become effective DMOADs. The selection of the factors has been based on their effect on cartilage repair and their role during meniscal development [16, 17, 35, 36].

In this study, we collected a whole set of meniscal samples at different time points from embryonic to adult periods and unveiled the patterns of cell proliferation, apoptosis and matrix production. Our study first establishes that organ culture of mouse menisci is a feasible way to test different factors for their potential in meniscal regeneration, cell proliferation, matrix production, angiogenesis, etc. Meniscal cells treated with different factors and overall evaluation indicates that EPO is a promising candidate to regenerate meniscus. In vivo study in mice underwent MIO demonstrates that EPO enhances meniscal repair and ameliorates OA severity. One mechanism is to inhibit MMP-13 expression in both menisci and articular cartilage.

Our first set of experiments aimed to characterize the developmental pattern of mouse meniscus. Some basic data were obtained including meniscal cell proliferation, apoptosis and the distribution of Col-1. Simultaneous proliferation and apoptosis of meniscal cells just before and after birth suggests an active tissue turnover in this period. One month after birth, the meniscal cells become quiescent as evidenced by few cells positive for PCNA and active caspase 3. Our follow-up study will focus on the potential changes in the pathways and molecules involved in angiogenesis, autophagy, senescence, etc. [37, 38]. We expect that these studies will lend novel insights into the meniscal development and regeneration, which may help us identify new therapeutic targets.

Primary meniscal cells can be easily isolated although they are heterogeneous, and they were treated with the selected factors that may be involved in joint formation and cartilage regeneration [39-41]. EPO shows the capability to enhance cell proliferation, matrix protein production and neoangiogenesis. In addition, it suppresses the expression of MMP-13, a major matrix degradation enzyme, and inhibits joint inflammation. These capabilities may facilitate regeneration of injured or degenerative menisci, and prevent OA formation. However, we don't exclude the possibility that optimal effect may be achieved by combined use of different factors.

Compared to cell culture, our organ culture model largely preserves the morphology and composition of mouse menisci. Sufficient amounts of RNA and protein may be harvested for multiple RT-PCR and western blotting after a few days of culture and treatments. In addition, tissue sections from cultured menisci may be prepared for histological and IHC staining. Although it is technically challenging to collect mouse menisci compared to large animals, it is less expensive, and more importantly, the findings made in mice facilitate future studies using genetically engineered mice.

Our *in vivo* study demonstrated that erythropoiesis was not a major issue after EPO administration. Early intervention with EPO in mice underwent MIO showed a superior efficacy to intermediate and late interventions. Early use of EPO enhanced meniscal regeneration and suppressed MMP-13 expression, thus, halting OA progression. *In vitro* experiments showed that EPO upregulated the expression of *Vegf-A*, a critical factor for angiogenesis. Formation of new blood vessels is pivotal for meniscal regeneration, especially for degenerative tears in the white zone. The major concern is that neoangiogenesis may affect synovial membrane, causing rheumatoid arthritis-like lesions. However, in our experiment, we did not find such pathological changes. Based on these finding, we postulate that satisfactory outcomes may be achieved in treating meniscal injury and ensuing OA after early application of EPO.

When used in a physiological dose, EPO induces erythropoiesis through its homodimer receptor with subsequent activation of different signaling pathways including JAK2-STAT and PI3K-AKT pathways [42]. However, a supra-physiological dose of EPO may signal through a heterodimeric receptor composed of an EPOR monomer and CD131 in non-elytroid cells. It is

through such a non-classical pathway that EPO exerts its tissue protective and regenerative effects with minimal hematopoietic activation [43-46]. In addition to tissue protective effect, EPO alleviates inflammation in collagen induced arthritis (CIA) and lowers serum level of TNF-α in CIA mice [47], which is consistent to our results. In a rat model of intervertebral disc herniation, asialo-EPO alleviates the pain-related behaviors [48].

In conclusion, we for the first time demonstrate that EPO is able to enhance meniscal cell proliferation, chondrogenesis and angiogenesis. Combined with its anti-inflammatory and pain-relieving properties, EPO is a promising agent that may become an effective DMOAD.

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Disclosure of conflict of interest

None.

Abbreviations

EPO, erythropoietin; OA, osteoarthritis; MIO, meniscus injury induced OA; Col, Collagen; DMOADs, disease modifying osteoarthtis drugs; WT, wild type mice; Veh, Vehicle; IHC, immunohistochemistry.

Address correspondence to: Dr. Tian-Fang Li, Department of Rheumatology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450000, China. Tel: +86-15713865716; E-mail: tfli@zzu.edu.cn

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