IGFBP5 enhances osteogenic differentiation potential of periodontal ligament stem cells and Wharton's jelly umbilical cord stem cells, *via* the JNK and MEK/Erk signalling pathways

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Abstract

Objectives: Mesenchymal stem cell (MSC)-mediated tissue regeneration represents a promising strategy for repair of tissue defects, but its molecular mechanisms remain unclear, restricting the use of MSCs. Our previous study indicated that insulin-like growth factor-binding protein 5 (*IGFBP5*) exerted a valuable effect on osteogenic differentiation of MSCs, but its molecular mechanisms underlying directed differentiation remained unclear. In this study, we have investigated the molecular role of *IGFBP5* in regulating this osteogenic differentiation potential.

Materials and methods: Periodontal ligament stem cells (PDLSCs) were isolated from periodontal ligament tissue. Wharton's jelly of umbilical cord stem cells (WJCMSCs) was obtained commercially. Lentiviral *IGFBP5* shRNA was used to silence *IGFBP5*. Retroviruses expressing wild-type *IGFBP5* were used to overexpress *IGFBP5* in the WJCMSCs. Recombinant human *IGFBP5* protein (rh*IGFBP5*) was used to treat PDLSCs for 24 h. Western blot analysis was used to detect the MAPK signalling pathway, and alkaline phosphatase (ALP) activity, Alizarin Red staining and quantitative calcium analysis were used to study osteogenic differentiation potentials.

Results: Overexpression of *IGFBP5* or rh*IGFBP5* increased expression levels of phosphorylated c-Jun N-terminal kinase (p-JNK), phosphorylated mitogen-activated protein kinase 1 and 2 (p-MEK1/2) and phosphorylated extracellular regulated protein kinases (p-Erk1/2) in both WJCMSCs and PDLSCs. Consistently, silenced *IGFBP5* was found to effectively inhibit expression of p-JNK, p-Erk1/2 and p-MEK1/2 in PDLSCs and WJCMSCs. Furthermore, inhibition of JNK by its inhibitor, SP600125, or MEK/Erk signalling by its inhibitor, PD98059, dramatically blocked *IGFBP5*-enhanced ALP activity and in vitro mineralization in both PDLSCs and WJCMSCs.

Conclusions: Our results demonstrated that *IGFBP5* promoted osteogenic differentiation potentials of PDLSCs and WJCMSCs *via* the JNK and MEK/Erk signalling pathways.

1 | INTRODUCTION

Mesenchymal stem cells (MSCs) were originally identified in the bone marrow. More recently, they have also been obtained from

numerous mesenchymal tissue types, including adipose tissue, umbilical cord tissue, vascular tissue, and the dental and craniofacial tissues.^{1–4} With their convenient isolation, self-renewal capability, and low immunogenicity, MSCs seem to provide a good therapeutic method for tissue regeneration applications. Recently, some MSCs have been isolated from the dental tissues, including stem cells from

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the periodontal ligament (PDLSCs), dental pulp stem cells (DPSCs) and stem cells from the apical papilla (SCAPs).^{5–7} In contrast to bone marrow stem cells, these cells are more intimately associated with dental tissues.^{5–7} Among studied stem cell populations, PDLSCs seem to be the most capable of regenerating typical cementum/periodontal ligament-like structures in vivo.^{8–10} However, the source of the PDLSCs is limited. WJCMSCs are of epiblast origin and can generate colonies of fibroblastic and osteogenic cells. They possess multipotent properties between those of embryonic stem cells and adult stem cells.^{11,12} Furthermore, they expand faster than adult-derived MSCs and are considered an ample seed cell source and are well recognized for their use in clinical applications, making them an ideal and reliable resource for tissue engineering.³ However, WJCMSCs possess a more limited osteogenic differentiation ability, and they, therefore, need to be further optimized for hard-tissue regeneration.^{3,13–15} This presents a challenge, as the molecular mechanisms of directed differentiation remain unclear. Thus, elucidating the mechanisms will be instructive for their further applications.

In a previous study, we found that the PDLSCs possessed superior properties of osteogenic differentiation. We compared the gene expressions of WJCMSCs and PDLSCs by microarray analysis to screen the candidate genes for their ability to enhance the osteogenic differentiation potential of WJCMSCs, and we identified the up-regulated expression of insulin-like growth factor-binding protein 5 (*IGFBP5*) in PDLSCs compared with WJCMSCs.¹⁵ Research has indicated that *IGFBP5* is a potential factor associated with bone metabolism.^{16–18} Our research also showed that *IGFBP5* enhanced the osteogenic differentiation potentials of MSCs.¹⁹ Previous reports found that *IGFBP5* could activate the mitogen-activated protein kinase (MAPK) and protein kinase B (Akt) signalling pathways in cell biological processes.^{20–26} *IGFBP5* also exerted an effect on the process of cell differentiation as an exocrine factor involved in the activated Akt or JNK/MAPK pathways.^{27,28} However, it is still unknown how *IGFBP5* regulates the osteogenic differentiation of the MSCs.

Here, we used WJCMSCs and PDLSCs to investigate the mechanism of *IGFBP5* in their osteogenic differentiation potential. Our results revealed that *IGFBP5* activated the JNK and MEK/Erk signalling pathways and that blocking JNK or MEK/Erk signalling limited the osteogenic differentiation potential of the MSCs. Our findings identified the underlying mechanism for the function of *IGFBP5*-mediated osteogenic differentiation in MSC biology.

2 | MATERIALS AND METHODS

2.1 | Cell cultures

All research involving human stem cells complied with the ISSCR “Guidelines for the Conduct of Human Embryonic Stem Cell Research.” Impacted third molars were collected from six healthy male patients (16–20 years old) under approved guidelines set by the Beijing Stomatological Hospital of the Capital Medical University, with informed consent. Teeth were first disinfected with 75% ethanol and

then washed with phosphate-buffered saline. PDLSCs were isolated, cultured and identified as previously described.^{15,28} Briefly, PDLSCs were separated from the periodontal ligament in the middle third of the root. Subsequently, the periodontal ligament tissue was digested in a solution of 3 mg/mL collagenase type I (Worthington Biochemical Corp., Lakewood, NJ, USA) and 4 mg/mL dispase (Roche Diagnostics Corp., Indianapolis, IN, USA) for 1 hour at 37°C. Single-cell suspensions were obtained by cell passage through a 70 µm strainer (Falcon, BD Labware, Franklin Lakes, NJ, USA). Human WJCMSCs were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA). MSCs were grown in a humidified incubator under 5% CO₂ at 37°C in DMEM alpha-modified Eagle’s medium (Invitrogen, Carlsbad, CA, USA), supplemented with 15% foetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 2 mmol/L glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen). The culture medium was changed every 3 days. MSCs at passage 3–5 were used in subsequent experiments (Fig. S1). The characterization of human WJCMSCs and PDLSCs was identified as previously described.¹⁵ Human embryonic kidney 293T cells were maintained in complete DMEM medium with 10% foetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. For SP600125, PD98059 (Cell Signaling Technology, Beverly, MA, USA) or rhIGFBP5 (R&D system, Minneapolis, MN, USA) treatment, MSCs were starved for 24 hours to synchronize the cells in the DMEM alpha-modified Eagle’s medium without serum, then changed to routine culture medium or osteogenic-inducing medium (StemPro Osteogenesis Differentiation Kit; Invitrogen) and treated with the SP600125, PD98059 or rhIGFBP5. All experiments were repeated three times.

2.2 | Plasmid construction and viral infection

The plasmids were constructed using standard methods; all structures were verified by the appropriate restriction digestion and/or sequencing. Human full-length *IGFBP5* cDNA from PDLSCs fused to a M2-FLAG tag was produced using a standard PCR protocol. This sequence (Flag-*IGFBP5*) was subcloned into the pQCXIN retroviral vector with AgeI and BamHI restriction sites. Short hairpin RNAs (shRNA) with the complementary sequences of the target genes were subcloned into the pLKO.1 lentiviral vector (Addgene, Cambridge, MA, USA). Viral packaging was prepared according to the manufacturer’s protocol (Clontech Laboratories, Addgene). For viral infections, MSCs were plated overnight and then infected with retroviruses or lentiviruses in the presence of polybrene (6 µg/mL; Sigma-Aldrich, St. Louis, MO, USA) for 6 h. After 48 h, infected cells were selected with different antibiotics. A scrambled shRNA (Scramsh) used as a control was purchased from Addgene. The target sequences for the shRNA were the *IGFBP5* shRNA (*IGFBP5*sh), 5′-CTCCCATTCTCCAATGACT-3′. Ectopic *IGFBP5* overexpression in WJCMSCs and the knockdown efficiency in the transduced PDLSCs were confirmed by real-time RT-PCR and Western blot analysis as previously described.¹⁹ Ectopic *IGFBP5* overexpression in WJCMSCs, the knockdown efficiency in the transduced PDLSCs and the knockdown efficiency in the transduced WJCMSCs were confirmed by Western blot analysis.

2.3 | Western blot analysis

Cells were lysed in RIPA buffer (10 mmol/L Tris-HCl, 1 mM EDTA, 1% sodium dodecyl sulphate [SDS], 1% NP-40, 1: 100 proteinase inhibitor cocktail, 50 mmol/L β -glycerophosphate, 50 mmol sodium fluoride). Twenty-five micrograms of protein of each sample was used for the Western blot analysis. The samples were separated on a 15% SDS polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes with a semi-dry transfer apparatus (BioRad, Hercules, CA, USA). The membranes were blotted with 5% dehydrated milk for 1 hour and then incubated with primary antibodies overnight. The immune complexes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Promega, Madison, WI, USA) and visualized with SuperSignal reagents (Pierce, Rockford, IL, USA). Primary antibodies, a monoclonal antibody against SAPK/JNK (Cat No. 9253), a monoclonal antibody against phospho-SAPK/JNK (Cat No. 4668), a monoclonal antibody against Akt (Cat No. 4685), a polyclonal antibody against phospho-Akt (Cat No. 9271), a monoclonal antibody against extracellular regulated protein kinase 1/2 (Erk1/2) MAPK (Cat No. 4695), a monoclonal antibody against phospho-Erk1/2 MAPK (Cat No. 4377), a monoclonal antibody against p38 MAPK (Cat No. 8690), a monoclonal antibody against phospho-p38 MAPK (Cat No. 4631), a monoclonal antibody against MEK1/2 (Cat No. 8727), a monoclonal antibody against phospho-MEK1/2 (Cat No. 9154), and a monoclonal antibody against M2-FLAG (Clone No. 9A3, Cat No. 8146) were purchased from Cell Signaling Technology. Other primary antibodies were purchased commercially, including the polyclonal antibody against IGFBP5 (Cat No. sc-13093; Santa Cruz Biotechnology, Dallas, TX, USA). We also used a primary monoclonal antibody to detect the housekeeping protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Clone No. GAPDH 71.1, Cat No. G8795; Sigma-Aldrich).

2.4 | Alkaline phosphatase and alizarin red detection

MSCs were grown in osteogenic-inducing medium using the StemPro osteogenesis differentiation kit (Invitrogen). MSCs with/without the inhibitor or rhIGFBP5 treatment were cultured for several days before collection. After 4 days of induction, the ALP activity was assayed with an ALP activity kit using 5 μ L protein lysate according to the manufacturer's protocol (Sigma-Aldrich). Signals were normalized based on the protein concentrations. For detecting mineralization, cells were induced for 2 or 3 weeks, fixed with 70% ethanol and stained with 2% Alizarin Red (Sigma-Aldrich). To quantitatively determine the calcium levels, Alizarin Red was destained with 10% cetylpyridinium chloride in 10 mmol/L sodium phosphate for 30 minutes at room temperature. The concentration was determined by measuring the absorbance at 562 nm on a multiplate reader and comparing it to a standard calcium curve with calcium dilutions in the same solution. The final calcium level in each group was normalized to the total protein concentration detected in a duplicate plate.

2.5 | Statistics

All statistical calculations were performed using SPSS10 statistical software. Student's *t* test or one-way ANOVA was utilized to determine the statistical significance; a *P* value of ≤ 0.05 was considered significant.

3 | RESULTS

3.1 | IGFBP5 increases JNK and MEK/Erk phosphorylation in WJCMSCs and PDLSCs

First, to elucidate the function of *IGFBP5* in MSCs, we inserted the Flag-*IGFBP5* sequence into a retroviral vector, which was then transduced into WJCMSCs via retroviral infection (Fig. S1a). We then used Western blot analysis to test for ectopic IGFBP5 overexpression in transduced WJCMSCs (Fig. 1a). To investigate how *IGFBP5* enhanced the osteogenic differentiation of MSCs, we performed Western blot analysis to examine the levels of proteins including p38, Erk1/2, JNK, Akt and MEK1/2. The results showed that the overexpression of *IGFBP5* could enhance the phosphorylation of JNK, Erk1/2 and MEK1/2 in the WJCMSCs (Fig. 1a,c), while the phosphorylation of p38 and Akt and the total protein levels of JNK, Erk1/2, p38 MAPK, Akt and MEK1/2 were not affected (Fig. 1a,b). Then, we used 0.5 ng/mL exogenous recombinant IGFBP5 (rhIGFBP5) to treat PDLSCs for 24 hours (Fig. S1b) and performed Western blot analysis to test the protein expression levels. The results showed that rhIGFBP5 could increase phospho-JNK, phospho-ERK1/2 and phospho-MEK1/2 in the PDLSCs (Fig. 1d,e), while the total protein levels of JNK, Erk1/2 and MEK1/2 were not affected (Fig. 1d).

To further confirm the signalling pathway involved in *IGFBP5* upon osteogenic differentiation in MSCs, we designed a short hairpin RNA (shRNA) to target *IGFBP5* and introduced it into the PDLSCs via lentiviral infection (Fig. S1d). After selection, the knockdown efficiency was verified by Western blot (Fig. 2a). Next, we detected the protein levels by Western blotting, and the results showed that the knockdown of *IGFBP5* inhibited phospho-JNK, phospho-ERK1/2 and phospho-MEK1/2 in the PDLSCs (Fig. 2a,c); however, the total amounts of JNK, Erk1/2, p38 MAPK, Akt and MEK1/2 proteins and amounts of phospho-p38 and phospho-Akt were not affected (Fig. 2a,b). We also silenced *IGFBP5* expression in the WJCMSCs via lentiviral infection, and the knock-down efficiency was verified by Western blot (Fig. S1c, Fig. 2d). Consistently, silencing *IGFBP5* decreased the expression levels of phospho-JNK, phospho-ERK1/2 and phospho-MEK1/2 in the WJCMSCs (Fig. 2d,e) and had no effect on the total amounts of JNK, ERK1/2 and MEK1/2 (Fig. 2d).

3.2 | IGFBP5-enhanced osteogenic differentiation of WJCMSCs or PDLSCs is repressed by a JNK inhibitor

First, MSCs were treated with 10, 20 or 50 μ mol/L SP600125, a specific inhibitor of JNK, for 48 hours to block the JNK signalling in the WJCMSCs. Western blot analysis indicated that 20 μ mol/L SP600125 effectively blocked the JNK signalling (Fig. 3a,b). Then, transduced WJCMSCs were cultured in osteogenic-inducing medium with

20 $\mu\text{mol/L}$ SP600125. Four days after induction, the results showed that 20 $\mu\text{mol/L}$ SP600125 could strongly suppress ALP activity in the WJCMSC-Flag-IGFBP5 cells (Fig. 3c). Three weeks after induction, Alizarin Red staining and quantitative calcium measurements revealed that the mineralization was significantly decreased after treatment of 20 $\mu\text{mol/L}$ SP600125 in WJCMSC-Flag-IGFBP5 cells (Fig. 3d,e).

To determine if IGFBP5 had similar functions in the PDLSCs, we used rhIGFBP5 to pretreat PDLSCs for 24 hours, after which PDLSCs

were cultured in osteogenic-inducing medium with 20 $\mu\text{mol/L}$ SP600125 and rhIGFBP5. Four days after induction, the ALP activity results showed that 20 $\mu\text{mol/L}$ SP600125 could markedly inhibit rhIGFBP5-enhanced ALP activity in the PDLSCs (Fig. 3f). After 2 weeks of induction, Alizarin Red staining and quantitative calcium measurements revealed that mineralization formation was decreased after treatment of 20 $\mu\text{mol/L}$ SP600125 in the PDLSC+rhIGFBP5 group (Fig. 3g,h).

FIGURE 1 IGFBP5 activates JNK and MEK/Erk signalling pathways in WJCMSCs and PDLSCs. (a–c) The overexpression of IGFBP5 activated JNK and MEK/Erk signalling pathways in WJCMSCs. Flag-IGFBP5-infected WJCMSCs showed IGFBP5 overexpression by Western blot analysis. Western blot analysis demonstrated that IGFBP5 overexpression caused incremental increases in the phosphorylated JNK (phospho-JNK), phosphorylated Erk (phospho-Erk) and phosphorylated MEK (phospho-MEK) in WJCMSCs. However, the total amounts of JNK, Erk1/2 and MEK1/2 proteins were not affected (a). Total and phosphorylated amounts of p38 and Akt proteins were not affected in WJCMSCs (b). Quantitative analysis of phospho-JNK, phospho-Erk and phospho-MEK based on Western blot results for the WJCMSC-Flag-IGFBP5 cells and WJCMSC-Vector cells (c). (d–e) rhIGFBP5 activated JNK and MEK/Erk signalling pathways in the PDLSCs. PDLSCs were treated with 0.5 ng/mL recombinant IGFBP5 for 24 hours. PDLSCs without rhIGFBP5 were used as a control. Western blot analysis showed that exogenous rhIGFBP5 could enhance the expression of phospho-JNK, phospho-Erk and phospho-MEK in the PDLSCs. The total amounts of the JNK, Erk1/2 and MEK1/2 proteins were not affected (d). Quantitative analysis of phospho-JNK, phospho-Erk and phospho-MEK based on Western blot results for the PDLSCs and PDLSCs treated with rhIGFBP5 (e). GAPDH was used as an internal control. Student's *t* test was used to determine the statistical significance. Error bars represent SD ($n=3$). ** $P \leq 0.01$. α , anti

3.3 | IGFBP5-enhanced osteogenic differentiation of WJCMSCs or PDLSCs is repressed by a MEK/Erk inhibitor

Then, we used a specific MEK/Erk inhibitor, PD98059, to treat the WJCMSCs for 1.5 hours and block the signalling. Western blot analysis suggested that 20 $\mu\text{mol/L}$ PD98059 blocked MEK/Erk signalling efficiently (Fig. 4a,b). Next, the transduced WJCMSCs were cultured in

osteogenic-inducing medium with 20 $\mu\text{mol/L}$ PD98059, and the results indicated that 20 $\mu\text{mol/L}$ PD98059 significantly decreased the ALP activity in WJCMSC-Flag-IGFBP5 4 days after induction (Fig. 4c). After 3 weeks of induction, Alizarin Red staining and quantitative calcium measurements indicated that 20 $\mu\text{mol/L}$ of PD98059 could effectively inhibit mineralization formation in WJCMSC-Flag-IGFBP5 cells (Fig. 4d,e).

To confirm whether IGFBP5 had similar functions in the PDLSCs, we used rhIGFBP5 to pretreat the PDLSCs for 24 hours, after which the

FIGURE 2 IGFBP5 silencing repressed the JNK and MEK/Erk signalling pathways in WJCMSCs and PDLSCs. (a–c) PDLSCs were infected with short hairpin RNAs (shRNAs) that silenced IGFBP5 (IGFBP5sh) or with scrambled shRNA (Scramsh). (a) IGFBP5 expression was evaluated by Western blot analysis after silencing IGFBP5 by IGFBP5 shRNA in PDLSCs. Compared to the Scramsh-infected cells, the expressions of phospho-JNK, phospho-Erk and phospho-MEK were inhibited in the PDLSC-IGFBP5sh cells by Western blot analysis. The total amounts of the JNK, Erk1/2 and MEK1/2 proteins were not affected. (b) Compared to PDLSC-Scramsh cells, the total and phosphorylated amounts of p38 and Akt proteins were not affected in the PDLSC-IGFBP5sh cells. (c) Quantitative analysis of phospho-JNK, phospho-Erk and phospho-MEK based on the Western blot results for the PDLSC-IGFBP5sh cells and PDLSC-Scramsh cells. (d–e) WJCMSCs were infected with short hairpin RNAs (shRNA) that silenced IGFBP5 (IGFBP5sh) or with scrambled shRNA (Scramsh). (d) The expression of IGFBP5 was evaluated by Western blot analysis after silencing IGFBP5 in the WJCMSCs. Compared with the Scramsh-infected cells, the expressions of phospho-JNK, phospho-Erk and phospho-MEK were depressed in the WJCMSC-IGFBP5sh cells by Western blot analysis. The total amounts of JNK, Erk1/2 and MEK1/2 proteins were not affected. (e) Quantitative analysis of phospho-JNK, phospho-Erk and phospho-MEK based on Western blot results for WJCMSC-IGFBP5sh cells and WJCMSC-Scramsh cells. GAPDH was used as an internal control. Student's *t* test was used to determine the level of statistical significance. Error bars represent SD (*n*=3). ***P*≤0.01. α, anti

FIGURE 3 JNK inhibition repressed the enhanced osteogenic differentiation of WJCMSCs and PDLSCs by IGFBP5. (a, b) Western Blot (a) and quantitative analysis (b) showed reduction in the phospho-JNK in WJCMSC-Flag-IGFBP5 after treatment with the JNK inhibitor, SP600125 (separately 10, 20 and 50 μ mol/L in DMSO) for 48 h. GAPDH was used as an internal control. (c–e) WJCMSCs were treated with 20 μ mol/L SP600125 for 48 h, then changed to osteogenic-inducing medium with 20 μ mol/L SP600125; an osteogenic-inducing medium without SP600125 was used for the control group. After 4 days of induction, an ALP activity assay showed that 20 μ mol/L SP600125 suppressed IGFBP5-mediated enhancement of ALP activity (c). After 3 weeks of induction, Alizarin Red staining (d) and calcium quantitative analysis (e) showed that 20 μ mol/L SP600125 repressed the IGFBP5-mediated enhancement of mineralization in WJCMSCs. (f) PDLSCs were treated with 0.5 ng/mL rhIGFBP5 for 24 hours or treated with 20 μ mol/L SP600125 for 48 hours, then changed to osteogenic-inducing medium with 0.5 ng/mL rhIGFBP5 or 20 μ mol/L SP600125. Osteogenic-inducing medium without rhIGFBP5 and SP600125 was used as a control. After 4 days of induction, the ALP activity assay showed that 20 μ mol/L SP600125 could inhibit rhIGFBP5-mediated enhancement of ALP activity in PDLSCs. (g, h) After 2 weeks of induction, Alizarin Red staining (g) and calcium quantitative analysis (h) showed that 20 μ mol/L of SP600125 repressed the rhIGFBP5-mediated enhancement of mineralization in PDLSCs. Student's *t* test was used to determine the level of statistical significance. Error bars represent SD ($n=3$). * $P \leq 0.05$; ** $P \leq 0.01$. α , anti

PDLSCs were cultured in osteogenic-inducing medium with 20 μ mol/L PD98059 and rhIGFBP5. Consistent with the above results, 20 μ mol/L PD98059 could markedly decrease the rhIGFBP5-enhanced ALP activity in the PDLSCs on 4 days after induction (Fig. 4f). Two weeks after induction, Alizarin Red staining and quantitative calcium measurements revealed that mineralization was inhibited after treatment of 20 μ mol/L PD98059 in the PDLSC+rhIGFBP5 group (Fig. 4g,h).

4 | DISCUSSION

Oral and craniofacial tissue defects can be part of severe and complicated disease, which is often associated with the loss of corbelled bone tissue and impaired appearances. To date, traditional methods, such as surgery and the use of prosthetic appliances and plastic operations, cannot efficiently rehabilitate the local damaged tissues.^{29–31}

FIGURE 4 Erk inhibition repressed the enhanced osteogenic differentiation of WJCMSCs and PDLSCs by IGFBP5. (a,b) Western blot (a) and quantitative analysis (b) showed a reduction in the phospho-MEK/Erk in WJCMSC-Flag-IGFBP5 after treatment with the MEK/Erk inhibitor, PD98059 (separately 10, 20 and 50 μmol/L in DMSO) for 1.5 hours. GAPDH was used as an internal control. (c–e) WJCMSCs were treated with 20 μmol/L PD98059 for 1.5 hours and then changed to an osteogenic-inducing medium with 20 μmol/L PD98059; an osteogenic-inducing medium without PD98059 served as the control. After 4 days of induction, an ALP activity assay showed that 20 μmol/L PD98059 suppressed the IGFBP5-mediated enhancement of ALP activity in WJCMSCs (c). After 3 weeks of induction, Alizarin Red staining (d) and calcium quantitative analysis (e) showed that 20 μmol/L PD98059 repressed the IGFBP5-mediated enhancement of mineralization in WJCMSCs. (f) PDLSCs were treated with 0.5 ng/mL rhIGFBP5 for 24 hours or treated with 20 μmol/L PD98059 for 1.5 hours, then changed to osteogenic-inducing medium with 0.5 ng/mL rhIGFBP5 or 20 μmol/L PD98059; osteogenic-inducing medium without the rhIGFBP5 and PD98059 group was used as a control. After 4 days of induction, an ALP activity assay showed that 20 μmol/L PD98059 could repress the rhIGFBP5-mediated enhancement of ALP activity in the PDLSCs. (g, h) After 2 weeks of induction, Alizarin Red staining (g) and calcium quantitative analysis (h) showed that 20 μmol/L PD98059 inhibited the rhIGFBP5-mediated enhancement of mineralization in the PDLSCs. Student's *t* test was used to determine the statistical significance. Error bars represent the SD (n=3). *P < 0.05; **P < 0.01. α, anti

MSC-based tissue engineering provides a promising therapy for the regeneration of hard tissue and the successful regeneration of tissues in large animal models or humans.^{8,29,32} However, some crucial issues remain to be studied, including the molecular mechanisms of cell differentiation. Previous studies have inferred that compared with the PDLSCs, WJCMSCs possess weakened properties of osteogenic differentiation, and the gene expression profiles revealed that one member of the insulin-like growth factor-binding protein family, IGFBP5, was

markedly less expressed in the WJCMSCs compared with the PDLSCs.¹⁵ Furthermore, we demonstrated that using the IGFBP5-overexpressed WJCMSCs dramatically enhanced the osteogenic differentiation of MSCs and promoted osteogenesis in vivo.^{3,15,28} Previous studies have also found that IGFBP5 was involved in the bone metabolism. Some previous research has found that IGFBP5 is one of the most abundant factors in bone tissue. Compared with cementoblasts, IGFBP5 was more highly expressed in osteoblasts.^{17,18} Moreover, in OVX mice, rhIGFBP5

reportedly stimulated osteoblast activity and bone accumulation.¹⁶ However, there is less evidence about the mechanisms underlying the effects of *IGFBP5*-enhanced osteogenic differentiation in MSCs.

Previous studies have reported that *IGFBP5* could activate the JNK and Akt pathways during the process of chondrocyte differentiation or myogenesis.^{27,28} In addition, *IGFBP5* can also activate the JNK, Erk1/2 and p38 pathways, thereby stimulating growth and proliferation of cells, including vascular smooth muscle cells, fibroblasts, osteoblasts, skeletal muscle cells and intestinal muscle cells.^{20,21,26,28,33–35} The Erk1/2 and p38 pathways are also involved in *IGFBP5*-mediated tumour cell survival.^{21,23} On the basis of these studies, we wondered if JNK, Akt, Erk1/2 and p38 were involved in the *IGFBP5*-mediated MSC osteogenic differentiation. Our results showed that the overexpression of *IGFBP5* could enhance the phosphorylation of JNK, Erk1/2 in the WJCMSCs and PDLSCs and silence the *IGFBP5*-restrained phosphorylation of JNK, Erk1/2 in PDLSCs and WJCMSCs. We then detected the expression of MEK1/2, the upstream effector of Erk, indicating that *IGFBP5* could activate MEK and then stimulate Erk signalling in *IGFBP5*-modified MSCs. Furthermore, applications of specific JNK or MEK/Erk inhibitors could abolish the *IGFBP5*-mediated enhancement of MSC osteogenic differentiation.

The regulation of osteogenesis is a complex process and is associated with several signalling pathways. The mitogen-activated protein kinases (MAPKs) are a group of serine/threonine kinases that transmit signals from the cell membrane into the nucleus, exerting an important role on the regulation of cell differentiation.^{36–38} The family includes three distinctly regulated groups: Erk1/2, JNK and p38 MAPKs.^{39–41} Several in vitro studies have inferred that JNK functions as a positive regulator of cell differentiation. Treatment with a JNK inhibitor could block late-stage osteoblast differentiation.⁴² Multiple growth factors could activate the JNK pathway, leading to a multitude of downstream alterations in the phosphorylation of c-Jun and the transcriptional activation. Bone morphogenetic protein (BMP) 2 caused osteoblastic cell differentiation through the activation of the JNK/MAPK pathway.⁴³ Insulin-like growth factor 1 (IGF1) could promote the osteogenic differentiation of PDLSCs through the Erk and JNK/MAPK pathways.⁴⁴ Erk has two isoforms, both of which are expressed in osteoblasts.^{38,39} Erk could activate and phosphorylate Runx2, the key transcriptional regulator for osteogenesis. Erk^{-/-} mice display substantially decreased bone

mineralization.⁴⁵ Moreover, active Erk could bind to the promoters of osteocalcin (OCN) and bone sialoprotein (BSP) in osteoblasts through the association between Erk and Runx2.⁴⁶ These findings are consistent with the results of our study showing the role of Erk and JNK in osteogenic differentiation. Researchers have found that Erk and JNK are both required for EPO-mediated or VEGF-induced cell proliferation.^{47–49} Rapid JNK activation is dependent on Erk activation, JNK is the final mediator for Erk, and these two MAP kinases mediate the VEGF-induced cell proliferation.^{40, 48} *RUNX2* and *OSX* are the two key transcription factors for osteogenic differentiation.⁵⁰ *RUNX2* is the first transcription factor for osteogenic differentiation, and *OSX* is a zinc finger-containing transcription factor that is essential for osteoblast differentiation and bone formation in bone homeostasis.⁵¹ Researchers have discovered the interaction domains in the *Osx* and *Runx2*, and these regions are phosphorylated by p38 and ERK MAPK. Blocking p38 and ERK disrupts the physical interaction between *Osx* and *Runx2* and cooperative transcriptional effects.⁵² Other researchers have found that exogenous IGF-1 can up-regulate the expression of phosphor-ERK and phosphor-JNK, enhance the osteogenic differentiation and increase the expression of *Osx* and *Runx2* in PDLSCs.³⁹ Mineral trioxide aggregate up-regulates the odonto/osteogenic capacity of BMSCs via JNK and ERK signalling pathways and promotes the expression of *Osx* and *Runx2*. The inhibition of JNK and ERK signalling dramatically blocked the expression of *Osx* and *Runx2*.⁵³ In addition, IGF-I-mediated *Osx* expression requires all three MAPK components (Erk, p38 and JNK).⁵⁴ Erk1/2 regulates a major transcription factor, Osterix, during osteoblast differentiation by increasing its protein stability and transcriptional activity.⁵⁵ Overall, our results confirmed that *IGFBP5* enhanced the osteogenic differentiation of MSCs by activating the JNK and MEK/Erk, and not the Akt and p38/MAPK, signalling pathways. In addition, our previous findings demonstrated that *IGFBP5* was able to activate the expression of one key transcription factor, *OSX*, but not *RUNX2*.¹⁹ These results indicated that *IGFBP5* enhances *OSX* expression through JNK and Erk (Fig. 5). Taken together, the results of our study suggest that both Erk and JNK signalling are involved in the *IGFBP5*-enhanced osteogenic differentiation of MSCs. Further study is required to investigate the relationship of JNK, MEK/Erk and *OSX* in this process.

In conclusion, our results demonstrated that *IGFBP5* can lead to activation of JNK and MEK/Erk signalling in the osteogenic

FIGURE 5 The diagram illustrated the relationships of *IGFBP5*, MAPK pathway and osteogenic differentiation in mammalian cells. The red colour indicates our discovery

differentiation of MSCs. Importantly, the application of specific inhibitors dramatically weakened the *IGFBP5*-enhanced osteogenic differentiation in MSCs. This study contributed to the molecular mechanisms underlying the osteogenic differentiation of MSCs, showing that *IGFBP5* prompted the osteogenic differentiation potentials of MSCs via the JNK and MEK/Erk signalling pathways, revealing the underlying mechanism of *IGFBP5*-enhanced osteogenic differentiation in MSCs, and identifying a potential target mediator for improving bone tissue regeneration based on MSCs.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

Yuejun Wang: data analysis and interpretation and manuscript writing; Zhi Jia: collection and/or assembly of data and financial support; Shu Diao, Xiao Lin, Xiaomeng Lian and Liping Wang: collection and/or assembly of data; Rui Dong: conception and design, collection and/or assembly of data, and financial support; Dayong Liu: collection and/or assembly of data, data analysis and interpretation, and financial support; Zhipeng Fan: conception and design, data analysis and interpretation, financial support, and final approval of the manuscript.

ABBREVIATIONS

a, anti; Akt, protein kinase B; ALP, alkaline phosphatase; ARS, Alizarin Red staining; BMP2, bone morphogenetic protein 2; BMSCs, bone marrow stromal stem cells; BSP, bone sialoprotein; Erk1/2, extracellular regulated protein kinases 1 and 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *IGFBP5*, insulin-like growth factor-binding protein 5; *IGFBPs*, insulin-like growth factor-binding proteins; IGF, insulin-like growth factor; IGF, insulin-like growth factor; IOD, integrated optical density; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK1/2, mitogen-activated protein kinases 1 and 2; MSCs, mesenchymal stem cells; OCN, osteocalcin; PDLSCs, periodontal ligament stem cells; rh*IGFBP5*, recombinant human *IGFBP5* protein; WJCMSCs, Wharton's jelly of umbilical cord stem cells; W, week.

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