Macrophage migration inhibitory factor takes part in the lumbar ligamentum flavum hypertrophy

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Abstract. The present study aimed to observe the content difference of macrophage migration inhibitory factor [MIF; novoprotein recombinant human MIF (n-6his) (ch33)], TGFβ1 and MMP13 in patients with and without ligamentum flavum (LF) hypertrophy and investigate the roles of MIF in LF hypertrophy. The concentration of MIF, TGFβ1 and MMP13 in LF were detected by ELISA in a lumbar spinal stenosis (LSS) group and a lumbar disc herniation (LDH) group. Culture of primary LFs and identification were performed for the subsequent study. Cell treatments and cell proliferation assay by CCK-8 was performed. Western blot and quantitative PCR analysis were used to detect the expression of TGFβ1, MMP13, type I collagen (COL-1) and type III collagen (COL-3) and Src which were promoted by MIF. The concentration of MIF, TGFβ1 and MMP13 were higher in the LSS group compared with the LDH group. Culture of primary LFs and identification were performed. Significant difference in LFs proliferation occurred with treatment by MIF at a concentration of 40 nM for 48 h ($P<0.05$). The gene and protein expression of TGFβ1, MMP13, COL-1, COL-3 and Src were promoted by MIF ($P<0.05$). Proliferation of LFs was induced by MIF and MIF-induced proliferation of LFs was inhibited by PP1 (a Src inhibitor). MIF may promote the proliferation of LFs through the Src kinase signaling pathway and can promote extracellular matrix changes by its pro-inflammatory effect. MIF and its mediated inflammatory reaction are driving factors of LF hypertrophy.

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Introduction

Ligamentum flavum (LF) is a connective tissue and can be affected by hypertrophy, which is the major pathogenic factor of degenerative lumbar spinal stenosis (LSS) (1). The pathology of LF hypertrophy is a common fibrosis process of this connective tissue. Upon fibrosis, LF shows decreased elasticity and increased thickness, proliferation of effector cells and component and structural change in the extracellular matrix (2). Symptoms such as lower back pain, bladder and rectal dysfunctions are induced by nerves and blood vessels stimulated by hypertrophic LF in the spinal canal. The mechanism of LF hypertrophy remains to be elucidated.

Recently, the major pathological elements of LF hypertrophy have been proved to be mechanical and metabolic (3,4). Pathologically, the hypertrophy of LF is associated with scar repair secondary to mechanically induced micro-injury and metabolic accumulation mediated biochemical reaction, among which the inflammatory reaction is important (5,6). Inflammatory factors serve a vital role in the hypertrophy process of LF (7). TGFβ1 can significantly promote the proliferation of LF fibroblasts and the overexpression of collagen fibers, while MMP13 has a distinct function of elastic fiber degradation in the LF extracellular matrix (8,9). A connection exists between macrophage migration inhibitory factor [MIF; novoprotein recombinant human MIF (n-6his) (ch33)] and TGFβ1 and MMP (10). As a multipotent pro-inflammatory factor, MIF and its mediated inflammatory reaction are driving factors of LF hypertrophy. MIF can be activated by several factors, including high glucose, infection and hypoxia (11-13). Our previous study found that the concentration of MIF was positively associated with the thickness of LF but the mechanism remains to be elucidated (14). Thus, the present study aims to explore the MIF effect on LF fibroblasts to explore the potential mechanism of LF hypertrophy.

Materials and methods

Ethics and study subjects. This project was approved by Wuhan Municipal Health Commission Medical Research Ethics Committee (Wuhan, Hubei; approval no. 672HREC2020N01B). Written informed consents were acquired from all patients. Random and consecutive patients underwent surgery for single segment (L4/5) LSS with the LF thickness >4 mm and lumbar disc herniation (LDH) between February 2021 and July 2021 were included. Patients with spinal tumors, tuberculosis, infection, L4 spinal instability or spondylolisthesis were excluded. LF specimens removed from these two groups [lumbar spinal stenosis (LSS; n=12) and lumbar disc herniation (LDH; n=15)] were initially processed to measure the concentration of MIF, TGFβ1 and MMP13 by ELISA. Basic data of patients are in Table I.

ELISA. The ligamentum flavum tissue was milled with PBS and centrifuged with 10,000 x g at 4˚C for 15 min. The supernatant was collected. Protein concentrations were measured by the BCA kit (Beyotime Institute of Biotechnology). ELISA kits (Human MIF, cat. no. JL11770; Shanghai Future Industry Co., Ltd.; Human TGF-β1, cat. no. JL10706; Shanghai Future Industry Co., Ltd.; Human MMP 13, cat. no. JL12202; Shanghai Future Industry Co., Ltd.) were used following the manufacturer's instructions. The sample was added to the well and covered to incubate 1 h at 37˚C. The enzyme-labeled plate was taken out, the liquid was discarded and the biotinylated antibody working solution was added before covering and incubating for 1 h at 37˚C. The liquid was discarded, it was wash three times. The enzyme conjugate working solution was added, and the plate-sealing film was covered to incubate 30 min at 37˚C. The liquid was discard again and washed 5 times. Substrate was added and incubated 15 min at 37˚C in the dark. The stop solution was added and the concentrations of MIF, TGF-β1 and MMP-13 were measured under the wavelength of 450 nm.

Culture of primary LFs. The LF tissue from a 35 year old male patient who was recruited in February 2021 without other history of disease in the LDH group was harvested. It was washed three times with sterile physiologic saline containing penicillin and streptomycin. LF tissue was cut into 0.5 mm^3 and placed into a 15 ml centrifuge tube. LF tissue was digested with 0.2% type I collagenase at 37˚C for 1 h. Tissue was washed with low-glucose DMEM and centrifuged at 118 x g for 5 min at room temperature. LF tissue was evenly inoculated in a culture dish (Fig. 1A) with high-glucose DMEM supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C, 5% CO_2 under controlled humidity. When the cell growth area reached 80% area, passage was performed.

Human primary LFs identification. The third generation cells were first observed under the light microscope (three fields of vision were randomly selected and the typical images were shown in the figure; magnification, x200). The cells were fully

Table I. Basic information of LSS and LDH groups.

Characteristic	LSS (n=12)	LDH (n=15)	t|χ2	P-value
Age (year)	60.40±4.27	56.67±8.56	1.479	0.152
BMI	23.88±1.96	25.27±1.99	1.819	0.081
Male/Female	4\8	6\9	0.127	0.722

LSS, lumbar spinal stenosis; LDH, lumbar disc herniation.

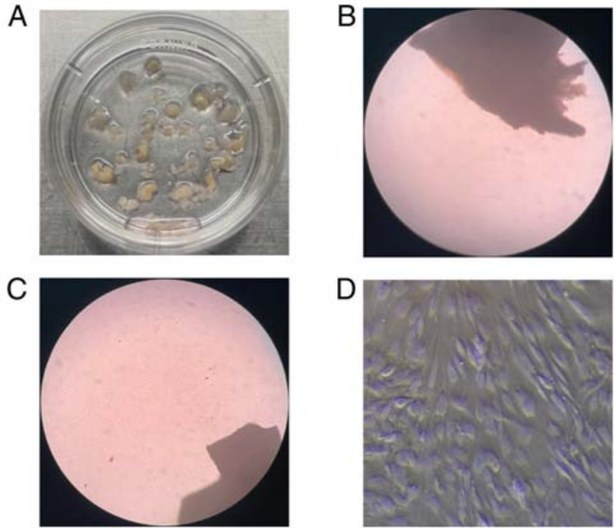

Figure 1. Growth process of primary cells. (A) LF tissues. (B) LFs migrated after 10 days (magnification, x40). (C) The cells reached ~80% confluence 14 days after LFs migration (magnification, x40). (D) Experimental LFs (magnification, x200). LF, ligamentum flavum.

digested by 0.25% trypsin and 0.02% EDTA (Dalian Meilun Biotech Co., Ltd.) The cells were transferred to a culture dish and the culture medium was removed when the cells grew to 90%. The cells were washed with PBS 3 times for 5 min each and then fixed with 4% paraformaldehyde solution for 10 min. The cells were washed with PBS for 3 times, 5 min per time. Then, the cells were permeabilized with 0.5% Triton (Beijing Biosynthesis Biotechnology Co., Ltd.) for 2 min and washed twice with PBS for 5 min per time. Cells were blocked with 1% BSA (Beijing Biosynthesis Biotechnology Co., Ltd.) for 30 mins at room temperature. Subsequently, the cells were incubated at 4˚C overnight with mouse anti vimentin monoclonal antibody (1:100; cat. no. bsm-33170M) and rabbit anti type I collagen (COL-1) polyclonal antibody (1:100; cat. no. bs-10423R), both from Beijing Biosynthesis Biotechnology Co., Ltd. Then the cells were incubated with FITC labeled Goat Anti-Mouse IgG and Goat Anti-rabbit IgG (1:50; cat. no. bs-0296G and bs-0295G respectively, Beijing Biosynthesis Biotechnology Co., Ltd.) for 1 h in the dark at room temperature. Nuclei were stained with 4', 6-diamino-2-phenylindole. Images were acquired using a laser confocal fluorescence microscope (magnification, x400).

Cell treatments. There was a two part cell treatment in the present study. In the first, LFs (50-60% confluence) were treated with 20 and 40 nM MIF at 37˚C for 24 or 48 h to observe

Table II. Primers used for reverse transcription-quantitative PCR.

Name		Primer Sequences (5'-3')	Melting Temperature, ˚	CG %
COL-1	Sense	AAGACAGTGATTGAATACAAAACCAC	58.6	34.6
	Antisense	GGGAGTTTACAGGAAGCAGACAG	60.9	52.1
COL-3	Sense	CAAGGCTGAAGGAAATAGCAAA	59.4	40.9
	Antisense	TCTCACAGCCTTGCGTGTTC	59.3	55
MMP13	Sense	CAGAACTTCCCAACCGTATTGAT	60.1	43.5
	Antisense	TGTATTCAAACTGTATGGGTCCG	59.5	43.5
TGFβ1	Sense	CAGCAACAATTCCTGGCGATA	61.2	47.6
	Antisense	GCTAAGGCGAAAGCCCTCAAT	62.7	52.4
Src	Sense	CCTCAACGTGAAGCACTACAAG	59.5	50
	Antisense	GGCGTGTTTGGAGTAGTAGGC	61	57
GAPDH	Sense	CATCATCCCTGCCTCTACTGG	59.4	57.1
	Antisense	GTGGGTGTCGCTGTTGAAGTC	60.1	57.1

COL, collagen.

the difference in the proliferation of the cells and record the MIF concentration and treatment time. For the second, LFs PP1 (5 μM; Dalian Meilun Biology Technology Co., Ltd.) was added to pretreat the cells for 1 h in a blank group (PP1 group) and MIF treatment group (MIF+PP1 group; treated by MIF at the concentration and time as the aforementioned). A control group was treated with the same amount of DMSO under basic medium.

Cell proliferation assay. Treated LFs were plated into a 96-well plate at a density of 4,500 per well and proliferation was measured by CCK-8 kit. (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. The absorbance at 450 nm was determined by a model 680 microplate reader (Bio-Rad Laboratories, Inc.). The experiment was repeated three times.

Western blot analysis. The cells were lysed in RIPA buffer with 1% phenylmethylsulfonyl fluoride. Total protein concentrations were measured by a BCA kit (Beyotime Institute of Biotechnology). Equal amounts of protein (25 μg) were separated on 8 or 10% SDS-PAGE gels (Dalian Meilun Biology Technology Co., Ltd.) and transferred onto polyvinylidene difluoride membranes (MilliporeSigma). The membrane was sealed with 5% skimmed milk powder for 1 h at room temperature. Then, the membranes were incubated with the corresponding primary antibodies (rabbit anti-human Src; cat. no. bs-1135R; rabbit anti-human TGFβ1; cat. no. bs-0086R; rabbit anti-humanMMP13; cat. no. bs-0575R; rabbit anti-human COL-1; cat. no. bs-7158R; rabbit anti-human COL-3; cat. no. bs-0549R; rabbit anti-human all from Beijing Biosynthesis Biotechnology Co., Ltd. Co., Ltd.) respectively, overnight at 4˚C, washed three times in Western Blot Wash Buffer (Beyotime Institute of Biotechnology) and incubated with the corresponding secondary antibody (anti-rabbit IgG-HRP linked antibody; 1:10,000; cat. no. 111-035-003 Jackson ImmunoResearch) for 1.5 h at room temperature. The membranes were washed three times in Wash Buffer and subjected to western blotting using ECL chemiluminescence kit (Beyotime Institute of Biotechnology). The immunoreactive protein was determined by chemiluminescent center detection, and software (image lab,6.1.0.07, Bio-Rad Laboratories) was used for densitometry.

Table III. Fibrotic factors in the LF between LSS and LDH groups. (pg/mg protein).

Factors	LSS (n=12)	LDH (n=15)	t	P-value
MIF	340.56±42.86	189.20±51.17	8.20	0.000
TGFβ1	3,045.60±595.79	2,185.50±734.57	3.28	0.003
MMP13	649.08±135.31	366.13±75.92	6.88	0.000

LF, ligamentum flavum; LSS, lumbar spinal stenosis; LDH, lumbar disc herniation; MIF, macrophage migration inhibitory factor [novoprotein recombinant human MIF (n-6his) (ch33)].

Reverse transcription-quantitative (RT-q) PCR. Total cell with density of $2x10^6$/well RNA was extracted using TRIzol reagent (cat. no. 15596026; Thermo Fisher Scientific, Inc.). According to the manufacturer's protocols, total RNA was reverse transcribed with mRNA reverse transcription kit (Bestar; cat. no. DBI-2220; DBI Bioscience) The resulting cDNA was used as template for RT-qPCR using SYBR-Green (cat. nos. QPK-201 and QPK-201T; Toyobo Life Science). Amplification with 95˚C 5 sec (denaturation), 55˚C 10 sec (annealing) and 72˚C 15 sec (extension) was followed by a melting curve analysis with continual fluorescence data acquisition during the 55-95˚C melt. The threshold cycle (CT) values were normalized to GAPDH and the relative expression was calculated by the $\Delta\Delta$Cq method (15). The RT-qPCR primer sequences are shown in Table II. The experiment was repeated three times.

Drugs and treatments. MIF was dissolved in DMEM incomplete culture medium. PP1 (cat. no. MB3961; Dalian Meilun

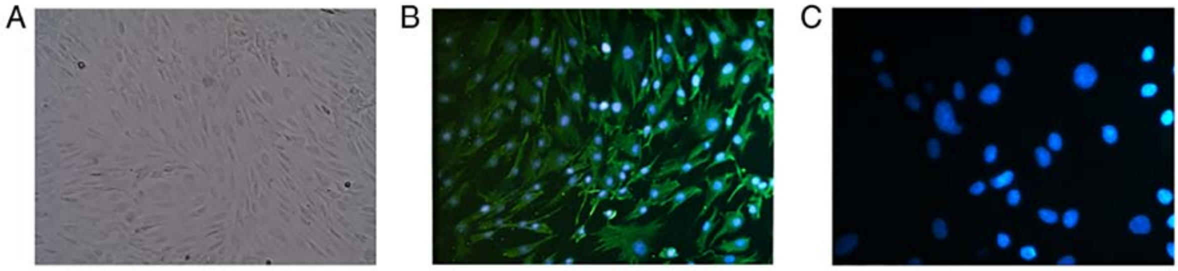

Figure 2. Experimental LF identification. (A) LF cells under an optical microscope (magnification, 200x) showing that most fibroblasts were long and spindle shaped with oval nucleus and abundant cytoplasm. (B) Vimentin and (C) type I collagen were visualized using immunofluorescence staining (magnification, x400). LF, ligamentum flavum.

Biotech Co., Ltd.), the Src family inhibitor, was dissolved in DMSO with the concentration of working solution <1.0%.

Statistical analysis. Statistical data was analyzed by GraphPad 6 (GraphPad Software, Inc.) and SPSS 16.0 (SPSS, Inc.) software. Mean ± standard deviation were applied to describe all quantitative data. Quantitative data of normal distribution was compared using independent t-tests. Chi-square test was applied for comparison of male/female rate between two groups. Comparison among the three and four groups was assessed using one-way ANOVA and LSD and Bonferroni post hoc tests were used following ANOVA. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Concentration of fibrotic factors in the LF specimens. The concentration of MIF, TGFβ1 and MMP13 were higher in LSS group compared with the LDH group. (Table III).

Primary cell culture and identification. The LF tissues (~0.5 mm^3) were placed onto culture dish (Fig. 1A). LF cells (LFs) migrated out of the tissues a week later (Fig. 1B). LF tissues was removed when the LFs growth occupied ~50% of the area of culture dish (Fig. 1C) and passage was performed. The LFs were identified on the third passage (Fig. 1D). LFs identification were performed by microscopic observation (Fig. 2A) and immunofluorescence (Fig. 2B and C) analysis.

Time and concentration of MIF treatment for LFs proliferation. Fibroblast proliferation is the vital pathological process in the hypertrophy of LF. The time of proliferation of LF cells under MIF treatment was unclear; two different MIF concentrations (20 and 40 nM) were used for 24 and 48 h. The proliferation condition of LFs was tested by CCK8 following MIF treatment. No significant difference in proliferation was observed in cells treated by MIF at the concentration of 20 and 40 nM for 24 h, while significant difference was observed with the concentration of 40 nM for 48 h (Fig. 3).

MIF promotes expression of TGFβ1, MMP13, COL-1 and COL-3 and Src. To investigate the effect of MIF on protein expression of LFs, LFs were stimulated with different concentrations of human recombinant MIF to observe the expression of important downstream LF fibrosis factors and the condition of collagen deposition. LFs were treated with

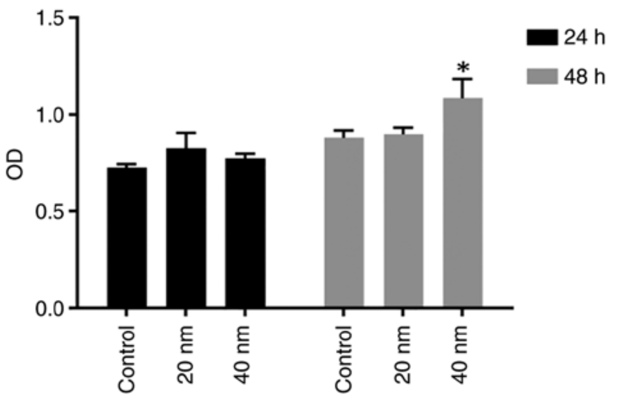

Figure 3. MIF promotes LFs proliferation at 40 nM concentration for 48 h. OD value of CCK-8 showed no significant differences for each concentration over 24 h. However, significant difference was found when stimulated with MIF at 40 nM concentration for 48 h (*$P<0.05$ vs. Control and 20 nM group in 48 h). OD, optical density; MIF, macrophage migration inhibitory factor [novoprotein recombinant human MIF (n-6his) (ch33)].

MIF at 20 and 40 nM for 48 h and, compared with the control groups, the expression of TGFβ1, MMP13, COL-1, COL-3 and Src in the MIF stimulated groups were significantly increased and those in 40 nM MIF treatment were more evident (Figs. 4 and 5).

Src kinase is involved in LFs proliferation induced by MIF. The experimental LFs were divided into control group, MIF group (40 nM recombinant MIF treatment), PP1 group (Src kinase specific antagonist 5 μM PP1 pretreatment for 1 h) and MIF+PP1 group (MIF combined with PP1) and were treated for 48 h. The purpose of the experiment was to explore whether Src kinase was involved in MIF-induced fibroblasts proliferation. Accordingly, the experiment demonstrated that the efficacy of MIF in promoting cell proliferation decreased following PP1 intervention (Fig. 6).

Discussion

MIF level in hypertrophic LF was higher compared with normal LF and the concentration of TGF β 1 and MMP13 also showed this trend in hypertrophic LF. MIF can promote LFs to secrete LF hypertrophy factors such as TGFβ1 and MMP13. The former can directly promote the expression of the main components of matrix collagen fibers, which were

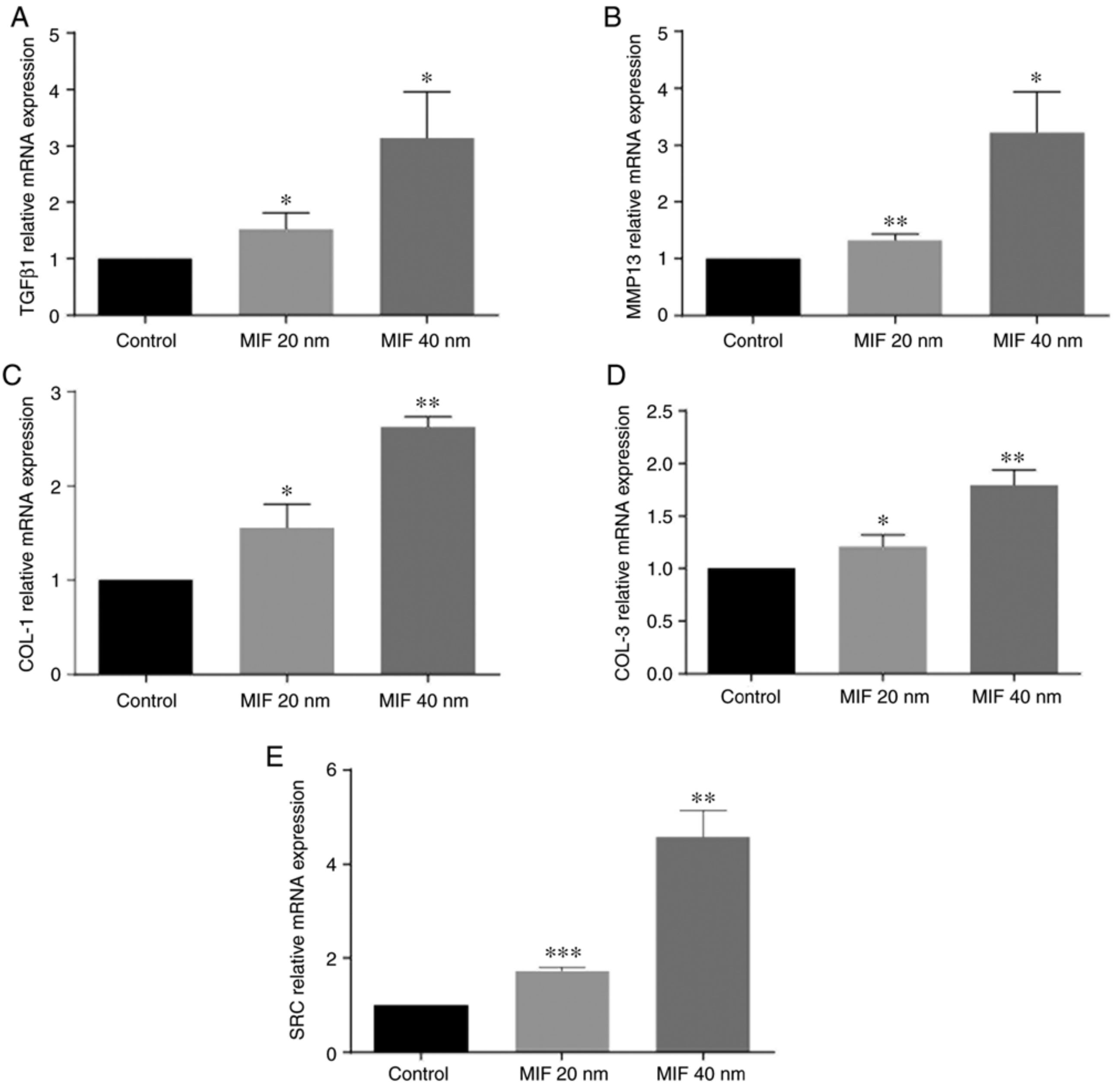

Figure 4. MIF promotes mRNA expression of fibrotic related factors. The gene expression levels of (A) TGFβ1, (B) MMP13, (C) COL-1, (D) COL-3 and (E) Src were significantly increased with increasing concentration of MIF treatment. (*P<0.05, **P<0.01, ***P<0.001 vs. Control). COL, collagen; MIF, macrophage migration inhibitory factor [novoprotein recombinant human MIF (n-6his) (ch33)].

composed of type I collagen (COL-1) and type III collagen (COL-3) and the latter can degrade the elastic fibers in LF extracellular matrix. MIF can promote LFs proliferation with concentration-dependent. The effect of MIF in promoting LFs proliferation was decreased by PTK inhibitor, so MIF may promote the proliferation of LFs through the Src kinase signaling pathway. MIF and related inflammatory reactions take part in the procedure of LF hypertrophy and MIF may occupy the dominant role.

The lumbar LF is a connective tissue with the thickness <4 mm. Anatomically, it connects the upper and lower vertebral lamina and covers the central spinal canal, the nerve root canal and the dorsal part of the intervertebral foramen (16). The LF mainly consists of fibroblast and extracellular matrix secreted by it (and most of the original secretory type fibroblasts) transform into stable fibroblast when LF tissue becomes mature (2). The extracellular matrix is mainly constituted of elastic fibers, collagen fibers, proteoglycans and glycoproteins. Elastic fibers and collagen fibers are mixed with a ratio of 4:1 (16). The collagen fibers are mainly comprised of type I collagen (COL-1) and type III collagen (COL-3) (16,17). The pathological manifestations of LF hypertrophy are stable fibroblast transforming into secretory type fibroblast to proliferate in the LF (16). Inflammatory reactions mediated by inflammatory cell infiltration cause overexpression of collagen fibers in the matrix, degradation of elastic fibers, aggregation of endothelial cells and microvascular neogenesis accelerated scar formation in the LF tissues (5). Specifically, elastic fibers are degraded, collagen fibers are increased and proliferous collagen fibers are arranged in disorder. These microstructural

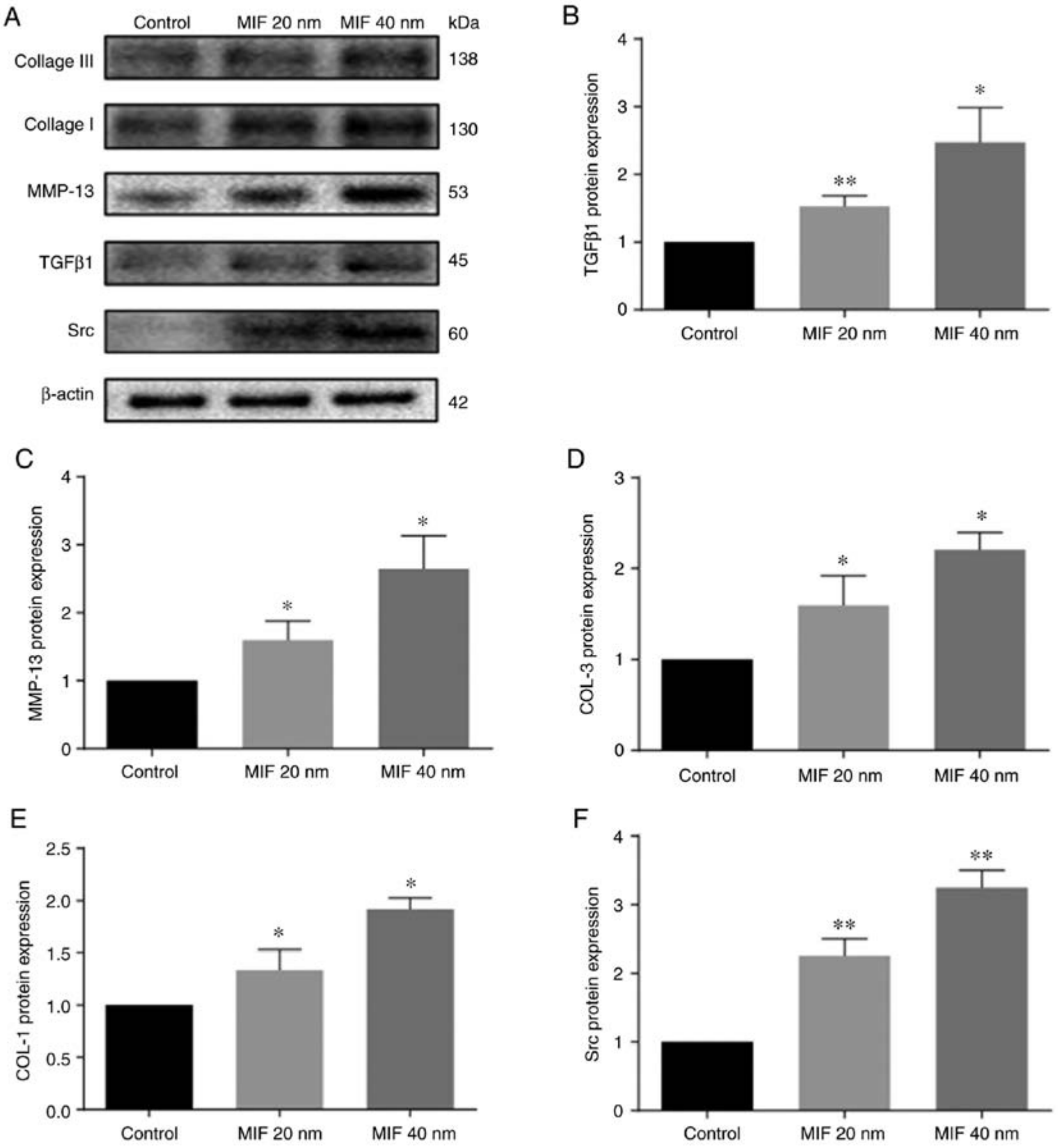

Figure 5. MIF promotes protein expression of fibrotic related factors. (A) Densitometric analysis of protein expression by western blot analysis. The protein expression of (B) TGFβ1, (C) MMP13, (D) COL-1, (E) COL-3 and (F) Src were significantly increased with higher concentration of MIF treatment. (*P<0.05, **P<0.01 vs. Control). COL, collagen; MIF, macrophage migration inhibitory factor [novoprotein recombinant human MIF (n-6his) (ch33)].

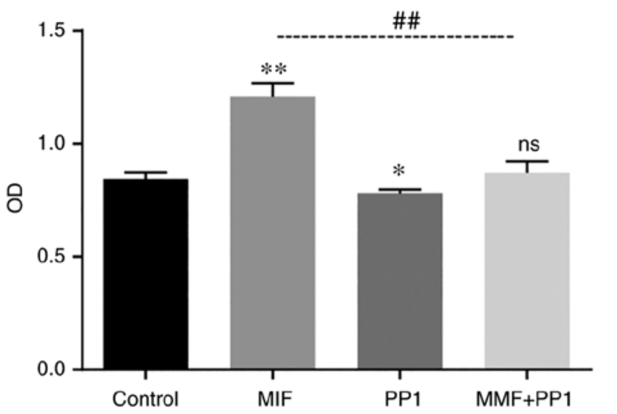

Figure 6. OD value increased in MIF group as compared with that in control group (**P<0.01 vs. Control). OD value decreased in the PP1 groups (*P<0.05 vs. Control). Furthermore, the OD value in MIF + PP1 groups showed decreased as compared with that in the MIF groups (##P<0.01), which showed no statistically different with that in control group (ns; P>0.05 vs. Control). OD, optical density; MIF, macrophage migration inhibitory factor [novoprotein recombinant human MIF (n-6his) (ch33)].

changes affect matrix remodeling, which result in an increase in the thickness of the LF tissue, decrease in elasticity and increase in brittleness (18). Hence, Lumbar, lower extremities pain and numbness and bladder and bowel disorder induced by intraspinal neurovascular stimulation secondary to the stenosis of the central spinal canal, nerve root canal (lateral recess) and intervertebral foramen, which have close association with the hypertrophy of lumbar LF (19).

Although sex, obesity, spinal mechanics and metabolic substances factors are influential elements in LF hypertrophy, studies on the effect of MIF on hypertrophy are rare (20-22). Other factors need to be first controlled as much as possible during the experimental design of MIF effects. Mechanics stress is a vital element during the LF hypertrophy procedure on lumbar region. LDH mostly occurs in L5/S1 and L4/5 and LSS mostly occurs in L4/5 segment (23). Therefore, in order to control the mechanics stress influence in present study, all LF samples were taken from the same segment (L4/5), excluding patients with instability or spondylolisthesis of L4. Despite

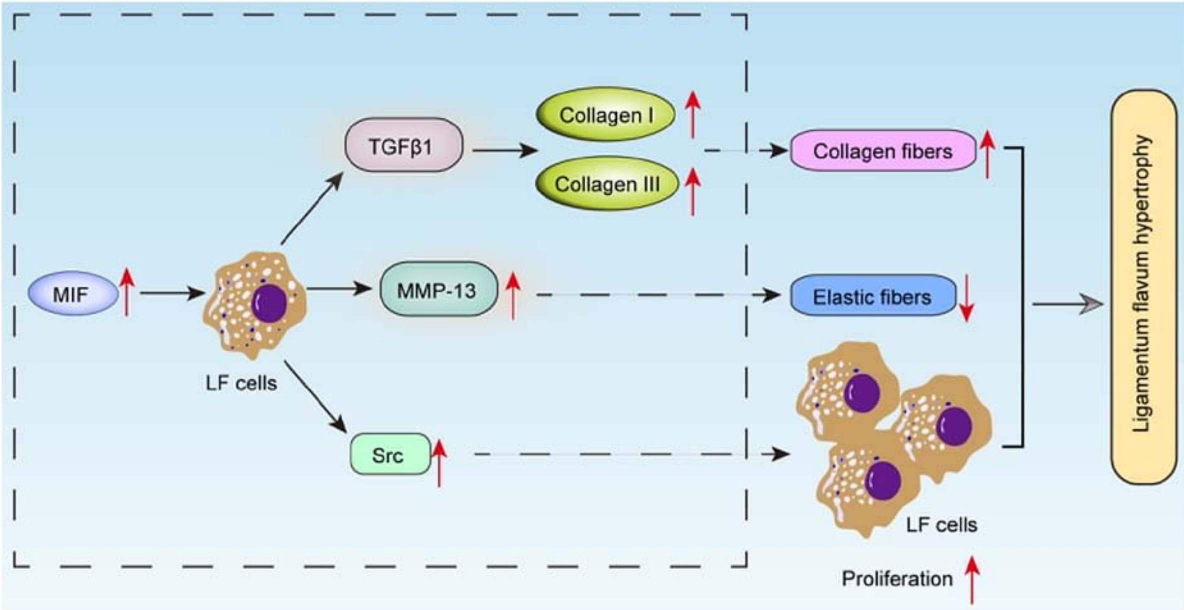

Figure 7. Mechanism schematic diagram showing how MIF involves in the pathogenesis of ligamentum flavum hypertrophy. MIF, macrophage migration inhibitory factor [novoprotein recombinant human MIF (n-6his) (ch33)]; LF, ligamentum flavum.

the ventral side of LF being furnished with little blood supply, the upper, lower and dorsal part can directly contact with the blood supplied periosteum and muscles. Therefore, patients with tumors, tuberculosis, infection or metabolic disease were excluded to reduce the hematogenous influence in this study. Coincidentally, participants revealed no statistically significant difference in sex and BMI between two groups in this study.

Inflammatory mediators serve major roles in the fibrosis of LF hypertrophy (7). The content of MIF measured by ELISA in the study tended to be higher in the hypertrophic LF samples. It has been confirmed that IL1, IL-6 and TNF-α are the downstream factors of MIF (24). Among them, IL1 and IL-6 can promote the expression of COL-1 and TNF-α promotes the expression of type III collagen of LFs (7). Theoretically, MIF also can affect the fibroblasts of LF. Furthermore, TGFβ1, one of the most closely related factors to LF hypertrophy, can not only promote the proliferation of fibroblasts but also contribute to the COL-1 and COL-3 collagen fibers in LF (8). MMP has the function of dissociating the extracellular matrix (9). Sugimoto *et al* (25) suggested that MMP is the major degradation factor for elastic fiber of LF, mainly MMP13, MMP2 and MMP9. Studies related to rheumatoid and cardiovascular explain that MIF can promote the expression of MMP and TGFβ1 (10,26). In the present study, higher concentration of TGFβ1 (3,045.60±595.79 vs. 2,185.50±734.57 pg/mg protein) and MMP13 (649.08±135.31 vs. 366.13±75.92 pg/mg protein) were found in the hypertrophic LF of patients. MIF concentration differences (340.56±42.86 vs. 189.20±51.17 pg/mg protein) were also discovered in LF specimens between the LSS and LDH groups. MIF concentration in hypertrophic LF of LSS patients was higher compared with that in LDH groups. TGFβ1and MMP13 also shared this characteristic. The effect of higher level MIF on the LF and its influence on the expression of TGFβ1 and MMP13 in LFs remains to be elucidated.

Classified as a multipotent cytokine with enzymatic, chemokine and hormonal properties, MIF can promote cell proliferation, directly mediate inflammation and participate in multiple organ fibrosis processes (27). A number of types of cell proliferation can be promoted by MIF. The MIF promoter region is a type of multipotent proinflammatory cytokine which contains DNA binding sites binding to a variety of transcription factors, so MIF is able to promote the release of a number of inflammatory factors such as TNF-α, IL-1β, IL-6, IL-8 and IL-12 and the synthesis of several matrix metalloproteinases, MMP-1, MMP-3, MMP-9 and MMP-13 (22,28). The effect of MIF on the proliferation and fibrosis factor expression of ligamentum flavum fibroblasts has not been reported to the best of the authors' knowledge. Given the reported biological abilities of MIF and the similar enhancement trend of TGFβ1 and MMP13 in LF samples of LSS group, it was hypothesized that MIF could promote LFs proliferation and the expression of the two typical fibrotic factors associated with the hypertrophy of LF tissues. Primary LFs were obtained from one patient of the LDH group (the aforementioned 35 year old). In order to explore the time of the reaction of LFs under the intervention of different concentrations of MIF, two different MIF concentrations (20 and 40 nM) were used for 24 and 48 h. The results showed that there was a significant difference in cell proliferation at the concentration of 40 nM for 48 h. Therefore, the intervention time for subsequent experiments was set at 48 h. In *in vitro* LFs experiments TGFβ1 and MMP13 showed concentration dependent increases at genetic and protein levels with subsequent increase of COL-I and COL-III following stimulation with MIF at 20 and 40 nM for 48 h (Figs. 5 and 6). In addition to the increase of fibrosis factor, it was also found that the Src kinase gene and protein level were closely associated with proliferation also increased significantly.

As a core family member of the Src family kinases (SFKs), Src kinase is a group of intracellular non-receptor tyrosine kinases involved in the regulation of cell proliferation, differentiation, angiogenesis and other biological behaviors (29). In normal tissues, Src kinase is the signal transduction center that coordinates cellular responses to extracellular stimulation (30). As a ligand for several growth factor receptors, including fibroblast growth factor receptors, epidermal growth factor receptor and platelet-derived growth factor receptor, Src can promote cell proliferation and differentiation (30,31). Furthermore, Src can be activated by MIF and high blood glucose (32,33). A study on cardiovascular diseases indicates that tyrosine kinases in SFKs are recruited to activate ERK1/2 by phosphorylation after MIF combines with its receptors (34). In the present study, LFs stimulated by MIF (40 nM) for 48 h *in vitro* showed the same phenomenon of LFs proliferation compared with the control group. From qPCR and western blotting results, MIF could promote the expression of Src kinase in LFs. In order to explore the relationship between Src kinase stimulated by MIF and the proliferation of LFS, the specific SRC antagonist PP1 was used. The results showed that the proliferation of LFS induced by MIF was also inhibited by PP1, which suggested that MIF promoted the proliferation of CFs through Src kinase signal transduction pathway.

It was concluded that MIF promoted LFs to express the vital fibrosis related factors TGFβ1 and MMP13 (Fig. 7). TGFβ1 could increase COL-I and COL-III of collagen fiber and MMP13 decreased the elastic fiber in LF. MIF also stimulated the expression of Src kinase to promote the LFs proliferation. These processes contributed to the LF hypertrophy. There were some experimental limitations in the present study. The sample size was small for lumbar spinal stenosis (LSS) and lumbar disc herniation (LDH) patients. The *in vitro* LFs experiments cannot exactly simulate MIF concentration and accurate time treatment for human LF fibroblasts. The mechanism of MIF promoting the expression of fibrosis TGFβ1 and MMP13 require further study. CD74, CXCR2, CXCR4 and CXCR7 are the receptors for MIF and CD74 is more common (35,36). The receptors blocked or knocked down will be a useful direction of further research. LFs can be directly affected by peripheral blood, so whether the higher level of MIF in hypertrophic LFs is exogenous or of local origination is also a direction worthy of exploration.

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Availability of data and materials

The raw data and materials generated and used during this study are available from the corresponding author upon reasonable request.

Authors' contributions

QLL, ZXZ and YHY designed the present study from literature search and manuscript preparation. JYL, YQZ and CS performed the clinical and experimental studies. CJX, FX and GXY designed the present study, performed data acquisition, data analysis and statistical analysis. FX and GXY confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by Wuhan Municipal Health Commission Medical Research Ethics Committee (Wuhan, Hubei; approval no. 672HREC2020N01B). Written informed consents were acquired from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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