Insulin-Like Growth Factor Binding Protein 5: A Novel Regulator of Early Osteogenic Differentiation of hMSCs

Zhimin Zhang

hu bei zhong yi yao da xue: Hubei University of Chinese Medicine

Min Li

Hubei University of Chinese Medicine

Daolong Jiang

Hubei University of Chinese Medicine

Zhengyang Han

Hubei University of Chinese Medicine

Lihua Wang (✉ lihuawang666@163.com)

hu bei yi yao xue yuan: Hubei University of Medicine <https://orcid.org/0000-0002-8986-2832>

Research Article

Keywords: Insulin-like growth factor-binding protein 5 (IGFBP5), human bone marrow mesenchymal stem cells(hMSCs), osteogenic differentiation, extracellular signal-regulated kinase 1/2 (ERK1/2).

Posted Date: February 19th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-212703/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

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. Herein, we determined that IGFBP5 expression was markedly increased during the early osteogenic differentiation of hMSCs. We then overexpressed and knocked down this gene in hMSCs and evaluated the impact of the manipulation of IGFBP5 expression on osteogenic differentiation based upon functional assays, ALP staining, and the expression of osteogenic markers. Together, these analyses revealed that IGFBP5 overexpression enhanced early osteogenic differentiation as evidenced by increased ALP staining and osteogenic marker induction, whereas knocking down this gene impaired the osteogenic process. Overexpressing IGFBP5 also markedly bolstered extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation level, while IGFBP5 knockdown suppressed this signaling activity. We additionally compared the impact of simultaneous IGFBP5 overexpression and ERK1/2 inhibitor treatment to the effect of IGFBP5 overexpression alone in these hMSCs, revealing that small molecule-mediated ERK1/2 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 signaling 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* [1], and that can differentiate into a range of cell types including osteoblasts and adipocytes [2–5]. 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 [6]. During osteogenesis, hMSCs upregulate specific genes in a defined manner while suppressing the activation of other genes to coordinate phenotypic changes [7], with the early stages of this differentiation process being particularly important as determinants of future cell development [8, 9]. *In vitro*-expanded hMSCs functioning as an optimal model system that can be used to explore the molecular regulation of osteogenesis [10].

Insulin-like growth factor-binding proteins (IGFBPs) are pivotal regulators of the mitogenic activity of Insulin-like growth factors (IGFs) [11], are closely linked to differentiation, proliferation and invasion [12, 13]. IGFBP5 is the most highly conserved IGFBP family member among vertebrates, and controls cellular growth, cell fate determination, and tumor cell metastasis [14]. When ovariectomized rats were injected daily with a subcutaneous dose of IGFBP5, this was shown to enhance osteoblast proliferation therein [15]. However, the specific role of this gene as a regulator of hMSC osteogenesis remains to be clarified.

Herein, we determined 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 determined that IGFBP5 controls early hMSC osteogenic differentiation via activating the extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway. Together, our data provide a new framework for the understanding of 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, China) were $\geq 95\%$ positive for CD105, CD73, and CD90 and $\leq 5\%$ positive for HLA-DR, CD45, CD19, CD11b, and CD35 expression. Cells were then cultured at $5 \times 10^4/\text{cm}^2$ in oriCell hMSC growth media (HUXMA-90011, Cyagen Biosciences) supplemented with 10% FBS, glutamine, and penicillin/streptomycin in a humidified 5% CO₂ incubator at 37°C. Every 3–4 days, 0.25% trypsin-EDTA solution (Gibco) was used to pass 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, MO, USA). Fresh differentiation media was added every 3 days.

Lentiviral transduction

Shanghai Genechem Co., Ltd produced IGFBP5-overexpression and IGFBP5-shRNA lentiviral vectors, which were tittered by serial dilution. The hMSCs were then plated in 6-well plates until 20–30% confluent, at which time 1×10^8 TU/ml virus (10 μl), 5 $\mu\text{g}/\text{ml}$ polybrene, and additional media was added per well. Following a 10 h incubation at 37°C, media was exchanged and cells were allowed to rest for 72 h. Media was then refreshed and supplemented with 0.5 $\mu\text{g}/\text{ml}$ puromycin, which was used to screen cells for 48 h. Media was then exchanged for fresh puromycine-containing media, and selection was maintained for 6 total days at which time surviving cells had begun 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 minutes using 4% formalin. Cells were then incubated two times in ALP buffer (0.1 M NaCl, 0.1 M Tris-HCl, 50 mM MgCl₂·6H₂O, pH 9.5) for 5 minutes each, followed by a 30-minute incubation with ALP substrate solution (5 μl BCIP and 10 μl NBT in 1 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 analyzing absorbance at 520 nm using a microplate reader (Bio-rad, CA, USA).

qRT-PCR

Trizol (Invitrogen) was employed to extract cellular RNA, after which a Reverse Transcription System and Oligo (dT) kit was utilized to prepare cDNA (Thermo 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, JAPAN) and a 7500 Real-Time PCR System (ABI, CA, USA), with relative gene expression being evaluated via the $2^{-\Delta\Delta CT}$ method.

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'- ggtcacaattgggcaggtag-3'	209
β -actin	5'- GCGAGAAGATGACCCAGATCATGT- 3'	5'-TACCCCTCGTAGATGGGCACA-3'	160

Western blotting

RIPA buffer was for lysing cells, after which a BCA protein assay kit (Thermo Fisher Scientific, Inc.) was employed to measure protein levels in individual samples. Isolated protein extracts were then diluted 10-, 20-, or 40-fold using 0.9% NaCl and were incubated for 2 h incubation at 37°C, after which absorbance was assessed with an iMark microplate reader (Bio-rad). Protein extracts were then combined with 5x SDS sample buffer, boiled for 5 minutes, and 15 μ g of protein per sample was separated via 10% SDS-PAGE and transferred onto PVDF membranes (EMD Millipore). 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). Protein band detection was then conducted with an ECL reagent (BeyoECL Plus; Beyotime Institute of Biotechnology).

Statistical analysis

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

Results

Assessment of IGFBP5 expression during hMSC osteogenesis

We started by evaluating IGFBP5 expression dynamics during osteogenic differentiation of hMSCs, revealing that this gene was gradually upregulated 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, 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. Consistent with positive staining, cells were stained a blue-violet color. Notably, staining intensity was significantly greater in IGFBP5-overexpressing 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-overexpressing 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 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 overexpression markedly enhanced levels of the osteogenic marker genes RUNX2, OCN and ALP at the RNA and protein levels, whereas IGFBP5 knockdown suppressed the induction of both of these genes on days 3 and 7 of the differentiation process (Fig. 2E, F and Fig. 2G, H).

IGFBP5 expression impacts ERK1/2 signaling in hMSCs

Next, we evaluated the impact of IGFBP5 expression on ERK1/2 signaling in the context of osteogenesis by Western blotting, revealing that both IGFBP5 overexpression 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 that end, cells were treated using 20 μ M ERK1/2 inhibitor U0126 (Selleck) to reduce its phosphorylation (Fig. 3C), revealing that the inhibition of ERK1/2 activity was sufficient to impair the osteoblastic differentiation of hMSCs overexpressing IGFBP5 (Fig. 4). Overall, these results indicate that IGFBP5 controls early hMSC osteogenic differentiation via controlling ERK1/2 signaling activity.

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 [16, 17]. Impairment of hMSC osteogenesis can impair bone formation, and such impairment is a common hallmark of osteoporosis [6, 18]. It is therefore essential that the molecular mechanisms regulating hMSCs be better understood in order to guide the treatment of osteoporosis and bone fractures.

Herein, 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 overexpressed or knocked down using lentiviral constructs (Fig. 1B-D).

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

Of the six known IGFBP family members, IGFBP5 is the most broadly bioactive and is expressed in many different cells and tissues [13, 19, 20]. The relationship between IGFBP5 and osteogenic differentiation, however, remains to be fully clarified. There is some evidence that IGFBP5 can enhance the osteogenic differentiation of umbilical cord stem cells and periodontal ligament stem cells (PDLSCs) [21], and recombinant human IGFBP5 (rhIGFBP5) can promote PDLSC migration, chemotaxis, and osteo/dentinogenic differentiation [22]. However, IGFBP5 overexpression has also been shown to

decrease *in vitro* osteoblastogenesis [12], and there is some evidence that this protein can also restrain skeletal growth [23]. 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 the coordinated simultaneous activation of many signaling pathways, making it essential to understand which of these pathways function downstream of IGFBP5. In prior research, IGFBP5 was shown to modulate dental pulp stem cell dentinogenesis by controlling the ERK signaling pathway [21]. Consistent with such activity, IGFBP5 also impacts the growth of pancreatic cancer cells by modulating ERK1/2 signaling [24]. As such, we hypothesized that IGFBP5 may also control osteogenesis via the ERK1/2 signaling pathway. Consistent with this model, we determined that overexpressing 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 overexpression, 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.

Declarations

Acknowledgements

The authors would like to acknowledge Shanghai Genechem Co., Ltd for the technical support.

Author contributions ZZ and ML performed the experiments and wrote the manuscript draft. ZZ, ML, JD, HZ and WL performed the experiments and participated in analysis. ZZ and WL conceived the experiments and revised the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

Research involving human and animal participants This article does not include studies using human participants or animals performed by any of the authors.

Consent to participate The authors consent to participate.

Consent for publication The authors consent for publication.

References

1. Pittenger MF, Mackay AM, Beck SC et al (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* 284:143–147
2. Barry F, Boynton RE, Liu B et al (2001) Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. *Exp Cell Res* 268:189–200
3. Arinzech TL (2005) Mesenchymal stem cells for bone repair: preclinical studies and potential orthopedic applications. *Foot Ankle Clin* 10:651–665
4. Helder MN, Knippenberg M, Klein-nulend J et al (2007) Stem cells from adipose tissue allow challenging new concepts for regenerative medicine. *Tissue Eng* 13:1799–1808
5. Rosen C, Karsenty G, MacDougald O (2012) Foreword: interactions between bone and adipose tissue and metabolism. *Bone* 50:429
6. Scheideler M, Elabd C, Zaragosi LE et al (2008) Comparative transcriptomics of human multipotent stem cells during adipogenesis and osteoblastogenesis. *BMC Genom* 9:340
7. Steward AJ, Kelly DJ (2015) Mechanical regulation of mesenchymal stem cell differentiation. *J Anat* 227:717–731
8. Park YH, Yun JI, Han NR (2013) Mass production of early-stage bone-marrow-derived mesenchymal stem cells of rat using gelatin-coated matrix. *Biomed Res Int* 2013:347618
9. Martino NA, Reshkin SJ, Ciani E et al (2014) Calcium-sensing receptor-mediated osteogenic and early-stage neurogenic differentiation in umbilical cord matrix mesenchymal stem cells from a large animal model. *PloS One* 9:e111533
10. Okolicsanyi RK, Camilleri ET, Oikari LE (2015) human mesenchymal stem cells retain multilineage differentiation capacity including neural marker expression after extended in vitro expansion. *PloS One* 10:e0137255
11. Pouriamehr S, Barmaki H, Rastegary M et al (2019) Investigation of insulin-like growth factors/insulin-like growth factor binding proteins regulation in metabolic syndrome patients. *BMC Res Notes* 12:653
12. Durant D, Pereira RM, Canalis E (2004) Overexpression of insulin-like growth factor binding protein-5 decreases osteoblastic function in vitro. *Bone* 35:1256–1262
13. Nishihara K, Suzuki Y, Roh S (2020) Ruminal epithelial insulin-like growth factor-binding proteins 2, 3, and 6 are associated with epithelial cell proliferation. *Anim Sci J* 91:e13422
14. Akkiprik M, Feng Y, Wang H (2008) Multifunctional roles of insulin-like growth factor binding protein 5 in breast cancer. *Breast Cancer Res* 10:212
15. Andress DL (2001) IGF-binding protein-5 stimulates osteoblast activity and bone accretion in ovariectomized mice. *Am J Physiol Endocrinol Metab* 281:E283–E288
16. Kim YH, Park M, Cho KA (2016) Tonsil-Derived Mesenchymal Stem Cells Promote Bone Mineralization and Reduce Marrow and Visceral Adiposity in a Mouse Model of Senile Osteoporosis.

17. Casado-Díaz A, Túnez-Fiñana I, Mata-Granados JM (2017) Serum from postmenopausal women treated with a by-product of olive-oil extraction process stimulates osteoblastogenesis and inhibits adipogenesis in human mesenchymal stem-cells (MSC). *Exp Gerontol* 90:71–78
18. Benisch P, Schilling T, Klein-Hitpass L (2012) The transcriptional profile of mesenchymal stem cell populations in primary osteoporosis is distinct and shows overexpression of osteogenic inhibitors. *PloS One* 7:e45142
19. Du Y, Wang P (2019) Upregulation of MIP regulates human breast cancer proliferation, invasion and migration by mediated by IGFBP2. *Pathol Res Pract* 215:152440
20. Xi G, Demambro VE, D'Costa S (2020) Estrogen Stimulation of Pleiotrophin Enhances Osteoblast Differentiation and Maintains Bone Mass in IGFBP-2 Null Mice. *Endocrinology* 161:bqz007
21. Wang Y, Jia Z, Diao S (2016) 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. *Cell Prolif* 49:618–627
22. Han N, Zhang F, Li G (2017) Local application of IGFBP5 protein enhanced periodontal tissue regeneration via increasing the migration, cell proliferation and osteo/dentinogenic differentiation of mesenchymal stem cells in an inflammatory niche. *Stem Cell Res Ther* 8:210
23. Mukherjee A, Rotwein P (2008) Insulin-like growth factor-binding protein-5 inhibits osteoblast differentiation and skeletal growth by blocking insulin-like growth factor actions. *Mol Endocrinol* 22:1238–1250
24. Johnson SK, Haun RS (2009) Insulin-like growth factor binding protein-5 influences pancreatic cancer cell growth. *World J Gastroenterol* 15:3355–3366

Figures

Figure 1

Assessment of IGFBP5 expression during hMSC osteogenesis and stably infected hMSC identification. (A) IGFBP5 during osteogenic differentiation was assessed via qRT-PCR over time. Data are means $\pm$ SD ($X \pm SD$, $n=3$). ** $P<0.01$ vs. Day 0. (B) Following infection, cells were assessed via light and fluorescence microscopy (10 $\times$; scale bar, 50 μ m), with representative images being shown. (C) IGFBP5 mRNA expression was evaluated via qRT-PCR. Data are means $\pm$ SD ($X \pm SD$, $n=3$). ** $P<0.01$ vs. Negative control. (D) IGFBP5 protein expression was evaluated via Western blotting. Note: NC: Negative control; OE:IGFBP5 overexpression; shRNA:IGFBP5 shRNA.

Figure 2

Modulation of IGFBP5 expression (IGFBP5 overexpression or knockdown) impacts hMSC osteogenesis. (A, B) Light microscopy was used to evaluate ALP staining in hMSCs at different stages of differentiation (10 $\times$; scale bar, 50 μ m). (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 $\pm$ 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 overexpression; sh: IGFBP5 shRNA.

A **B** **C** **Figure 3**

Analysis of ERK1/2 phosphorylation during hMSC osteogenesis. (A) IGFBP5 overexpression promoted ERK1/2 phosphorylation. (B) IGFBP5 knockdown suppressed p-ERK1/2 levels. (C) ERK1/2 inhibitor treatment decreased intracellular p-ERK1/2 levels. Note: NC: negative control; OE:IGFBP5 overexpression; sh:IGFBP5-shRNA; OE+IN:IGFBP5 overexpression+inhibitor.

Figure 4

IGFBP5 controls hMSC osteogenesis via regulating ERK1/2 activation. Analysis of the osteogenic differentiation of hMSCs following IGFBP5 overexpression and ERK1/2 signaling inhibitor treatment. (A) Osteogenesis was evaluated via ALP staining (10 $\times$; scale bar, 50 μ m). Cells in which IGFBP5 was overexpressed that were treated with an ERK1/2 signaling inhibitor exhibited decreased osteogenesis relative to cells in which IGFBP5 was overexpressed but no inhibitor was added. (B) Significant differences in ALP activity were observed when comparing the overexpression IGFBP5+inhibitor and the overexpression IGFBP5 groups. (C) RUNX2, OCN and ALP expression in different groups was compared via qRT-PCR. Data are means $\pm$ SD ($\bar{X}\pm$ SD, n=3). **P<0.01 vs. Overexpression IGFBP5. (D) RUNX2 and OCN protein levels were evaluated via Western blotting. Note: OE: IGFBP5 overexpression; OE+IN: IGFBP5 overexpression+inhibitor.