Original Article

Insulin-Like Growth Factor Binding Protein 5: an Important Regulator of Early Osteogenic Differentiation of hMSCs

(insulin-like growth factor binding protein 5 / osteogenic differentiation / human bone marrow mesenchymal stem cells)

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Abstract. Insulin-like growth factor binding protein 5 (IGFBP5) is broadly bioactive, but its role in osteogenic differentiation of human bone marrow-derived mesenchymal stem cells (hMSCs) remains to be clarified. Here, we demonstrated that IGFBP5 expression was markedly increased during the early osteogenic differentiation of hMSCs. We then over-expressed and knocked down this gene in hMSCs and evaluated the impact of manipulation of *IGFBP5* expression on osteogenic differentiation based upon functional assays, ALP staining, and expression of osteogenic markers. Together, these analyses revealed that *IGFBP5* over-expression enhanced early osteogenic differentiation, as evidenced by increased ALP staining and osteogenic marker induction, whereas knocking down this gene impaired the osteogenic process. Over-expression of *IGFBP5* also markedly bolstered the extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation level, while *IGFBP5* knockdown suppressed this signalling activity. We additionally compared the impact of simultaneous *IGFBP5* over-expression and ERK1/2 inhibitor treatment to the effect of *IGFBP5* over-expression alone in these hMSCs, revealing that small molecule-mediated EKR1/2 inhibition was sufficient to impair osteogenic differentiation in the context of elevated IGFBP5 levels. These findings indicated that IGFBP5 drives the early osteogenic differentiation of hMSCs via the ERK1/2 signalling pathway. Our results offer value as a foundation for future efforts to study and treat serious bone-related diseases including osteoporosis.

Introduction

Human bone mesenchymal stem cells (hMSCs) are multipotent cells that can self-renew *in vivo* (Pittenger et al., 1999) and differentiate into a range of cell types, including osteoblasts and adipocytes (Barry et al., 2001; Arinzeh, 2005; Helder et al., 2007; Rosen et al., 2012). When the normal homeostatic balance controlling hMSC differentiation into these two cell types is disrupted such that osteoblastic differentiation is impaired and/or adipogenic differentiation is enhanced, individuals can suffer from significant bone loss, contributing to the development of osteoporosis (Scheideler et al., 2008). During osteogenesis, hMSCs up-regulate specific genes in a defined manner while suppressing activation of other genes to coordinate phenotypic changes (Steward and Kelly, 2015), with the early stages of this differentiation process being particularly important as determinants of future cell development (Park et al., 2013; Martino et al., 2014). *In vitro* expanded hMSCs function as an optimal model system that can be used to explore the molecular regulation of osteogenesis (Okolicsanyi et al., 2015).

Insulin-like growth factor-binding proteins (IGFBPs) are pivotal regulators of the mitogenic activity of insulin-like growth factors (IGFs) (Pouriamehr et al., 2019), are closely linked to cell differentiation, proliferation and invasion (Du et al., 2019; Nishihara et al., 2020). IGFBP5 is the most highly conserved IGFBP family member among vertebrates and controls cellular growth, cell fate determination, and tumour cell metastasis

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Abbreviations: ALP – alkaline phosphatase, ERK1/2 – extracellular signal-regulated kinase 1/2, hMSCs – human mesenchymal stem cells, HRP – horseradish peroxidase, IGFBP5 – insulin-like growth factor binding protein 5, MOI – multiplicity of infection, OCN – osteocalcin, PDLSC – periodontal ligament stem cells, PVDF – polyvinylidene difluoride, qRT-PCR – quantitative real-time PCR, RUNX2 – runt-related transcription factor 2.

(Akkiprik et al., 2008). When ovariectomized rats were injected daily with a subcutaneous dose of IGFBP5, this was shown to enhance osteoblast proliferation (Andress, 2001). However, the specific role of this protein as a regulator of hMSC osteogenesis remains to be clarified.

Here, we demonstrated that IGFBP5 is markedly upregulated during the early stages of hMSC osteogenesis, leading us to hypothesize that this IGFBP family member is a key regulator of this differentiation process. Specifically, we demonstrated that IGFBP5 controls early hMSC osteogenic differentiation via activating the extracellular signal-regulated kinase 1/2 (ERK1/2) signalling pathway. Together, our data provide a new framework for understanding how IGFBP5 can contribute to early hMSC osteogenesis.

Material and Methods

Cell culture and osteogenic induction

Flow cytometry was utilized to determine that hMSCs (HUXMA-01001, Cyagen Biosciences, Guandong, China; cell lot No. of three donors: 150724I31, 161125R41, and 160202I31) were ≥ 95 % positive for CD105, CD73, and CD90 and ≤ 5 % positive for HLA-DR, CD45, CD19, CD11b, and CD35 expression. Cells were then cultured at $5 \times 10^4/cm^2$ in oriCell hMSC growth media (HUXMA-90011, Cyagen Biosciences) which contained 10 % FBS, 0.4% glutamine, 100 IV/ml penicillin and 100 μg/ml streptomycin in a humidified 5% CO_2 incubator at 37 °C. Every 3–4 days, 0.25% trypsin-EDTA solution (Gibco, Thermo Fisher Scientific, Waltham, MA) was used to passage cells, and cells were used for experimentation when between passages 3 and 6.

Cells were grown until 70% confluent, at which time they were stimulated to undergo osteogenic differentiation by culturing them in media supplemented with 50 mM ascorbic acid, 10 mM β-glycerophosphate and 100 nM dexamethasone (all from Sigma-Aldrich, St Louis, MO). These cells were then cultured in this media for 0, 3, 7, or 9 days, with media being changed every three days. In addition, cells were collected to assess the expression of ALP, OCN, RUNX2, ERK1/2 and IGFBP5 at the mRNA and protein levels.

Lentiviral transduction

In order to knockdown or over-express *IGFBP5*, appropriate lentiviral vectors were obtained from Shanghai Genechem Co., Ltd (Shanghai, China). A short hairpin RNA (shRNA) targeting human *IGFBP5* was designed (target sequence, 5′-GCAGATCTGTGAATATGAA-3′). In addition, a negative control (NC) shRNA was used (sequence, 5′-TTCTCCGAACGTGTCACGT-3′). Chemically synthesized DNA oligonucleotides (Shanghai Genechem Co., Ltd.) were respectively cloned into the GV493 (for shRNA) and GV492 (for over-expression)-green fluorescent protein (GFP) lentiviral vector (Shanghai Genechem Co., Ltd.). Cells were transfected to achieve either over-expression of *IGFBP5*, over-expression of a control construct, to express an IGFBP5-specific shRNA, or express a control shRNA. Lentiviral titres were assessed via serial dilution, and hMSCs were then plated in 6-well plates until 20–30% confluent, at which time 1×10^8 TU/ml virus (10 μl), 5 μg/ml polybrene, and additional media was added per well. Following 10-h incubation at 37 °C, medium was exchanged and cells were allowed to rest for 72 h. Medium was then refreshed and supplemented with 0.5 μg/ml puromycin, which was used to screen cells for 48 h. Medium was then exchanged for fresh puromycin-containing medium, and selection was maintained for six total days, at which time surviving cells began proliferating.

ALP staining and activity analyses

ALP activity was assessed with a staining kit (Beyotime Institute of Biotechnology, Shanghai, China) based on provided directions. Briefly, after two washes with PBS, cells were fixed for 20 min using 4% formalin. Cells were then incubated two times in ALP buffer (0.1 M NaCl, 0.1 M Tris-HCl, 50 mM $MgCl_2$. $6H_2O$, pH 9.5) for 5 min each, followed by 30-min incubation with ALP substrate solution (5 μl BCIP and 10 μl NBT in l ml ALP buffer) at room temperature protected from light. Distilled water was then added to terminate staining, and cells were assessed via microscopy (Olympus, Tokyo, Japan).

An ALP Detection Kit (Nanjing Jiancheng Bioengineering Ltd., Nanjing, China) was additionally used to assess ALP activity based upon provided directions. Briefly, cells were freeze-thawed four times to release endogenous ALP, after which lysates were added to 96-well plates containing ALP substrate and were incubated at 37 °C, after which a stop buffer was added to terminate the reaction. The p-nitrophenol product levels in each well were then assessed by analysing absorbance at 520 nm using a microplate reader (Bio-Rad, Hercules, CA).

qRT-PCR

Trizol (Invitrogen, Carlsbnad, CA) was employed to extract cellular RNA, after which a Reverse Transcription System and Oligo (dT) kit was utilized to prepare cDNA (Thermo Fisher Scientific). For normalization, we utilized β-actin, and primers used for this study are compiled in Table 1. All qRT-PCR reactions were conducted using a SYBR Premix Ex Taq kit (TOYOBO, Otsu, Shiga, JAPAN) and a 7500 Real-Time PCR System (ABI, Foster City, CA), with relative gene expression being evaluated via the $2^{-\Delta\Delta CT}$ method.

Western blotting

RIPA buffer was used for lysing cells, after which a BCA protein assay kit (Thermo Fisher Scientific, Inc.) was employed to measure the protein levels in individual samples. Isolated protein extracts were then diluted 10-, 20-, or 40-fold using 0.9% NaCl and incubated at 37 °C for 2 h, after which absorbance was assessed with an iMark microplate reader (Bio-Rad). Protein extracts

Table 1. qRT-PCR primers

Gene symbol	Forward primers	Reverse primers	Length (bp)
RUNX2	5‘-GGACGAGGCAAGAGTTTCACC-3‘	5‘-GGTTCCCGAGGTCCATCTACT-3‘	161
OCN	5‘-TGAGAGCCCTCACACTCCTC-3‘	5‘-CGCCTGGGTCTCTTCACTAC-3‘	151
ALP	5’-CCCCGTGGCAACTCTATCTTT-3‘	5’-GCCTGGTAGTTGTTGTGAGCATAG-3‘	161
IGFBP5	5‘-AGTGAAGAAGGACCGCAGAA-3'	5‘-GGTCACAATTGGGCAGGTAC-3'	209
β-actin	5‘-GCGAGAAGATGACCCAGATCATGT-3‘	5‘-TACCCCTCGTAGATGGGCACA-3‘	160

were then combined with 5× SDS sample buffer, boiled for 5 min, and 15 μg of protein per sample was separated via 10% SDS-PAGE and transferred onto PVDF membranes (EMD Millipore, Burlington, MA). Blots were subsequently blocked using 5% non-fat milk for 2 h at room temperature, and blots were then incubated overnight with rabbit anti-RUNX2 (1 : 1,000; ab23981), rabbit anti-OCN (1 : 1,000; ab133612), rabbit anti-IGFBP5 (1 : 1,000; CST 10941), rabbit anti-ERK1/2 (CST 9102), rabbit anti-p-ERK1/2 (CST 9101), or mouse anti-β-actin (1 : 2,000; 173838) at 4 °C. Blots were then probed for 1 h using HRP-linked anti-mouse or anti-rabbit IgG (1 : 5,000; 7076P2 and 7074P2; Cell Signaling Technology, Danvers, MA). Protein band detection was then conducted with an ECL reagent (BeyoECL Plus; Beyotime Institute of Biotechnology, Shanghai, China).

Statistical analysis

Data are given as means ± SD, and all experiments were conducted in triplicate. Data were compared via one-way analysis of variance (ANOVA), with $P < 0.05$ as the significance threshold.

Results

Assessment of IGFBP5 expression during hMSC osteogenesis

We started by evaluating *IGFBP5* expression dynamics during the osteogenic differentiation of hMSCs, revealing that this gene was gradually up-regulated over time until reaching a maximal expression level on day 7 (Fig. 1A). This indicated that IGFBP5 may be a key regulator of early osteogenic differentiation of hMSC.

hMSC transduction

At 6 days post-lentiviral transduction, the remaining hMSCs were puromycin-resistant, indicating good transduction efficiency. These cells grew effectively and exhibited GFP expression when evaluated via fluorescent microscopy (Fig. 1B). The efficacy of lentiviral transduction was additionally confirmed via qRT-PCR and Western blotting (Fig. 1C and D).

ALP staining and activity

The osteoblastic differentiation of hMSCs was evaluated on days 3 and 7 post-induction via ALP staining. Positively stained cells were manifested by a blue-violet colour. Notably, the staining intensity was significantly greater in *IGFBP5*-over-expressing cells (Fig. 2A), whereas it was significantly decreased in cells in which *IGFBP5* was knocked down (Fig. 2B). This result was also confirmed via quantifying intracellular ALP activity, again revealing that ALP staining intensity was increased in *IGFBP5*-over-expressing cells and decreased in cells transduced with an *IGFBP5*-specific shRNA relative to control cells (Fig. 2C and D).

Modulation of IGFBP5 expression impacts hMSC osteogenesis

In an effort to more fully understand the impact of *IGFBP5* expression on hMSC osteogenic differentiation, we additionally assessed osteogenic marker gene expression patterns in cells prepared as above. We found that *IGFBP5* over-expression markedly enhanced the levels of osteogenic marker genes *RUNX2*, *OCN* and *ALP* at the RNA and protein levels, whereas *IGFBP5* knockdown suppressed induction of both these genes on days 3 and 7 of the differentiation process (Fig. 2 E, F and Fig. 2 G, H).

IGFBP5 expression impacts ERK1/2 signalling in hMSCs

Next, we evaluated the impact of *IGFBP5* expression on ERK1/2 signalling in the context of osteogenesis by Western blotting, revealing that both *IGFBP5* over-expression and knockdown were linked with increased and decreased p-ERK1/2 levels (Fig. 3A and B).

IGFBP5 controls hMSC osteogenesis via regulating ERK1/2 activation

Lastly, we assessed the role of ERK1/2 in the regulation of IGFBP5-mediated hMSC osteogenesis. To this end, cells were treated using 20 μM ERK1/2 inhibitor U0126 (Selleck, Pittsburgh, PA) to reduce its phosphorylation (Fig. 3C), revealing that the inhibition of ERK1/2 activity was sufficient to impair the osteoblastic differentiation of hMSCs over-expressing *IGFBP5* (Fig. 4). Overall, these results indicate that IGFBP5 controls early hMSC osteogenic differentiation via controlling ERK1/2 signalling activity.

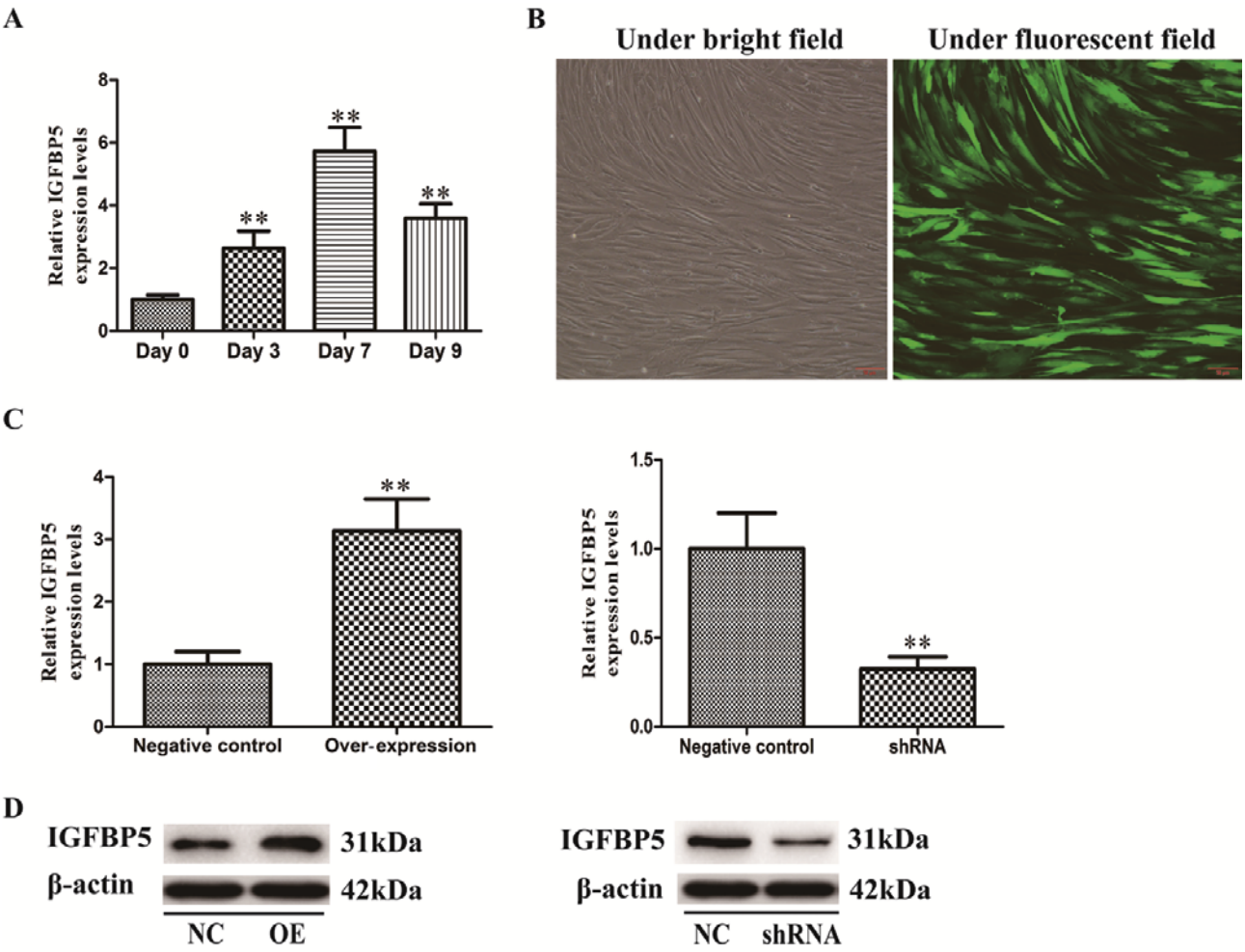

Fig. 1. Assessment of *IGFBP5* expression during hMSC osteogenesis and evaluation of the efficacy of lentiviral transduction at 6 days post-lentiviral transduction. (**A**) *IGFBP5* expression during osteogenic differentiation was assessed via qRT-PCR over time. Data are means ± SD (X ± SD, N = 3). **P < 0.01 vs. Day 0. (**B**) Following infection, cells were assessed via light and fluorescence microscopy (10×; scale bar, 50 μm), with representative images being shown. (**C**) IGFBP5 mRNA expression was evaluated via qRT-PCR. Data are means ± SD (X ± SD, N = 3). **P < 0.01 vs. negative control. (**D**) IGFBP5 protein expression was evaluated via Western blotting.
NC – negative control, OE – IGFBP5 over-expression, shRNA – IGFBP5 shRNA.

Discussion

Osteoporosis is a disease that causes progressive bone loss, increasing the susceptibility of affected patients to bone fractures. The ability of hMSCs to differentiate into osteoblasts, osteocytes, and adipocytes ultimately controls the development of bone and fat tissues (Kim et al., 2016; You et al., 2016; Casado-Díaz et al., 2017). Impairment of hMSC osteogenesis can impair bone formation, and such impairment is a common hallmark of osteoporosis (Scheideler et al., 2008; Benisch et al., 2012). It is therefore essential that the molecular mechanisms regulating hMSC differentiation be better understood in order to guide the treatment of osteoporosis and bone fractures.

Here, we found that *IGFBP5* expression in hMSCs increased over time during osteoblastic differentiation, with maximum expression levels being reached on day 7 of this process (Fig. 1A). This showed that IGFBP5 may be a key regulator of the early phases of the osteogenic differentiation process in these cells. To test this possibility, we generated hMSCs in which *IGFBP5* was stably over-expressed or knocked down using lentiviral constructs (Fig. 1B-D).

We found that cells over-expressing *IGFBP5* exhibited more robust ALP staining and activity, whereas the opposite was true in cells in which this gene was knocked down (Fig. 2 A,C; Fig. 2 B,D). In line with these findings, osteogenic marker gene expression was markedly increased during hMSC osteogenesis in cells over-expressing *IGFBP5* (Fig. 2 E,G), while the expression of these marker genes was suppressed following *IGFBP5* knockdown (Fig. 2 F,H). These data suggest that IGFBP5 functions as a positive regulator of early hMSC osteoblastogenesis.

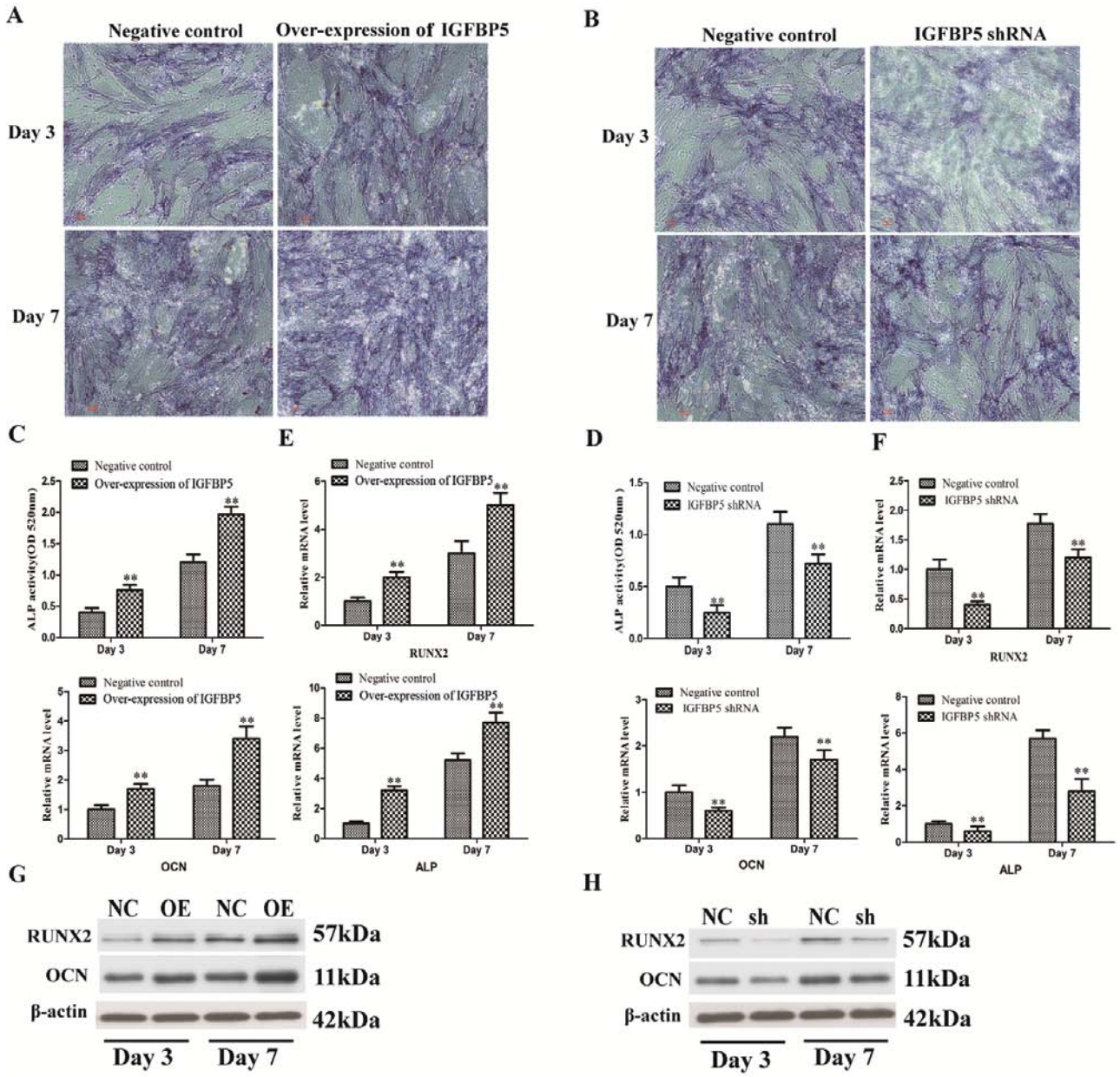

Fig. 2. Modulation of *IGFBP5* expression (*IGFBP5* over-expression or knockdown) impacts hMSC osteogenesis. (**A**, **B**) Light microscopy was used to evaluate ALP staining in hMSCs at different stages of differentiation (10×; scale bar, 50 μm). (**C**, **D**) Absorbance readings were utilized to quantify ALP activity. (E-F) *RUNX2*, *OCN*, and *ALP* expression were evaluated via qRT-PCR. Data are means ± SD (N = 3). **P < 0.01 vs. negative control, respectively. (G-H) RUNX2 and OCN levels were measured via Western blotting.
NC – negative control, OE – *IGFBP5* over-expression, sh – *IGFBP5* shRNA.

Of the six known IGFBP family members, IGFBP5 is the most broadly bioactive and is expressed in many different cells and tissues (Du et al., 2019; Nishihara et al., 2020; Xi et al., 2020). The relationship between IGFBP5 and osteogenic differentiation, however, remains to be fully clarified. There is some evidence that IGFBP5 can enhance osteogenic differentiation of umbilical cord stem cells and periodontal ligament stem cells (PDLSCs) (Wang et al., 2016), and recombinant human IGFBP5 (rhIGFBP5) can promote PDLSC migration, chemotaxis, and osteo/dentinogenic differentiation (Han et al., 2017). However, IGFBP5 over-expression has also been shown to decrease *in vitro* osteoblastogenesis (Durant et al., 2004), and there is some evidence that this protein can also restrain skeletal growth (Mukherjee et al., 2008). As such, IGFBP5 may play cell- and tissue-specific roles in regulating physiological activities. As such, in the present study, we specifically evaluated the impact of IGFBP5 on hMSC osteogenesis.

The mechanistic basis by which IGFBP5 controls osteogenesis has yet to be clarified. The differentiation of hMSCs into osteoblasts is controlled by coordinated

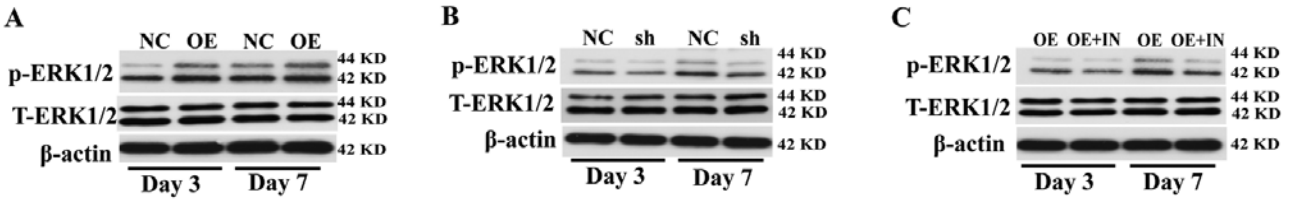

Fig. 3. Analysis of ERK1/2 phosphorylation during hMSC osteogenesis. (**A**) *IGFBP5* over-expression promoted ERK1/2 phosphorylation. (**B**) *IGFBP5* knockdown suppressed p-ERK1/2 levels. (**C**) ERK1/2 inhibitor treatment decreased intracellular p-ERK1/2 levels.
NC – negative control, OE – *IGFBP5* over-expression, sh – *IGFBP* shRNA, OE+IN – *IGFBP5* over-expression + inhibitor.

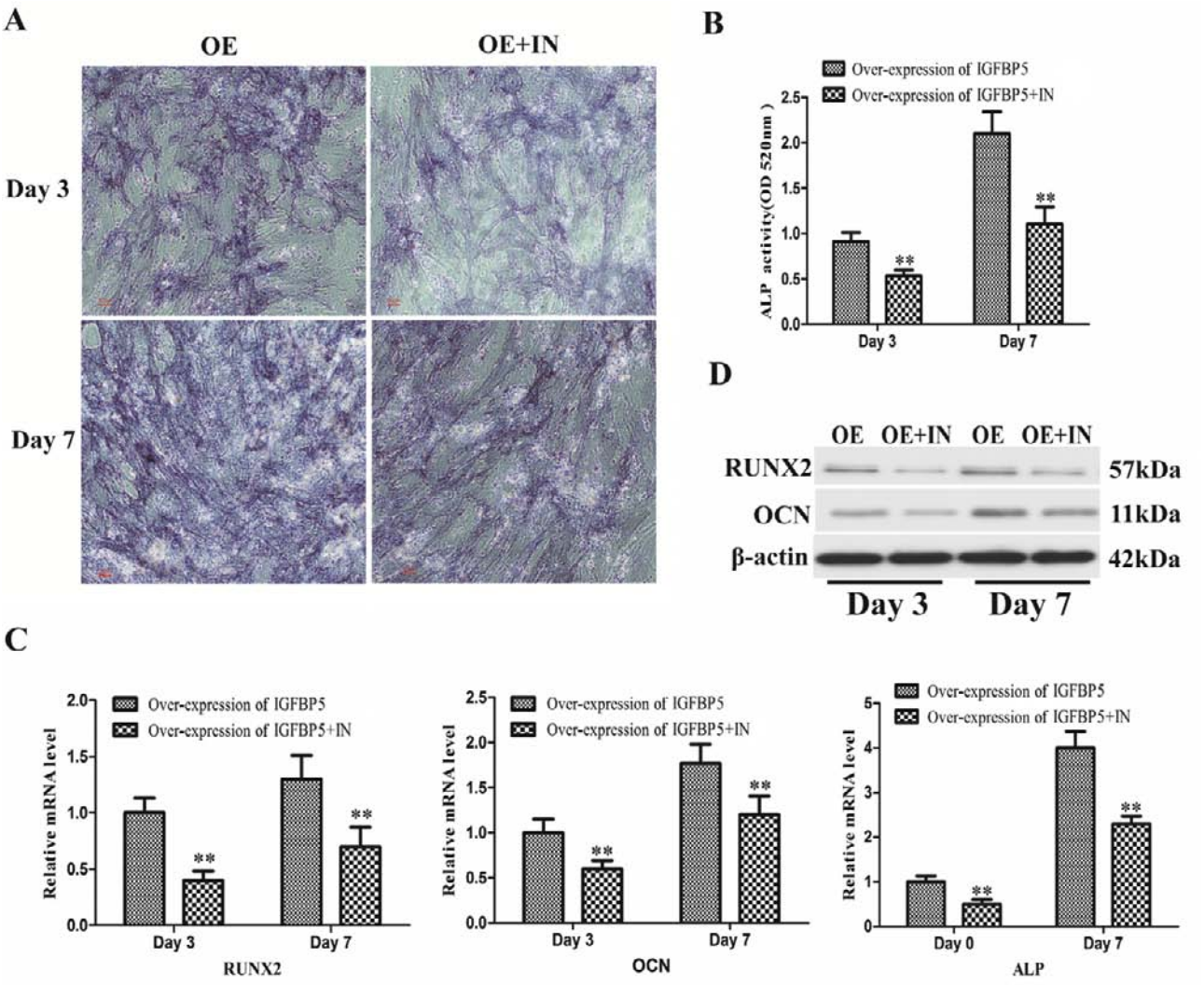

Fig. 4. IGFBP5 controls hMSC osteogenesis via regulating ERK1/2 activation.
Analysis of osteogenic differentiation of hMSCs following *IGFBP5* over-expression and ERK1/2 signalling inhibitor treatment. (**A**) Osteogenesis was evaluated via ALP staining (10×; scale bar, 50 μm). Cells in which *IGFBP5* was over-expressed that were treated with an ERK1/2 signalling inhibitor exhibited decreased osteogenesis relative to cells in which *IGFBP5* was over-expressed but no inhibitor was added. (**B**) Significant differences in ALP activity were observed when comparing the *IGFBP5* over-expression + inhibitor and the *IGFBP5* over-expression groups. (**C**) *RUNX2*, *OCN* and *ALP* expression in different groups was compared via qRT-PCR. Data are means ± SD (X ± SD, N = 3). **P < 0.01 vs. over-expression of *IGFBP5*. (**D**) RUNX2 and OCN protein levels were evaluated via Western blotting.
OE – *IGFBP5* over-expression, OE+IN – *IGFBP5* over-expression+inhibitor.

simultaneous activation of many signalling pathways, making it essential to understand which of these pathways function downstream of IGFBP5. There are several signalling pathways related to IGFBP5, such as JNK, MEK/ERK and MAPK signalling pathways (Rousse et al., 2001; Yasuoka et al., 2009; Wang et al., 2016). In prior research, IGFBP5 was shown to modulate dental pulp stem cell dentinogenesis by controlling the ERK signalling pathway (Wang et al., 2016). Consistent with such activity, IGFBP5 also impacts the growth of pancreatic cancer cells by modulating ERK1/2 signalling (Johnson and Haun, 2009). As such, we hypothesized that IGFBP5 may also control osteogenesis via the ERK1/2 signalling pathway. Consistent with this model, we demonstrated that over-expressing or knocking down *IGFBP5* was sufficient to alter ERK1/2 phosphorylation levels during hMSCs osteogenesis (Fig. 3A and B). The treatment of these cells with ERK1/2 inhibitors was also sufficient to reverse the impact of *IGFBP5* over-expression on hMSC osteogenic differentiation (Fig. 3C and Fig. 4). These data therefore confirmed that IGFBP5 signals via ERK1/2 in order to control the early osteogenic differentiation of hMSCs.

Together, our data indicate that IGFBP5 serves as a key regulator of early stage osteoblastogenesis in hMSCs. As a novel positive regulator of this important differentiation process, IGFBP5 may thus be a viable target for future studies of the treatment of osteoporosis and other bone-related diseases.

Disclosure of conflict of interest

The authors declare no financial or commercial conflict of interest.

Acknowledgments

Z. M. Z.[#] and L. M.[#] contributed equally. We thank Prof. Tao Wang, Key Laboratory of System Bio-medicine of Jiangxi Province, Jiujiang University, for reading the manuscript and for his helpful comments and suggestions.

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