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Nuclear Factor I transcription factors regulate IGF binding protein 5 gene transcription in human osteoblasts

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Abstract

Insulin-like growth factor binding protein 5 (*IGFBP5*) is expressed in many cell types including osteoblasts and modulates IGF activities. *IGFBP5* may affect osteoblasts and bone formation, in part by mechanisms independent of binding IGFs. The highly conserved *IGFBP5* proximal promoter within 100 nucleotides of the start of transcription contains functional *cis* regulatory elements for C/EBP, Myb and AP-2. We report evidence for a functional Nuclear Factor I (NFI) *cis* element that mediates activation or repression of *IGFBP5* transcription by the *NFI* gene family. All four *NFI* genes were expressed in human osteoblast cultures and osteosarcoma cell lines. Co-transfection with human *IGFBP5* promoter luciferase reporter and murine Nfi expression vectors showed that Nfib was the most active in stimulating transcription. Nfix was less active and Nfia and Nfic were inhibitory. Knockdown of *NFIB* and *NFIC* expression using siRNA decreased and increased *IGFBP5* expression, respectively. Analysis of *IGFBP5* promoter deletion and mutation reporter constructs identified a functional NFI *cis* element. All four NFI proteins bound the NFI site in electrophoretic mobility shift experiments and NFI-B bound in Chromatin Immunoprecipitation assays. Results suggest that NFI proteins are important regulators of *IGFBP5* expression in human osteoblasts and thus in modulating *IGFBP5* functions in bone.

Keywords

Osteoblasts; IGF Binding Proteins; Nuclear Factor I; *IGFBP5* promoter

1. Introduction

The Insulin-like Growth Factors (IGFs) are important paracrine and autocrine regulators of cellular growth, differentiation and apoptosis [1-3]. IGFs are produced by osteoblasts and have important functions in regulating osteoblast proliferation, differentiation and survival, and bone development, growth, repair and continuous renewal [4-6]. IGF functions are modulated by a family of binding proteins referred as Insulin-like growth factor binding proteins

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(IGFBPs). These binding proteins prolong the serum half-life of the IGF peptides, transport the IGFs to target cells, and modulate the interaction of IGFs with their surface membrane receptors [7-9]. IGFBP5 possesses a high specific affinity for bone matrix proteins and bone mineral [10], and is the most abundant IGFBP stored in bone [11]. It is expressed in osteoblasts and osteoblast precursors, stimulates osteoblast proliferation, and protects osteoblasts from apoptosis *in vitro*. IGFBP5 has been reported to stimulate or inhibit osteoblast differentiation and bone formation *in vivo* [3,11-14]. While extracellular IGFBP-5 actions to inhibit IGF activity is mediated by binding to IGFs [3], IGFBP5 effects on bone cell growth have also been found to be IGF independent through binding to specific cell surface sites [15-17] and phosphorylation of putative IGFBP receptors [18]. Moreover, recent data shows that IGFBP5 contains a nuclear localization sequence that mediates transport of IGFBP5 into the nucleus [19], interacts with nuclear proteins FHL2 [20] and vitamin D receptor [21], and contains a functional transactivation domain localized in the N terminus [22], suggesting that IGFBP5 could act as a regulator of transcription.

Previous studies reported that *IGFBP5* transcription is positively regulated by prostaglandins, progesterone, testosterone, retinoids, cAMP, PTH, and IGFs [23-29], and negatively regulated by glucocorticoids and OP-1/BMP-7 [30-32]. The proximal few hundred bp of rat, human and mouse *IGFBP5* gene promoters are highly conserved [1] and contains a TATA box and functional *cis*-acting sites for MN1, C/EBP, Myb, and AP-2 transcription regulators [28, 33-36]. The proximal 70 bp of the promoter contains a putative NFI binding site overlapping the AP-2 binding site, but the role of NFI transcription factors in regulation of *IGFBP5* transcription has not previously been reported. The aim of the present study was to determine whether the putative NFI *cis* element mediates transactivation by NFI transcription factors in human osteoblasts.

The *NFI* gene family is composed of four closely related members in vertebrates, *NFIA*, *NFIB*, *NFIC* and *NFIX*. These proteins play wide reaching roles in viral DNA replication, regulation of gene transcription, cell proliferation, and development. In germline gene knockout studies *Nfia*^{-/-} mice had mainly neuroanatomical defects [37], *Nfib*^{-/-} mice had lung and brain defects, and embryonic lethality [38], *Nfic* knockout inhibited odontoblast differentiation and caused disruption of tooth root and underlying mandibular bone development [39,40], and *Nfix*^{-/-} mice had brain malformation [41] and developed a deformation of the spine, exhibited poor growth postnatally, and had impaired endochondral ossification and decreased mineralization [42]. Alternative mRNA splicing in all *NFI* genes generates multiple isoforms, most of which retain the conserved DNA binding and dimerization domains [43]. Transcriptional activation or repression of genes by *NFI* gene family members varies depending on cell type and gene promoter. In this study we found that *IGFBP5* transcription is differentially modulated in human osteoblastic cells by members of the *NFI* transcription factor family through binding to the consensus NFI element in the proximal promoter region, suggesting that NFI proteins have a potential role in regulating osteoblast proliferation and differentiation.

2. Experimental Procedures

2.1 Cell Culture

Human osteosarcoma cell lines U2OS (hTB96), MG63, and TE85 with osteoblastic properties were from American Type Culture Collection (ATCC). Normal human osteoblasts were originally isolated as described [30] from calvaria of a 52 year old female (HBC 157), rib of a 32 year old male (HBR 112), and vertebrae of a 38 year old male (HBV 163) and 33 old male (HBV6408) obtained from Cooperative Human Tissue Network, which is supported by the National Cancer Institute or from non-identified discarded surgical waste (HBV 6408) with approved exemption from the VA Loma Linda Health Care Network Institutional Review

Board. All cells were cultured in DMEM (Mediatech, Inc.) plus 10% Fe-supplemented bovine calf serum (CS, Atlanta Biologicals) as described previously [23]. Human choriocarcinoma cell line JEG-3 was obtained from ATCC and cultured in DMEM plus 10% Fetal Bovine Serum (FBS, Atlanta Biologicals). Cell culture plasticware was from Costar/Corning or Greiner Bio-One.

2.2 RNA extraction and Quantitative RT-PCR

Three independent samples of total RNA per group were isolated from 80 % confluent cells using a Gentra Systems Versagene™ kit (Fisher Scientific), from which 300 ng RNA were reverse transcribed in duplicate using SuperScript III (Invitrogen) or iScript (BioRad). Real time PCR was performed with an Opticon 2 Fluorescent Detector (MJ Research/BioRad), using SYBR Green Master Mix (Qiagen) and sequence specific primers for human NFI-A, NFI-B, NFI-C, NFI-X, IGFBP5 and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1). PCR primers (Table 1) and other oligodeoxynucleotides were from Integrated DNA Technologies Inc. Initially, the PCR products were cloned into pCR-Blunt II TOPO plasmids (Invitrogen), sequences were verified, and the plasmids were used to verify linearity of amplification as a function of template concentrations. Relative mRNA levels normalized to endogenous GAPDH mRNA levels are presented relative to NFI-C mRNA levels in each cell line (Table 2) or relative to non-targeting control in siRNA knockdown experiments (Figure 2) using the $2^{-\Delta\Delta CT}$ Method [44].

2.3 Plasmid Constructs

Wild type and mutant *IGFBP5* promoter sequences (GenBank accession No. **U20271**) were inserted in the pGL3 basic luciferase reporter vector (Promega). In a previous study, a series of promoter sequences were inserted into the CAT reporter gene vector pJFCAT1 [23]. The sequences from bp -252 to +24 and -124 to +24 were sub-cloned from the respective pJFCAT1 vectors into pGL3-basic, resulting in plasmids BP5p252-Luc and BP5p124-Luc. Promoter bp -252 corresponds to bp 1139 of GenBank U20271 sequence. BP5p106-Luc containing bp -106 to +24 was made by PCR with BP5p252-Luc as the template using forward primer 5'-TAGCACTCGAGCATCCTTGCTGAGTTGGG-3' containing an XhoI site (underlined) and reverse primer 5'-GGCGTCTTCCATGGTGGC-3' corresponding to the pGL3 vector sequence containing an NcoI site. After digestion with XhoI and NcoI, the fragment was sub-cloned into pGL3-basic. BP5p70-Luc containing bp -70 to +24 was constructed by introducing an XhoI site at bp -70 in p252-Luc by PCR based site directed mutagenesis (Quick Change™, Stratagene) with primer 5'-GTTGGGTGTTGGGAAGCTCgAgTTGCAGCTACAACTGG-3' and its complement (altered nucleotides in lower case). The XhoI/NcoI fragment from bp -70 to +98 was sub-cloned from the mutant BP5p252-Luc into the pGL3-basic vector. To delete bp -70 to -35 from BP5p252-Luc to make the BP5p252 Δ 70-35-Luc construct, a PCR product was made encoding bp -35 to +24 of the *IGFBP5* promoter using forward primer 5'-TAGCACTCGAGGCTATTTAAAAGCGCCTGC-3' containing an XhoI site and reverse primer 5'-GGCGTCTTCCATGGTGGC-3' containing the pGL3 NcoI site. This XhoI/NcoI digested PCR fragment was used to replace the XhoI/NcoI fragment in the mutant BP5p252-Luc that contained the added XhoI site at bp -70.

BP5p106m1-Luc with a deletion of the overlapping NFI and AP2 binding sites (bp -54 to -37) was made with a synthetic DNA duplex 5'-CCGCTCGAGGCATCCTTGCTGAGTTGGTGTGGGAA GCTCAAATTGCAGCTACAACT [deletion] CGGCTATTTAAAAGCGCCTGCTCTCCCGGAGCC CCGTAGTCTCTTTGGAACTTACTAGTCTGCAGGTCG-3' containing XhoI and PstI sites (underlined sequences). The fragment was digested with XhoI and PstI and cloned into the BP5p106-Luc plasmid after removal of the wild type fragment. Mutation of the NFI site

at its 5' end to make BP5p106m2-Luc used a synthetic DNA duplex 5'-
 AGCCCGGGCTCGAGGCATCCTTGCCTGAGTTG
 GGTGTTGGGAAGCTCAAATTGCAGCTACAAACTaGtTGGCAGCCAGGGGCCGGC
 TATTTAAA
 AGCGCCTGCTCTCCCGGAGCCCCGTAGTCTCTTTGGAAACTTCTGCAGGTCGACT
 C-3' to replace the wild type sequence in BP5p106-Luc (altered nucleotides in the NFI site are in lowercase, XhoI and PstI sites are underlined). BP5p106m3-Luc containing proximal and distal mutations in the NFI binding site of the *IGFBP5* proximal promoter (bp -55 to -41) was made by site directed mutagenesis using primer 5'-
 GCAGCTACAAACTtGCTGGCAGCaAGGG GCCGGC-3' and its complement (altered nucleotides in lower case), with BP5p106-Luc as template. DNA sequences of all constructs were verified by automated DNA sequencing.

Mouse HA-tagged pCH-A (*Nfia-4*) [45], pCH-B (*Nfib2*), pCH-C (*Nfic2*), and pCH-X (*Nfix2*) plasmid expression vectors [46] that encode mouse NFI proteins; as well as the empty pCMV β vector pCH were provided by Dr Richard M. Gronostajski (Department of Biochemistry, SUNY at Buffalo, Buffalo, NY, USA). A pCMV6-XL5 plasmid vector expressing human NFIB2 was obtained from Origene (Catalogue No. SC116683).

2.4 Luciferase Reporter Assay

TE85 and MG63 cells were transiently transfected at 50% confluency in 6 well plates with 0.5 μ g of *IGFBP5* promoter-Luc plasmid DNA and 0.125 to 1 μ g of each murine Nfi expression vector, using SuperFect (Qiagen) for TE85 cells or TurboFectin 8.0 (OriGene Technologies) for MG63 cells. Total plasmid DNA was adjusted by addition of pCH. TE85 and MG63 culture medium was changed to 1 mg/ml BSA in DMEM after 5 h or 18 h, respectively, and cells were extracted in reporter lysis buffer (Promega) after an additional 24 h for luciferase assay as described [23]. Luciferase activities were normalized to protein concentrations determined by bicinchoninic acid (BCA) assay (Pierce Biotechnology) and are presented as fold of control. Sample groups consisted of 6 replicate wells of cells. Each transient transfection experiment was performed at least two times. JEG-3 cells were transiently transfected with 2 μ g of each HA-tagged pCH expression vector (Nfi) and TurboFectin 8.0 (OriGene Technologies, Rockville, MD).

2.5 siRNA Transfection

RNAi experiments were performed with ON-TARGET plus SMART pools (4 targets each) targeting NFIB (L-008456-00), NFIC (L-008362-00) or NFIX (L-009250-00) mRNAs and ON-TARGET plus siCONTROL Non Targeting siRNA #1 (Dharmacon). MG63 cells (2×10^5 per well of 6-well plate) were transfected in Opti-MemI (Gibco-Invitrogen) with 200 pmol of SMARTpool or siCONTROL using DharmaFECT-1 reagent. MG63 cells (2×10^5 per well of 6-well plate) were transfected in Opti-MemI (Gibco-Invitrogen) with 200 pmol of SMARTpool or siCONTROL using DharmaFECT-1 reagent. Human vertebral derived cells (HBV6408 passage 4) were trypsinized and resuspended in siPORT siRNA electroporation buffer (Ambion, Austin, TX) at a density of 1.8×10^7 cells/ml. The cells were electroporated with 74 pmol of siCONTROL non targeting siRNA #1 or ON-TARGET plus NFIB Duplex 5 (Dharmacon J-008456-05) in 0.1 ml using a Bio Rad Gene pulse Xcell instrument and 2 mm cuvettes. The cells received a single 140 V square wave pulse of 15 ms duration. Total RNA was extracted at 24 and 48 h for qRT-PCR assay. MG63 cell lysates were collected at 72 h for protein analysis by western blot. To determine if overexpression of Nfib can reverse the effects of NFIB siRNA knockdown on *IGFBP5* transcription, MG63 cells were trypsinized and resuspended in siPORT buffer at a density of 3×10^7 cells/ml. Cells were electroporated with 74 pmol of siCONTROL non targeting siRNA #1 or ON-TARGET plus NFIB Duplex 5 (Dharmacon J-008456-05) together with 2.5 μ g of BP5p252-luc and 2.5 μ g of pCH-B (mouse

Nfib) or pCH-parental plasmid using the same parameters as for normal vertebral cells. The single IGFBP5 siRNA duplex targets a sequence in endogenous human NFIB mRNA that is not present in mouse *Nfib* mRNA. Luciferase reporter activity was determined 48 h after electroporation.

2.6 Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared from confluent MG63 and JEG-3 cells utilizing the Active Motif Nuclear Extract Kit, as per manufacturer's instructions. A double stranded synthetic oligonucleotide corresponding to *IGFBP5* promoter sequence bp -67 to -34 containing the NFI binding site wt BP5⁶⁷⁻³⁴: (5'-GCAGCTACAACTGGCTGGCAGCCAGGGGCCG-3') (Figure 3A), was labeled with dCTP[α -³²P] (111 Tbq/mmol, MP Biomedical), by fill in with DNA polymerase Klenow fragment (New England Biolabs). The gel shift reaction was performed with 10 μ g of nuclear extract protein using the Nushift™ Kit (Active Motif) without addition of poly dI:dC. For supershift 5 μ g HA.11 antibody (Covance), NFIB2 antibody (Active Motif) or Normal Rabbit IgG (Jackson ImmunoResearch) were incubated with nuclear extract for 20 min at 4°C prior to addition of IGFBP5 probe for 20 min. DNA-protein complexes were separated by electrophoresis on 5% polyacrylamide gels run in TGE (0.05M Tris, 0.4M Glycine, and 0.002M EDTA) buffer, pH 8.5 at 4°C. Complexes were imaged using a Storm 820 Phosphorimager (GE Healthcare Bio-sciences).

In binding competition experiments increasing concentrations (2.5X, 5X, 10X and 20X) of unlabeled double stranded oligonucleotides were incubated with the nuclear extract prior to addition of the [³²P]-BP5⁶⁷⁻³⁴ wt probe. Binding competitors were BP5⁶⁷⁻³⁴ wt containing the NFI binding site, NFI consensus sequence (5'-CAGCCTTTGGCATGCTGCCAATA-3'), BP5⁶⁷⁻³⁴ mNFI with mutations in the NFI binding site (5'-GCAGCTACAA ACctCTGGCAGtaAGGGGCCG-3'), BP5⁶⁷⁻³⁴ mAP2 with mutation of the AP-2 site (5'-GCAGC TACAAACTGGCTGGCAGCCAGGactaGGC-3'), and Col1a2 from the proximal promoter of the human type I α 2 procollagen gene, containing no NFI binding sequences (NCBI Gene symbol COL1A2, 5'-CCTCCTCTGCGCCCCCGCAGG CTCCTCCCAGCTGTG GCT-3').

2.7 Chromatin Immunoprecipitation (ChIP)

MG63 cells were formaldehyde fixed and chromatin DNA complexes were enzymatically sheared using a ChIP-IT™ Express Enzymatic Kit (Active Motif). Chromatin was immunoprecipitated with 3 μ g of mouse NFI-B2 or Negative Control Rabbit IgG (Active Motif). PCR with precipitated chromatin and input DNA templates was performed with 2X GoTaq Master Mix (Promega) using primer pairs for the *IGFBP5* gene bp -119 to + 54 (For: 5'-GGCATCCTTGCTGAGTTG-3', Rev: 5'-CTGCTTTGCAGCTCTTTCCTA-3') and for two negative controls, NFI-X coding sequence (Exon 4 – 6, For: 5'-CCACTGCCCCAACGGACACTT-3', Rev: 5'-CCGGGATAGAACACGTCATCA-3') and GAPDH gene proximal promoter (For: 5'-TACTAGCGGTTTTACGGGCG-3', Rev: 5'-TCGAACAGGAGGAGCAGAGAGCGA-3'). To compare relative amounts of IGFBP-5 promoter amplicons precipitated by NFIB2 and control antibodies, the precipitated chromatin DNA was amplified by real time PCR from duplicate precipitated chromatin samples of each, using the same IGFBP5 promoter primers and SYBR as described for RT-PCR.

2.8 Western Blots

MG63 cells were lysed in RIPA Buffer containing protease inhibitors (Sigma). Extracts (20 μ g protein) were assessed with NFIB2 (Active Motif), IGFBP5 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), HA.11 (Covance, Emeryville, CA), and Actin (Sigma, St. Louis, MO) primary antibodies followed by IRDye secondary antibodies. Bands were imaged with an Odyssey Infrared Imaging system (Li-Cor).

2.9 Statistical Analysis

All of the statistical analyses were performed using the Microsoft Excel data analysis program for Student's *t* test analysis with an assigned significance level of $p < 0.01$. All of the experiments were repeated at least three times. The values are expressed as the means $\pm$ SEM.

3. RESULTS

3.1 Nuclear Factor I mRNA Expression in Osteoblasts and Osteoblast-like Cells

We sought to determine the potential roles of NFI transcription factors in regulation of *IGFBP5* transcription in human osteoblasts. As a first step, the relative mRNA expression levels of *NFIA*, *NFIB*, *NFIC* and *NFIX* genes were determined in early passage subconfluent cultures of human osteoblasts and osteoblast-like cell lines using qRT-PCR (Table 2). RT and realtime PCR reactions were performed under conditions in which relative mRNA levels are linearly proportional to RNA input. *NFIC* mRNAs were consistently the most abundant in each cell sample, therefore these were used as baseline for the quantification of relative expression of the other *NFI* genes. The *NFIC* ΔC_t values ranged from 6.3 (U2OS) being the most abundant to 8.51 (MG63) the least abundant, with a difference of 4 fold in relative expression levels. Although *NFIC* was the most highly expressed gene in the majority of cell lines, in MG63 osteoblast-like cells *NFIB* mRNA levels were 16 fold higher than *NFIC* levels. *NFIA* mRNA levels were least abundant in all cell samples except the normal osteoblasts and U2OS cells in which *NFIX* expression was also low. These data suggested that *NFIB* and *NFIC* might have the most important roles in regulating *IGFBP5* transcription in human osteoblast cells.

3.2 NFI transcription factors modulate IGFBP5 Promoter Activity

We examined the effects of NFI proteins on *IGFBP5* promoter activity by transiently co-transfecting MG63 and TE85 cells with mouse *Nfi* expression vectors (pCH-A, pCH-B, pCH-C and pCH-X) and a promoter construct containing bp -252 to +24 of the human *IGFBP5* gene in pGL3.1 luciferase reporter (BP5-p252-luc) as described [23]. *Nfib* was the most active in stimulating *IGFBP5* promoter activity (Figure 1A, 1B), and showed a biphasic dose response in TE85 cells with maximal stimulation of approximately 10 fold. Western blot from a representative experiment indicates that all four *Nfi* genes were expressed after transfection (Figure 1C). Human *NFIB2* was as effective as mouse *Nfib2* in stimulating *IGFBP5* transcription (Figure 1D). *Nfix* was less active than *Nfib* and increased promoter activity with dose dependent maximal 3 fold stimulation in TE85 cells (Figure 1A). *Nfia* and *Nfic* modestly increased *IGFBP5* promoter activity at lower doses and inhibited promoter activity at the higher doses tested. The effect of *Nfic* on *IGFBP5* promoter activity was surprising since its relative mRNA expression was high in both early passage human osteoblasts and osteoblast-like osteosarcoma cells. In contrast to TE85 cells, fold activation of the *IGFBP5* promoter by *Nfib* and *Nfix* was less in MG63 cells (Figure 1B), possibly because high endogenous expression of *NFIB* in this cell line already supported strong basal promoter activation, and increased the sensitivity to inhibition by exogenous *Nfi* expression.

3.3 Effects of NFI siRNA transfection on NFIB and IGFBP5 expression levels

To complement the NFI overexpression and *IGFBP5* promoter activity experiments, siRNA was used to determine if knockdown of endogenous *NFIB* expression would decrease *IGFBP5* expression. MG63 cells were transiently transfected with 200 pmol of non-targeting control siRNA or a pool of 50 pmol each of 4 different siRNAs targeting human *NFIB*. Analysis of mRNA levels by qRT-PCR indicated that *NFIB* mRNA levels were significantly reduced by 60% and 70% at 24 and 48 h respectively, compared to non-targeting control (Figure 2A, $p < 0.01$). *NFIB* protein expression was also reduced by 76% at 72 hours post transfection (Figure 2B). Importantly, *IGFBP5* expression was also inhibited by 50% and 55% at 24 and

48 h after transfection with NFIB siRNA, and IGFBP5 protein levels were inhibited 33% at 72 h compared to the control ($p < 0.01$). To determine whether knockdown of NFIB expression also reduces IGFBP-5 expression in non-transformed human osteoblasts, vertebral bone derived cells were transfected with non-targeting or NFIB targeting siRNA using electroporation. NFIB mRNA levels were significantly reduced by 96 % at 48 h, compared to non-targeting control and IGFBP5 mRNA levels were reduced by 63 % (Figure 2A). NFIC and NFIX mRNA levels in MG63 cells did not significantly change when NFIB expression was inhibited by siRNA (Figure 2A). Similar results were obtained with 200 pmol of individual NFIB siRNAs compared to the mixture (data not shown).

Because NFIC was highly expressed in human osteoblasts and was apparently inhibitory to the *IGFBP5* promoter activity, we determined whether knocking down NFIC expression would affect NFIB and IGFBP5 mRNA levels. MG63 cells were transfected with a specific pool of siRNAs targeting human NFIC, (Figure 2C) resulting in a decrease in NFIC mRNA expression of 90% ($p < 0.01$). NFIC knockdown increased IGFBP5 mRNA levels 1.6-fold ($p < 0.01$), but did not significantly affect NFI-B mRNA levels. NFIX siRNA knockdown reduced NFI-X mRNA levels 90 %, but did not significantly affect IGFBP5 or NFI-B mRNA levels (Figure 2C). These results are consistent with *IGFBP5* promoter experiments showing that NFIC inhibited *IGFBP5* transcription.

While use of an optimized mixture of gene targeting SMART pool siRNAs minimizes non-specific off-target effects [47], to further confirm the specificity of NFIB siRNA knockdown, we performed a rescue of function experiment as a control for off target effects [48]. Mouse *Nfib* was overexpressed to counteract effects of endogenous NFIB knockdown on *IGFBP5* promoter activity. MG63 cells were co-transfected by electroporation with non-targeting siRNA or a siRNA duplex targeting endogenous human NFIB that does not target mouse *Nfib*, plus BP5-p252-luc and pCH-B mouse *Nfib* expression vector or pCH empty vector (Figure 2D). *IGFBP5* promoter activity was determined after 48 h. In cells transfected with siRNA targeting NFIB, and control pCH vector, BP5-p252-luc promoter activity was decreased by 74% compared to the control group that contained the non-targeting siRNA ($p < 0.01$). Effects of siRNA on promoter activity were thus similar to effects on IGFBP5 mRNA levels. Overexpression of mouse *Nfib* increased *IGFBP5* promoter activity in cells receiving NFIB-targeting siRNA by approximately 2 fold ($p < 0.01$). This rescue of the effects of NFIB siRNA on *IGFBP5* expression supports the conclusion that the siRNA knockdown was not the result of off target siRNA effects.

3.4 NFIB Activation of IGFBP5 Promoter Deletion and Mutation Constructs

To identify elements of the *IGFBP5* promoter region between bp -252 and +24 that are involved in NFI-mediated transactivation, we created a series of *IGFBP5* promoter deletion constructs beginning at different 5' positions (Figure 3A). Results indicated that basal promoter activity and activation of *IGFBP5* promoter by *Nfib* in TE85 cells progressively decreased as 5' regions of the promoter were deleted (Figure 3B). Internal deletion of bp -70 to -35 from BP5p252-Luc strongly decreased basal promoter activity, but it did not completely abolish *Nfib* mediated activation (Figure 3B). These results suggest that while the proximal promoter region containing the putative NFIB binding site mediates significant regulation of promoter activity and NFI responsiveness, upstream *cis*-acting elements may also contribute to *Nfib* activation. Subsequent experiments focused on the proximal promoter region bp -106 to +24, containing the palindromic consensus NFI binding site sequence TGGC(N)₅GCCA [43], adjacent to other *cis* elements that were previously reported to participate in modulating *IGFBP5* gene transactivation [28, 33-36]. Deletion of the overlapping NFI/AP-2 binding site sequence in the BP5p106-Luc construct (BP5p106m1 in Figure 3A) or mutation of two bases in the 5' side of the NFI binding sequence, without altering the 3' side that overlaps the AP-2 site

(BP5p106m2), strongly inhibited Nfib activation of promoter activity in both TE85 and MG63 cell lines (Figure 3D and Figure 3E). Mutation of one base in the 5' side and one base in the 3' side of the NFI binding sequence (BP5p106m3) decreased Nfib activation of the promoter, although not as strongly.

3.5 NFIB Binds to the IGFBP5 Promoter Region

In order to establish if NFIB activation of *IGFBP5* transcription was mediated by direct binding to the consensus binding sequence in the proximal promoter, we performed Electrophoretic mobility shift assays (EMSA) and Chromatin Immunoprecipitation (ChIP) assay. Nuclear extracts were made from the MG63 cell line, because it contains high endogenous NFIB mRNA and protein levels. Nuclear extract formed a major complex with the BP5⁶⁷⁻³⁴ probe containing putative binding sequences for NFI as well as AP-2, and probe binding was competed by excess unlabeled wild type BP5⁶⁷⁻³⁴ oligonucleotide (Figure 4A, lanes 3-6). Unlabeled BP5⁶⁷⁻³⁴ oligonucleotide containing mutations in the NFI binding site (lane 8-11), and an unrelated alpha 2 (I) collagen oligonucleotide competitor, not containing any consensus NFI *cis* elements (lane 12), did not compete with the BP5⁶⁷⁻³⁴ probe. Addition of an alternative NFI consensus oligonucleotide competitor, not identical to the probe sequence, and BP5⁶⁷⁻³⁴ oligonucleotide competitor with mutation of the AP-2 binding site, not overlapping the NFI site, also reduced probe binding to the nuclear protein complex (data not shown). To determine if NFIB was present in the probe-nuclear protein complex, NFIB2 antibody was added to the reaction mixture. NFIB2 antibody, but not control normal rabbit IgG, produced a supershift (Figure 4B), indicating that NFIB indeed binds to the putative binding site in the proximal *IGFBP5* promoter.

Due to unavailability of specific antibodies to NFIA, NFIC and NFIX proteins for use in EMSA, supershift experiments for these endogenous NFI proteins could not be done. To determine if these proteins bind to the *IGFBP5* proximal promoter region containing the NFI site, binding of HA-tagged Nfia, Nfib, Nfic, and Nfix to the BP5⁶⁷⁻³⁴ probe was tested using the human choriocarcinoma cell line JEG-3, which expresses low levels of endogenous NFI proteins [46]. JEG-3 cells were transiently transfected with pCH-A, pCH-B, pCH-C, or pCH-X and nuclear extracts were tested by EMSA. Nuclear extracts from cells transfected with all four HA-tagged NFI proteins formed complexes with the BP5⁶⁷⁻³⁴ probe (only Nfib is shown, Figure 5 lane 4), but nuclear extracts from JEG-3 control cells transfected with pCH empty control vector did not (lane 3). Addition of HA antibody produced a supershift with nuclear extracts from cells transfected with each of the Nfi expression vectors (lanes 5-8), as previously demonstrated with a probe from a well characterized NFI binding site consensus sequence [49]. This data shows that all four Nfi transcription factors bind to the NFI *cis* element in the *IGFBP5* proximal promoter.

NFIB binding to the *IGFBP5* promoter *in vivo* was assessed utilizing the ChIP assay. Sheared MG63 chromatin was immunoprecipitated with NFIB2 polyclonal antibody. PCR was performed on the precipitated DNA fragments by using specific *IGFBP5* promoter primers to amplify fragments of approximately 150 bp. We found that PCR amplification of the proximal *IGFBP5* promoter region with DNA template chromatin precipitated by the NFIB2 antibody produced a strong band, while chromatin precipitation with a control rabbit IgG did not (Figure 6). Relative amounts of *IGFBP5* promoter DNA precipitated by NFIB specific antibody and control IgG were compared by real-time PCR with the same primers. A difference of 3.0 cycles was observed between C_T values, corresponding to 8 fold higher abundance in the NFIB specific ChIP complex. Negative control primer sets amplifying exon 4-6 of the *NFIX* gene and *GAPDH* proximal promoter were used to determine specificity of chromatin precipitation. Both *GAPDH* promoter and *NFIX* coding region were amplified from input DNA, but not from

DNA precipitated by NFIB2 antibody or control IgG. These experiments corroborate data from *in vitro* binding of NFIB to the *IGFBP5* promoter in EMSA experiments.

4. Discussion

In the present study we demonstrated that all four Nfi proteins can bind to the *IGFBP5* proximal promoter region containing a NFI binding site. Murine Nfib and Nfix increased *IGFBP5* promoter activity in a biphasic manner with Nfib being the most active, while Nfia and Nfic modestly increased, but mostly decreased promoter activity depending on amount of expression vector used. Results of Nfib and Nfic expression on promoter activity were corroborated by siRNA mediated gene knockdown experiments in which knockdown of endogenous NFIB expression decreased *IGFBP5* mRNA and protein levels and decreased *IGFBP5* promoter activity, while NFIC knockdown increased *IGFBP5* expression. Relative expression levels of the four *NFI* genes, and effects of each on transcription of different target genes varies between species and cell type [43]. We found that the *NFIC* gene was the most highly expressed based on mRNA levels in non-transformed normal human osteoblasts and osteoblast-like cell lines, except in the MG63 cell line where NFIB was highly expressed. The PCR primers for NFIB could amplify NFIB1 and NFIB2 that are produced by alternate mRNA splicing in mouse, but not NFIB3 which is truncated after the DNA binding domain [43]. NFIB1 isoform expression has not been reported in human cells, and NFIB3 isoform had very low expression levels in MG63 cells by qRT-PCR with NFIB3 specific primers (data not shown). NFIC relative expression was highest in all three normal human osteoblast cultures, while NFIX and NFIA expression were lowest, which is similar to expression patterns in the TE85 and U2OS cell lines. While expression levels of NFIA, NFIB and NFIX varied between each of the three normal cell preparations, correlation of these differences with osteoblastic phenotype, or with tissue donor sex and age was not apparent. Observation that siRNA knockdown of NFIB had similar effects on *IGFBP5* expression in MG63 cells and normal vertebral bone derived cells suggests that the role of NFIB is similar in normal osteoblastic cells and cells that have undergone tumor transformation and immortalization.

Comparison of the effects of Nfib and Nfic on *IGFBP5* promoter activity, and effects of NFIB and NFIC siRNA on expression of *IGFBP5*, suggest that they could have opposing effects on *IGFBP5* transcription. The relative abundance of these and the other *NFI* gene products could play a significant role in modulating *IGFBP5* expression levels in osteoblasts and other cell types. Knockdown of NFIB by siRNA did not increase expression of NFIC and NFIX mRNA levels, and knockdown of NFIC or NFIX did not alter NFIB expression, suggesting that there may not be compensation of expression among the four genes. NFI proteins resulting from expression of the four genes and alternate mRNA splicing have been reported to have diverse relative effects on different gene promoters and in different cell types [43,50-53]. Heterodimer formation between NFIB and other NFI family members may produce different effects depending on the associated NFI proteins [43]. Differential expression, mRNA splicing and heterodimer formation likely function to integrate diverse biological signals in modulating NFI effects on expression of *IGFBP5*, other genes and associated cellular functions.

EMSA and ChIP experiments support the conclusions from the functional assays of mutant promoter constructs that NFIB transactivates the promoter by binding to the NFI consensus binding site in the *IGFBP5* proximal promoter region. However, deletion of bp -70 to -35 in the BP5-p252Δ70-35-Luc construct did not completely prevent NFI activation, suggesting that, in addition to the NFI site at bp -55 to bp -42, sequence elements upstream of the NFI site may also be involved in mediating activation of the promoter. The NFI binding site overlaps an AP-2 site (bp -46 to -37), previously demonstrated to mediate promoter activation by AP-2α [34]. A previous study found that mutation of the overlapping sequence reduced basal *IGFBP5* promoter activity in rat osteoblasts, while mutation of the AP-2 site alone did not, but effects

of NFI overexpression or siRNA knockdown on IGFBP-5 promoter activity were not investigated [35]. The present study provides more direct evidence that the NFI site does function in transcriptional regulation. Whether NFI and AP-2 interact directly in regulation of *IGFBP5* promoter activity remains to be determined. A Myb binding site on the 5' side of the NFI site (bp -60 to -56) was reported to mediate some, but not all activation of the *IGFBP5* promoter by c-Myb and B-Myb in neuroblastoma cells. The Myb response element was also reported to mediate inhibition of *IGFBP5* promoter activity in fetal rat osteoblasts by BMP-7 and cortisol [31,32]. However, we found that deletion of the NFI site, leaving the Myb site intact, abolished NFI activation, suggesting that activation of *IGFBP5* promoter by NFI does not involve the adjacent Myb response element. However, the results do not rule out potential interactions between NFI and Myb, C/EBP, AP-2 or other transcription factors. Competition of AP-2 and NFI for the overlapping binding site, for example, may be important in modulating *IGFBP5* gene transactivation, and is being investigating in ongoing studies.

NFI transcription factors have been implicated in diverse cellular and molecular processes. For instance, NFI proteins stimulate initiation of adenovirus DNA replication [43], and have been associated with the regulation of many genes including cyclin-dependent kinase inhibitor 1A (p21), TR2 orphan receptor, alpha 1 (I) collagen, p53, whey acidic protein (WAP), and GLUT4 [52,54-58]. NFI proteins functionally interact with and/or bind other TFs and co-activators including GR, AP-2, SRC-1 and CBP/p300 [35,45,59].

Recent studies indicate that NFI plays important roles during bone development and chondrocyte differentiation. *Nfix* deficient mice displayed severe growth retardation and deformation of the spine that was associated with delayed endochondral ossification and decreased mineralization [42], although the growth retardation was reduced in another study by feeding a soft diet [41]. *Nfic* deficiency in mice inhibited odontoblast differentiation and tooth root development [39,40]. Nfib overexpression in murine mesenchymal (ATDC5) cells transactivated the murine alpha 2 (I) collagen promoter, suggesting that this transcription factor is involved in the early stage of chondrocyte differentiation [60]. In contrast, Nfia overexpression was reported to repress Runx2/Cbfa1 promoter activity in rat osteosarcoma cells by a mechanism involving an osteoblast specific enhancer region, while the other NFI family members had no effect on activity [61].

Observations of the present study suggest that NFIB modulation of *IGFBP5* expression in human osteoblasts may have a role in support or regulation of osteoblast proliferation and differentiation, given that IGFBP5 is an important modulator of these events [8]. IGFBP5 stimulated osteoblast proliferation and differentiation *in vitro* and bone formation *in vivo*. For example, human IGFBP5 protein increased alkaline phosphatase activity and osteocalcin expression [13] in MG63 cells and increased proliferation in mouse calvaria cells [13]. *In vivo* studies found that systemic administration of IGFBP5 to rats increased the effects of IGF-I to stimulate calvaria bone formation [62], and IGFBP-5 treatment of mice increased serum osteocalcin levels (bone formation marker) and femoral bone ALP activity [13]. IGFBP-5 actions on osteoblast differentiation and bone formation were found, in part, to be IGF independent. IGFBP-5 stimulated proliferation and alkaline phosphatase activity of mouse osteoblasts from IGF I knockout mice, in which IGF I and IGF II production were undetectable, and *in vivo* IGFBP5 injection increased parietal bone alkaline phosphatase activity [11]. However, other studies reported that overexpression of IGFBP5 in transgenic mice [63,64] or by viral vector expression in cultured osteoblasts inhibited osteoblast differentiation [65]. These inhibitory IGFBP5 actions may arise from inhibition of IGF activities by high extracellular IGFBP5 concentrations [3,66,67], while other actions of IGFBP-5 may be mediated by intracellular mechanisms [8,12,22,68]. Yin et al. for example found that siRNA inhibition of IGFBP-5 expression increased apoptosis in U2OS cells and that IGFBP5 expression from a viral vector reduced apoptosis while addition of extracellular IGFBP5

protein did not [68]. It is possible that NFI and other transcription regulators modulate intracellular IGFBP5 levels and activities while external factors such as blood levels, extracellular matrix and proteases function more to modulate extracellular IGFBP5 levels to provide a complex balance between stimulatory and inhibitory IGFBP5 actions on osteoblasts and other cells.

In summary, we report novel evidence for *IGFBP5* transcriptional regulation by NFI proteins in human osteoblasts. NFIB activates *IGFBP5* promoter mainly through direct binding to a response element in the proximal promoter, although interaction with other *cis*-acting elements upstream of this sequence may also play a role in NFI regulation of *IGFBP5* promoter activation. These results suggest that differential regulation of *IGFBP5* expression by *NFI* gene family transcription factors may be significant to modulation of osteoblast proliferation and differentiation.

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Figure 1.

Effects of *Nfi* family transcription factors on *IGFBP5* promoter activity. Cells were co-transfected with 0.5 μ g of BP5-252-Luc promoter luciferase reporter and the indicated amounts of mouse *Nfia*, *Nfib*, *Nfic*, or *Nfix* HA tag expression vector DNA, or empty expression vector. A) TE85 cells B) MG63 cells. Promoter activity determined as luciferase light units/protein is expressed as fold activation compared to control activity (co-transfection of empty expression vector), mean $\pm$ SEM $n = 6$ replicates. Data from each graph are representative of one experiment, which was repeated two or more times. C) Western blot with HA antibody of 20 μ g of MG63 whole cell lysates transfected with each NFI expression vector demonstrates expression of the transgenes, and is similar to those previously published [45,46]. The 51 kDa

band is non-specific, and is present in non-transfected control cell extracts. D) TE85 cells were co- transfected with BP5-252-Luc and the indicated amounts of mouse Nfib or human NFIB expression vector DNA, or empty expression vector. * indicates significantly different from control ($p<0.01$).

Figure 2.

Effects of NFI siRNA transfection on NFIB and IGFBP5 mRNA levels. MG63 cells were transfected in with 200 pmol of non-targeting siRNA or Smart Pool siRNA targeting NFIB, NFIC or NFIX. Normal vertebral bone derived osteoblasts (HBV6408) were transfected by electroporation with 74 pmol of non targeting siRNA or siRNA duplex 5 targeting NFIB A) NFIB siRNA. Relative mRNA levels were quantified by qRT-PCR at 24 or 48 h. NFIB and IGFBP5 mRNA levels were significantly decreased in the NFIB siRNA groups compared to non-targeting control (* $p < .05$) but NFIC and NFIX mRNA levels were not changed. B) NFIB siRNA. NFIB and IGFBP5 protein levels were detected by Western Blot 72 hours post transfection as in A. C) NFIC or NFIX siRNA. NFIC and NFIX mRNA levels were significantly decreased by respective siRNAs. IGFBP5 mRNA levels were significantly increased by NFIC siRNA, but not by NFIX siRNA. NFIB mRNA levels not significantly affected by NFIC or NFIX siRNA. D) Effects of mouse *Nfib* expression rescue on IGFBP5

expression were determined by assessing IGFBP5 promoter activity. MG63 cells were co-transfected by electroporation with 74 pmol of non-targeting or NFIB targeting siRNA (single siRNA duplexes) plus 2.5 µg of BP5p252-Luc reporter plasmid and 2.5µg of either Nfib expression vector or control vector. Mouse Nfib mRNA was not targeted by the human NFIB siRNA duplex. *IGFBP5* promoter activity was determined at 48 hours (n = 6 wells per group). Relative mRNA levels and promoter activities in all groups are reported as % of the non-targeting siRNA group. * = significantly different from non-targeting siRNA, $p < 0.01$; In C, # = significantly different from control expression vector, $p < 0.01$.

Figure 3.

NFIB activation of *IGFBP5* promoter activity in promoter deletion and mutation constructs. A) *Cis* elements in the human *IGFBP5* promoter, sequences of proximal promoter containing NFI and AP-2 sites, and deletion and mutant sequences tested in BP5p106-Luc. B) TE85 cells were co-transfected with 0.25 μ g pCH control or pCHB Nfib expression plasmid plus 0.5 μ g of promoter reporter BP5-252-Luc, 5' deletions (p124, p106, p70) or bp -35 to -70 deletion in BP5-252-Luc. C), D) TE85 and MG63 cells were transiently co-transfected with 0.5 μ g of wt, m1, m2 and m3 BP5p106-luc constructs and 0.25 μ g of pCH control or Nfib HA tag expression vector. Altered nucleotides in m2 and m3 are indicated in lower case.

Figure 4.

Endogenous NFIB binds *in vitro* to a NFI binding site in the *IGFBP5* proximal promoter region. Equal amounts of double stranded BP5⁶⁷⁻³⁴ oligonucleotide probe were incubated with 7 μ g of MG63 nuclear extract (NE). DNA-protein complexes were resolved in 5% polyacrylamide gels and analyzed with a Storm Phosphorimager. A) Increasing amounts (2.5X, 5X, 10X, and 20X) of BP5⁶⁷⁻³⁴ wt and NFI mutant (BP5⁶⁷⁻³⁴ mNFI) or 20 fold excess of unrelated $\alpha 2$ (I) collagen (Col) unlabeled competitors (Comp) were added to the reaction. B) Equal amounts (5 μ g) of NFIB2 and non-immune IgG (IgG) antibodies (Ab) were added to the probe/nuclear extract reaction. Arrow: supershifted complex. Lanes 1-3 and lanes 5-6 were separated by other samples on the same gel and were joined to produce this figure.

NE -	Con	Con	Nfib	Nfia	Nfib	Nfic	Nfix
Ab -	-	HA	IgG	HA	HA	HA	HA

Figure 5.

Nfi proteins bind *in vitro* to the *IGFBP5* proximal promoter. JEG-3 cells were transfected with mouse Nfia, Nfib, Nfic, or Nfix HA tag expression vectors, or empty control vector. 10 μ g of nuclear extract from each transfection were incubated with BP5⁶⁷⁻³⁴ probe plus anti-HA antibody or non-immune control (IgG) as indicated. Protein-DNA complexes were analyzed as in Figure 4. As shown in lanes 2 and 3, JEG-3 cells have very low endogenous NFI/AP-2 binding to the probe.

Figure 6.

NFI proteins bind *in vivo* to the *IGFBP5* proximal promoter. Formaldehyde-fixed MG63 chromatin (6 μ g) was precipitated with 3 μ g of non-immune IgG1 (lane 1) or NFIB2 (lane 3) antibodies and the precipitated DNA was analyzed by PCR. Equal amounts of input chromatin DNA were also amplified (lane 2). Amplicons stained with ethidium bromide are shown as negative images. *IGFBP5* proximal promoter primers amplified a product from DNA precipitated by NFIB2 antibody which was 8 fold more abundant in NFIB2 compared to non-immune IgG ChIP as determined by quantitative real time PCR of duplicate samples. Negative control primers that target the human *NFIX* exon 4 - 6 coding sequence or the human *GAPDH* proximal promoter only produced PCR products from input DNA templates.

Table 1**Primer sequences utilized in qRT-PCR**

mRNA levels were assessed by reverse transcriptase cDNA synthesis using random primers, followed by real-time PCR using the primers listed and SYBR Green quantitation.

Gene	Forward primer (5' - 3')	Reverse primer (5'-3')	Product size
NFIA NM_005595	CAGCCAAGTGACGCTGACA	CCTCATTGCTCCTGGACTCAT	204 bp
NFIB NM_005596	GCCACAATGATCCTGCCAAGAA	GGTGGAGAAGACAGAGACCTCTGA	214 bp
NFIC NM_005597	GGACAGGGATGGGCTCTG	CGTTCTTCTGAGGCCAGTGC	224 bp
NFIX NM_002501	CCACTGCCCAACGGACACTT	CCGGGATAGAACACGTCATCA	279 bp
GAPDH NM_002046	CGAGCCACATCGCTCAGACA	GTGGTGAAGACGCCAGTGGA	333 bp
IGFBP5 NM_000599	CTCTGGCCTCCTCTCTGAA	GGTGTGACAGTGGCGGTAGA	156 bp

Table 2**Human *NFI* family gene expression in human osteoblasts and osteoblast-like cell lines**

Relative mRNA levels for each *NFI* family were estimated by qRT-PCR using primers in Table 1. Each value of ΔC_T (C_T *NFI* gene – C_T GAPDH) is average of at least 3 determinations. Abundance relative to NFIC in each cell type was determined by the $2^{-\Delta\Delta C_T}$ Method [43]. NFIC was the most abundant mRNA in all cells except MG63, in which NFIB was highly expressed.

NFI Gene	Cell line	ΔC_T	Relative abundance % of NFIC
NFIA	HBC157	9.81	14.3
	HBR112	11.26	3.9
	HBV163	8.35	28.3
	MG63	16.37	0.4
	TE85	15.63	0.4
	U2OS	8.49	21.9
NFIB	HBC157	7.65	64.1
	HBR112	10.34	7.5
	HBV163	7.89	38.9
	MG63	4.46	1656.4
	TE85	10.55	14.1
	U2OS	7.82	34.9
NFIC	HBC157	7.01	100
	HBR112	6.61	100
	HBV163	6.53	100
	MG63	8.51	100
	TE85	7.73	100
	U2OS	6.30	100
NFIK	HBC157	9.09	23.6
	HBR112	11.5	3.3
	HBV163	12.22	1.9
	MG63	10.68	22.2
	TE85	13.54	1.7
	U2OS	8.54	21.2