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Regulation of IGFBP-5 and Osteoblast Functions by Nuclear Factor I

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Regulation of IGFBP-5 and Osteoblast Functions by Nuclear Factor I

by

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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ABBREVIATIONS

AP-2	activating enhancer binding protein 2
bp	base pairs
BP5	insulin-like growth factor-5
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CS	calf serum
CT	cycle threshold
DMEM	Dulbucco's modified Eagle medium
dNTP	deoxynucleotide triphosphate
EMSA	electrophoretic mobility shift assay (gelshift)
FBS	fetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC	glucocorticoid
GIOP	glucocorticoid induced osteoporosis
GR	glucocorticoid receptor
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
kDa	kilodalton
αMEM	minimum essential medium alpha modification
MSC	mesenchymal stem cells
NFI	Nuclear Factor I
OCN	osteocalcin

PCR	polymerase chain reaction
qRT-PCR	quantitative reverse transcriptase PCR
Runx-2	runt-related transcription factor-2
SEM	standard error of the means
TF	transcription factor

ABSTRACT OF THE DISSERTATION

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by

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Doctor of Philosophy, Graduate Program in Microbiology and Molecular Genetics
Loma Linda University, March 2013
Dr. Thomas A. Linkhart, Chairperson

The Insulin-like Growth Factor (IGF) system is a major target of GC inhibition in bone. We found that GCs inhibit expression of IGF binding protein-5 (IGFBP5) which binds IGFs and stimulates osteoblast activity by IGF dependent and independent mechanisms. GC-induced inhibition of IGFPB5 promoter activity was mediated by a composite response element that has binding sites for transcription factor activator protein 2 (AP-2) and nuclear factor I (NFI). The work in this dissertation identifies the NFI gene family as an important regulator of IGFBP-5 transcription primarily in human, as well as murine osteoblasts. The mechanism of IGFBP5 gene regulation involves direct binding of the NFI members to its *cis* element located in the IGFBP5 promoter region. Knockdown of NFI mRNA expression had diverse effects on IGFBP5 expression depending on the gene isoform member, suggesting that NFI isoforms have different roles regulating this gene. NFI had important roles during the process of osteoblast differentiation and mineralization, in the MCT3T3-E1 cell culture model. Knockdown of murine *Nfix* gene expression delayed the mineralization of this cell line, and also decreased mRNA expression levels of early and late osteoblastogenesis makers, in particular osteocalcin. An understanding of the role of the NFI gene family and the role of this family in the process of osteoblastogenic maker gene regulation can provide a new

alternative for pharmacological target genes for the treatment of osteoporosis, a condition that widely affects Americans.

CHAPTER ONE

INTRODUCTION

Skeletal System

The skeleton is a complex and dynamic system that was initially thought to have a passive role of providing support and protection to the internal organs. Through the years, the functions of the skeleton have been redefined and expanded so that the skeletal system is now considered a complex organ system composed of multiple cell types. Comprising a total number of 213 bones, this system provides shape and form to the body allowing locomotion and protection of internal organs, and also has important roles in mineral homeostasis and hematopoietic cell production.

The process of cartilaginous on long bone formation, from an initial cartilaginous anlagen, takes place by two different pathways, endochondral and perichondral ossification. In long bones, perichondral (cortical) bone is developed first directly within the soft connective tissue (perichondrium). Later, bone begins to form at the proximal and distal ends within the anlagen, creating primary ossification centers, or growth plates (Yang, 2008) .

Osteogenesis

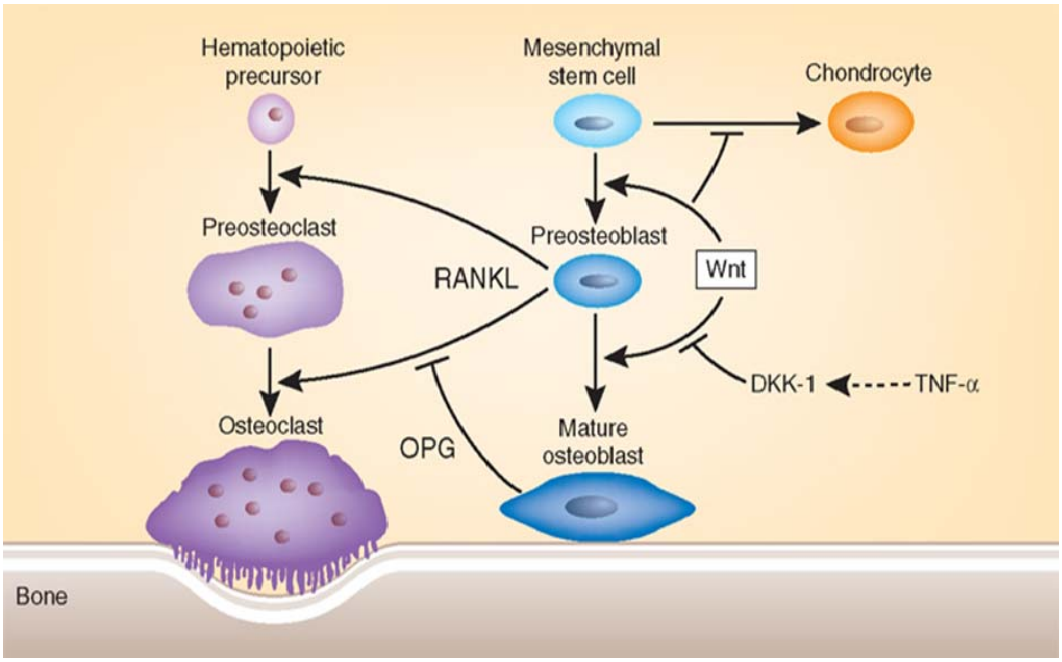
The osteoblastogenesis process requires a wide array of signaling proteins including hormones, cytokines, growth factors, and extracellular matrix proteins. Appropriate stimulation of signaling pathways will determine the commitment and fate of

the mesenchymal stem cells (MCSs) to differentiate into osteoblasts. Among these molecules the most relevant to controlling MSC commitment and diferentiation are osterix (Osx), Runx2 (Cbfa1/AML3), bone morphogenetic proteins (BMPs), Wnts and insulin like growth factors (IGFs) (McCarthy, Ji, & Centrella, 2000).

Osteoblast Lineage Commitment

The bone formation process is a very complex one and requires several signaling mechanisms. Osteoblasts arise from MSC. Postnatal bone marrow stroma cells have significant proliferative potential and when differentiated can give rise to, adipocytes, myoblasts, fibrous tissue forming cells, and chondrocytes (Figure 1) (Krebsbach, Kuznetsov, Bianco, & Robey, 1999) under specific signaling pathways. Commitment of MSCs towards the osteoblastic lineage is mediated by several molecules, in particular the bone morphogenic (BMP) 2/4/7 and Wnt proteins. This specifically happens during the process of condensation, which is the pivotal stage in skeletal development and takes place when a previously dispersed population of mesenchymal cells forms an aggregation of cells, thereby facilitating the selective regulation of genes specific for either chondrogenesis or osteogenesis (Wu, Shi, & Cao, 2007).

The BMP and Wnt pathways induce transcription factors that mediate commitment of early stem cell progenitors towards the osteoblastic lineage (Lian et al., 2006). BMP signaling induces Indian hedgehog (Ihh) promoting osteo/chondrogenic differentiation of a human chondrocytic cell line both *in vivo* and *in vitro* (Wu et al., 2007). Another important transcription factor stimulated by these pathways is Runx2/Cbfa1. Runx2 may be thought of as a master switch for osteogenic differentiation,



(Goldring & Goldring, 2007)

Figure 1. Commitment of Mesenchymal Stem Cells. A schematic of MSC commitment to different cell lineages. Some of the known transcription factors playing key regulatory roles in the mesenchymal lineages are indicated.

but it also interacts with many other transcription regulators that are involved in affecting its expression (Ducy, 2000; Lian et al., 2006). For example, Wnt signaling also increases osteoblast differentiation in part through increasing Runx-2 expression and by interacting with Runx2 protein to stimulate transcription of genes such as OCN (Kahler & Westendorf, 2003; Lian et al., 2006). As part of its pro differentiation actions, Runx-2 suppresses pre-osteoblast proliferation targeting CDK inhibitors (CKIs) p21 and p27 (Galindo et al., 2005; Pratap et al., 2005; Thomas et al., 2004; Westendorf et al., 2002). Runx-2 expression is stimulated by homeodomain proteins such as Dlx3/5 and HOXA10 and inhibited by Msx2 at early stages of differentiation. Binding sites for Msx/Dlx, HOxA10, Runx2, LEF1/TCF (which mediate Wnt/βcatenin signaling), ATF4 and NFI reside in the OCN and Runx2 proximal promoter regions (Lian et al., 2006). The switch from adipogenesis to osteogenesis is also induced by stimulation of Runx-2 expression through BMP signaling. The transcription factor peroxisome proliferator-activated receptor gamma (PPARγ2), and Runx-2 have opposing effects on the OCN promoter activity, which supports a mechanism for directing the fate of MSCs towards adipogenesis or osteogenesis (Jeon et al., 2003).

Osteoblast and Mineralization

The osteoblast and bone lining cells are in close contact with each other, joined by adhering junctions. Osteoblast differentiation is accelerated by interaction of the type I collagen matrix with α1β1, α2β1 integrins, which activate the MAPK signaling pathway. The α1 Integrin increases expression of osteogenesis, and the MAPK pathway phosphorylates Runx-2 and induces its activity (Franceschi & Xiao, 2003). The process

of osteoblast differentiation involves three main characteristic stages that are characterized by unique sets of osteoblastic genes at each particular stage: proliferation, extracellular matrix development and mineralization. Several molecules have relevant roles during these processes and control each one. Expression of the most frequently assayed osteoblast-associated genes collagen type I (COLLI), alkaline phosphatase (ALP), osteopontin (OPN), osteocalcin (OCN), bone sialoprotein (BSP), and PTH/PTHrP receptor (PTH1R) is asynchrononously upregulated, acquired, and/or lost as the progenitor cells differentiate and matrix matures and mineralizes. In general, ALP increases during extracellular matrix development and then decreases when mineralization is well progressed; OPN peaks twice, first during proliferation and then again later during mineralization, well before certain other matrix proteins, including BSP and OCN peak; BSP is transiently expressed very early and then expression peaks in differentiated osteoblasts forming mineral; OCN expression appears and peaks with mineralization. Mineralization results in upregulated expression of several non-collagenous enriched proteins, thereby providing markers of the mature osteoblast (OCN, OPN, BSP). These calcium and phosphate binding proteins may function in regulating the ordered deposition of mineral, the amount of hydroxyapatite crystals, or crystal size (Kobayashi & Kronenberg, 2005).

IGF System and Bone

IGFs are the most abundant growth factors produced by bone cells and stored in Bone (Canalis, McCarthy, & Centrella, 1989); (Mohan, Jennings, Linkhart, & Baylink, 1988) . IGFs regulate osteoblast proliferation, differentiation and survival (T. A.

Linkhart, Mohan, & Baylink, 1996; Mohan & Baylink, 1996). Moreover, IGFs are important for bone growth, fracture healing, and maintenance of bone mass (Hayden, Mohan, & Baylink, 1995). IGFs stimulate osteoblast proliferation, bone matrix protein synthesis, alkaline phosphatase activity and differentiation (Mohan et al., 1988);(S. Linkhart, Mohan, Linkhart, Kumegawa, & Baylink, 1986; Matsuyama, Lau, & Wergedal, 1990); (McCarthy, Centrella, & Canalis, 1989). IGFs also enhance osteoclast formation and activity. The IGF system is composed of IGF-I/II, growth hormone, type I and type II IGF-receptor, six high affinity binding proteins (IGFBPs), and IGFBP proteases (Govoni, Baylink, & Mohan, 2005; Mohan & Baylink, 1996). IGF-I and IGF-II are important paracrine and autocrine regulators of bone development, growth, repair and continuous renewal (Mohan et al., 2003; Yakar & Rosen, 2003).

IGF-I/II depletion adversely affects bone growth. IGF-I/II knockout mice display impaired bone growth (Baker, Liu, Robertson, & Efstratiadis, 1993);(J. P. Liu, Baker, Perkins, Robertson, & Efstratiadis, 1993) (Woods, Camacho-Hubner, Barter, Clark, & Savage, 1997); (DeChiara, Efstratiadis, & Robertson, 1990); (Wolf, Rapp, Blum, Kolb, & Brem, 1995); (Powell-Braxton, Hollingshead, Giltinan, Pitts-Meek, & Stewart, 1993). IGF-I knockout mice model has severe deficit on bone mineral density as well as, skeletal disruptions exemplified by a reduction in craniofacial size (McAlarney, Rizos, Rocca, Nicolay, & Efstratiadis, 2001). IGF-II null mice also demonstrate reduced birth weight (DeChiara et al., 1990). In these mice, only the paternal Igf2 allele is expressed in most tissues; therefore, the phenotype was only observed in progeny of males with an inactivated IGF-II gene. Post-natal growth was unaffected in IGF-II null mice unlike in

IGF-I null mice. This is believed to be due to the decreased importance of IGF-II in rodent post-natal development.

IGFBP5 Function in Bone

IGFBPs decrease or increase actions of IGFs on bone and other tissues (Baxter, 2000; Clemmons, 1998; Ferry, Katz, Grimberg, Cohen, & Weinzimer, 1999; M. M. Rechler, 1993; M.M. Rechler & Clemmons, 1998). In addition to binding extracellular IGF-I and IGF-II, IGFBPs affect cells by IGF-independent mechanisms that involve binding to extracellular matrix or transmembrane proteins (Jones, Gockerman, Busby, Camacho-Hubner, & Clemmons, 1993) or by IGFBP actions within the nucleus (Amaar et al., 2002; Schedlich et al., 2000).

IGFBP-5 possesses a high specific affinity for extracellular matrix proteins such as Type III and IV collagen, laminin and fibronectin and bone mineral (Jones et al., 1993), and is the most abundant IGFBP stored in bone (Miyakoshi, Richman, Linkhart, Baylink, & Mohan, 2001). It is expressed in osteoblasts and osteoblast precursors during skeletal development, and stimulates osteoblast proliferation *in vitro* and bone formation *in vivo* (Richman, Baylink, Lang, Dony, & Mohan, 1998; Wang, Wang, Chin, Zhou, & Bondy, 1995). IGFBP-5 has complex effects on IGF actions that are cell type and localization specific (Mohan & Baylink, 2002; Schneider, Wolf, Hoeflich, & Lahm, 2002). For instance, high extracellular IGFBP-5 concentrations inhibit IGF-stimulated vascular smooth muscle cell proliferation by sequestering free IGFs, while lower concentrations or extracellular matrix-bound IGFBP-5 enhance IGF activities (Xu et al., 2004). In contrast, high extracellular IGFBP-5 concentrations increased proliferation of

human intestinal smooth muscle cells and prostate cancer cells by IGF dependent and IGF independent pathways (Kuemmerle & Zhou, 2002; Miyake, Pollak, & Gleave, 2000). Inhibition of IGFBP-5 expression with siRNA in U2-OS osteosarcoma cells increased apoptosis (Yin, Xu, & Duan, 2004), suggesting that IGFBP-5 prevents cell death. Apoptosis was prevented by overexpressing IGFBP-5 in the cells, but addition of extracellular IGFBP-5 protein increased apoptosis (Yin et al., 2004), which was reversed by addition of excess IGF-I protein. However, in other studies Addition of IGFBP-5 increased proliferation and differentiation in osteoblast cultures through binding to specific cell surface sites (Andress, 1995; Andress & Birnbaum, 1992; Mohan et al., 1995) and phosphorylation of putative IGFBP-5 receptors (Andress, 1998; Mukherjee & Rotwein, 2008). On the basis of these findings, IGFBP-5 concentration and localization may influence effects of IGFBP5 on IGF activity. Excess extracellular IGFBP-5 may inhibit IGF activity by sequestering extracellular IGFs and preventing receptor binding, while moderate extracellular IGFBP-5 levels may enhance IGF activity, and excess intracellular IGFBP-5 may enhance IGF activity or have IGF independent effects.

In vivo studies have shown that systemic administration of IGFBP-5 to mice enhanced the effects of IGF-I to increase calvaria bone formation (Kling et al., 1996), serum osteocalcin levels (bone formation marker) and femoral bone ALP activity (Richman, Baylink, Lang, Dony, & Mohan, 1999). Also, treatment of ovariectomized mice with a C-terminal truncated form of IGFBP-5, which binds IGFs with reduced affinity, augmented spinal and femural bone densities, bone formation rates and osteoblast numbers in the absence of IGF-I (Andress, 2001). Furthermore, local injections of IGFBP-5 in the outer periosteum of the parietal bone of IGF-I knockout mice

increased markers of bone formation to concentrations comparable to those seen in wild-type mice (Miyakoshi et al., 2001), suggesting that these IGFBP-5 actions are IGF independent.

Glucocorticoids

The IGF system is one of the major targets of GC inhibition in bone (Rosen & Donahue, 1998; Rosen, Donahue, & Hunter, 1994). GCs inhibit IGF anabolic activity in bone by changing expression of IGFBPs, for example by decreasing IGFBP-5 and IGFBP-3 and increasing IGFBP-4 mRNA levels in human osteoblasts (Chevalley, Strong, Mohan, Baylink, & Linkhart, 1996). In rodents, GCs inhibited osteoblast IGF I expression, but in human bone marrow osteoblast precursors, GCs did not affect IGF I or IGF II expression (Cheng et al., 1998).

Therapeutic GCs are steroid hormone analogs that are frequently used to treat auto-immune and inflammatory diseases (Boumpas, Chrousos, Wilder, Cupps, & Balow, 1993). These drugs can cause multiple adverse side effects, the most severe of which is bone loss and GC-induced osteoporosis (GIOP) (Lukert & Raisz, 1990, 1994). The principal deleterious effect of GCs on the skeleton is to inhibit bone formation by inhibiting proliferation and differentiation of osteoblast lineage cells, and increasing osteoblast apoptosis (Adler & Rosen, 1994; Rackoff & Rosen, 1998). These adverse effects are mediated mainly by glucocorticoid receptor (GR) activation or inhibition of transcription of specific genes, many of which encode growth factors, transcription factors or cell cycle regulators. Almost all inhibitory actions of GR on gene expression

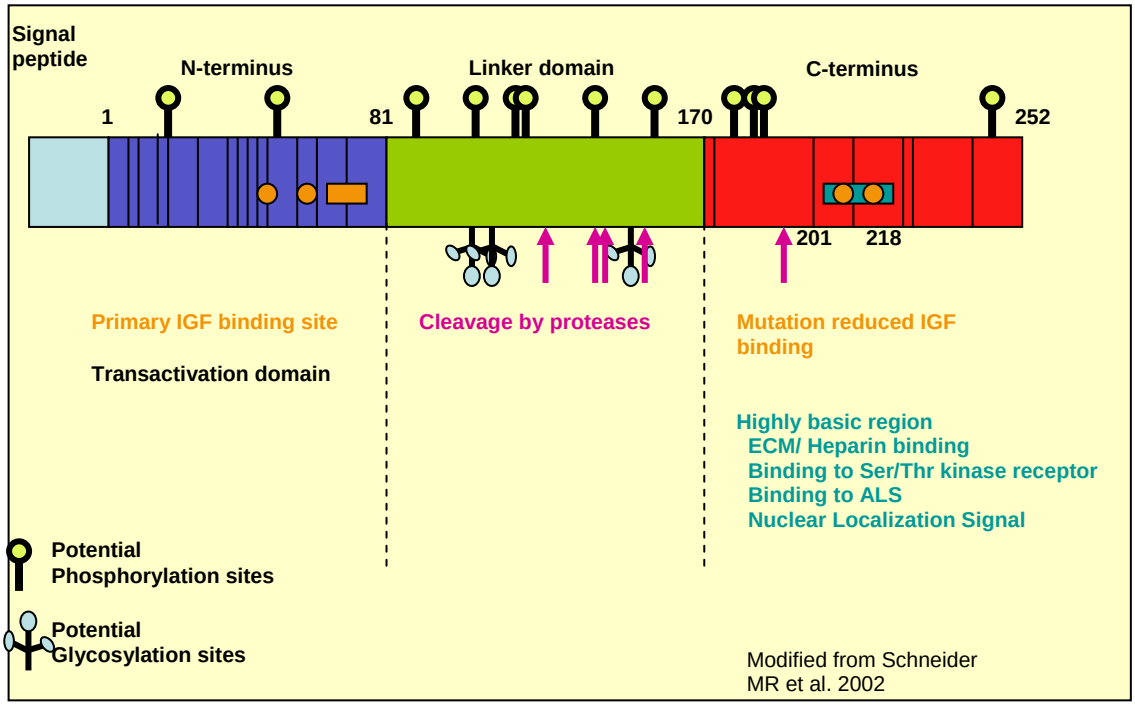

Signal peptide
N-terminus
Linker domain
C-terminus
1
81
170
252
201
218
Primary IGF binding site
Transactivation domain
Cleavage by proteases
Mutation reduced IGF binding
Highly basic region
ECM/ Heparin binding
Binding to Ser/Thr kinase receptor
Binding to ALS
Nuclear Localization Signal
Potential Phosphorylation sites
Potential Glycosylation sites
Modified from Schneider MR et al. 2002

Figure 2. Insulin-like growth factor 5 protein structure. IGFBP5 can be divided into 3 main protein domains. The N-terminus is the primary IGF binding site. The linker domain is susceptible to proteolytic cleavage. The C-terminus is involved in extracellular cell matrix protein interaction, and contains a putative nuclear localization signal.

are mediated by interactions of GR with other TFs rather than GR binding to DNA sequences. For this reason, discovery of TFs inhibited by GR in osteoblasts will not only reveal mechanisms of GC inhibition but will also identify TFs that are important in regulating osteoblast functions.

IGFBP5 Gene Promoter

IGFBP-5 transcription is positively regulated by prostaglandins, progesterone, cAMP, PTH, IGF-I and II and inhibited by GCs and BMP-7 (Boonyaratanakornkit et al., 1999b; Gabbitas & Canalis, 1998; Gabbitas, Pash, Delany, & Canalis, 1996; Ji, Chang, Centrella, & McCarthy, 2003; Ji, Chen, Centrella, & McCarthy, 1999; Yeh & Lee, 2000). The proximal few hundred bp of rat, human and mouse IGFBP-5 gene promoters are highly conserved and contain several putative transcription factor binding sites, including a TATA box, CAAT box, cEBP responsive element, Myb/E-box, and overlapping AP-2 and NFI sites (Allander et al., 1994; Duan & Clemmons, 1995; Ji et al., 1999; Kou, Mittanack, Fu, & Rotwein, 1995). Deletion of the segment containing the AP-2 and NFI sites reduced basal promoter activity but not PGE_2 and cAMP activation, while mutation of the c/EBP binding site reduced PGE_2 activation. Mutation of the Myb site completely inhibited promoter activity. The proximal promoter region containing the closely associated Myb, NFI and AP-2 sites may function as a composite response element, although the role of the Myb site is unclear.

Cortisol inhibited mouse IGFBP-5 transcription in rat osteoblasts, and this was prevented by mutation of the Myb/E-box motif (Gabbitas et al., 1996). However, our group found that mutation of a slightly different sequence (having one extra A) in the

human IGFBP-5 promoter, in the composite response element, did not prevent Dex inhibition. We identified a composite response element in the proximal IGFBP-5 gene promoter that contributes to basal promoter activation in human osteoblasts and is required for Dex inhibition. This element contains overlapping binding sequences for AP-2 and NFI. Although GR did not bind to the IGFBP-5 promoter directly, Dex induced GR binding to AP-2 and AP-2 overexpression prevented GC inhibition of IGFBP-5 promoter activity. This suggests that ligand dependent GR binding to AP-2 is involved in GR inhibition of IGFBP-5 transcription. Recently, we found that NFI activates BP-5 promoter activity to a greater degree than AP-2, NFI overexpression also reverses inhibition by GCs, and that GR binds to NFIB.

Nuclear Factor I Gene Family

The NFI gene family is composed of four members in vertebrates, NFIA, NFIB, NFIC and NFIX. These proteins play wide reaching roles in viral DNA replication, regulation of gene transcription and development. Gene knockout studies of the Nfi family in mice have shown that *Nfia* -/- mice display mainly neuroanatomical defects (Shu, Butz, Plachez, Gronostajski, & Richards, 2003), *Nfib* -/- mice show lung and brain defects (Steele-Perkins et al., 2005), *Nfic* knockout caused primarily a disruption of tooth root and underlying mandibular bone development (Steele-Perkins et al., 2003). The first *Nfix* -/- mouse knockout model reported showed severe brain malformation that was mainly due to impaired endochondral ossification and decreased mineralization in the vertebrae and long bones (Driller et al., 2007). A second *Nfix* knockout model showed altered prenatal brain development that suggested a defect in cell migration in specific

parts of the brain during development, as previously demonstrated by Driller et al (Campbell et al., 2008) .

The NFI family members contain a highly conserved N-terminal DNA binding domain and a heterogeneous proline rich transactivation and repression domain. Several studies reported that NFI proteins bound as dimers to the dyad symmetric consensus sequence TTGGC (N5) GCCAA on duplex DNA (Gronostajski, Adhya, Nagata, Guggenheimer, & Hurwitz, 1985). Alternative splicing in all NFI genes generates multiple isoforms, most of which retain the conserved DNA binding and dimerization domains. NFIB3 is a naturally truncated isoform derived from the human NFIB gene that does not contain the transactivation domain and was reported to function as a transcriptional repressor (Y. Liu, Bernard, & Apt, 1997). Transcriptional regulation by the four NFI gene members varied depending on the cell type and the specific gene promoter. NFIA inhibited Cbfa1/Runx2 promoter activity specifically in mouse non-osteoblasts, and based on its absence in osteoblasts was proposed to allow osteoblast specific expression (Zambotti, Makhluf, Shen, & Ducy, 2002). However, the study was based on comparison of a rat osteosarcoma cell line as the only osteoblast model, to mouse fibroblast cell lines, so relevance of these observations to regulation of osteoblast differentiation is unclear. Recently, a microarray analysis of MC3T3-E1 mouse osteoblasts, demonstrated that the GC, Dexamethasone induced expression of several transcription factors including NFIB (Leclerc et al., 2004). These findings are intriguing considering our findings that Dexamethasone inhibited NFIB activation of IGFBP-5 transcription. Perhaps this could be a dose-dependant feedback mechanism of Dexamethasone treatment.

NFI proteins functionally interact with and/or bind other transcription factors and co-activators including GR, AP-2, SRC-1, AP-1, Oct-1 and CBP/p300 (Chaudhry, Vitullo, & Gronostajski, 1999; Ji et al., 1999; Mukhopadhyay, Wyszomierski, Gronostajski, & Rosen, 2001). NFI has also been reported to affect chromatin remodeling on the mouse mammary tumor virus promoter (Hebbar & Archer, 2007). NFI and AP-2 interact in regulating expression of several genes that are relevant to osteoblasts, but have not previously been reported to bind each other. NFI/ AP-2 motifs have been identified in promoters of Type I α1 and α2 collagen genes, and may mediate TGF-β stimulation and TNF-α inhibition of mouse α2 (I) collagen transcription (Miao, Potter, Anania, Rennie-Tankersley, & Mezey, 1999; Rossi et al., 1988). In some genes like stellate cell type I collagen, binding of NFI and another transcription factor (including AP-2) to overlapping sites of promoter sequence is mutually exclusive (Chen, Beno, & Davis, 1996).

CHAPTER TWO

NUCLEAR FACTOR I TRANSCRIPTION FACTORS REGULATE IGF BINDING PROTEIN 5 GENE TRANSCRIPTION IN HUMAN OSTEOBLAST

This manuscript was accepted for publication by *Biochimica et Biophysica Acta* on August 26, 2008 and published online September 10, 2008* authored by: Laura A. Pérez-Casellasa,[b], Xiaoying Wanga,[b(1)], Kristy Howarda, Mark W. Rehagea, Donna D. Strongа,[b,c], Thomas A. Linkhartа,[b,d]

* The numerical reference system in this chapter is from the published paper and is separate from the references listed at the end of the thesis.

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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.

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 (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.

Experimental Procedures

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% Fe5 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.

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 4) using the $2^{-\Delta\Delta CT}$ Method [44].

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'TAGCACTCGAGCATCCTTGCCTGAGTTGGG-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 sub6 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 ChangeTM, Stratagene) with primer 5'-GTTGGGTGTTGGGAAGCTCgAgTTGCAGCTACAAACTGG-3' and its compliment (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 Δ70-35-Luc construct, a PCR product was made encoding bp –35 to +24 of the *IGFBP5* promoter using forward primer 5'-TAGCACTCGAGGCTATTT AAAAGCG CCTGC-3' containing an XhoI site and reverse primer 5'-GGCGTCTTCCATGGTG GC-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'-CCGCTCGAGGCATCCTTGCCTGAGTTG GGTGTTGGGAAGCTCAAATTGCAGTACAAACT [deletion]CGGCTATTTAAA AGCGCCTGCTCTC CCGGA GCCCCGTAGTCTCTTTGG AAACTTACTAGTCT GCAGGTCG-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'-AGCCCGGGC TCGAGGCAT CCTTGCCTGAGTTGGGTGT TGGGAAG CTCAAATTGCAGCTACAAAC TaGtTGGCAG CCAGGGGCCGGCTAT TTAAAAGCGCC TGCTCTCCCGGAGCCCCG TAGTCTCTTTGGAAACTTCTGCAGGTCGACTC-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 compliment (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 pCMVβ 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).

Luciferase Reporter Assay

TE85 and MG63 cells were transiently transfected at 50% confluency in 6 well plates with 0.5 μg of *IGFBP5* promoter-Luc plasmid DNA and 0.125 to 1μg 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 1mg/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 2µg of each HA-tagged pCH expression vector (Nfi) and TurboFectin 8.0 (OriGene Technologies, Rockville, MD).

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 si*CONTROL* Non Targeting siRNA #1 (Dharmacon). MG63 cells (2 X 105 per well of 6-well plate) were transfected in Opti-MemI (Gibco-Invitrogen) with 200 pmol of SMARTpool or si*CONTROL* using DharmaFECT-1 reagent. MG63 cells (2 X 105 per well of 6-well plate) were transfected in Opti-MemI(Gibco-Invitrogen) with 200 pmol of SMARTpool or si*CONTROL* 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 X 107 cells/ ml. The cells were electroporated with 74 pmol of si*CONTROL* 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 $X10^7$ cells/ ml. Cells were electroporated with 74 pmol of si*CONTROL* non targeting siRNA #1 or ONTARGET

plus NFIB Duplex 5 (Dharmacon J-008456-05) together with 2.5 μg of BP5p252-luc and 2.5 μg 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.

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'-GCAGCTACAAACTGGCTGGCAGCCAGGGGCCGG-3') (Figure 5A), was labeled with dCTP[α-^{32}P] (111 Tbq/mmol, MP Biomedical), by fill in with DNA polymerase Klenow fragment (New England Biolabs). The gel shift reaction was performed with 10 μg of nuclear extract protein using the Nushift™Kit (Active Motif) without addition of poly dI:dC. For supershift 5 μg 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. DNAprotein 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 [^{32}P]-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 ACcttCTGGCAGtaAGGGGCCGGC-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 α2 procollagen gene, containing no NFI binding sequences (NCBI Gene symbol COL1A2, 5'-CCTCCTCTGCGCCCCCGCAGG CTCCTCCCAGCTGTG GCT-3').

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 μg 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'-GGCATCCTTGCCTGAGTTG-3', Rev: 5'-CTGCTTTGCAGCTCTTTCCTA-3') and for two negative controls, NFI-X coding sequence (Exon 4 – 6, For: 5'-CCACTGCCCAACGGACACTT-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.

Western Blots

MG63 cells were lysed in RIPA Buffer containing protease inhibitors (Sigma). Extracts (20 μg 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).

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 ± SEM.

Results

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 ΔCt 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.

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 3A, 3B), 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 3C). Human NFIB2 was as effective as mouse Nfib2 in stimulating *IGFBP5* transcription (Figure 3D). Nfix was less active than Nfib and increased promoter activity with dose dependent maximal 3 fold stimulation in TE85 cells (Figure 3A). 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 3B), 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.

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	CTCTGGCCTCCTCTCCTGAA	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 (ΔC_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 $2^{-\Delta\Delta CT}$ Method [43]. NFIC was the most abundant mRNA in all cells except MG63, in which NFIB was highly expressed.

NFI Gene	Cell line	ΔCt	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
NFIX	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

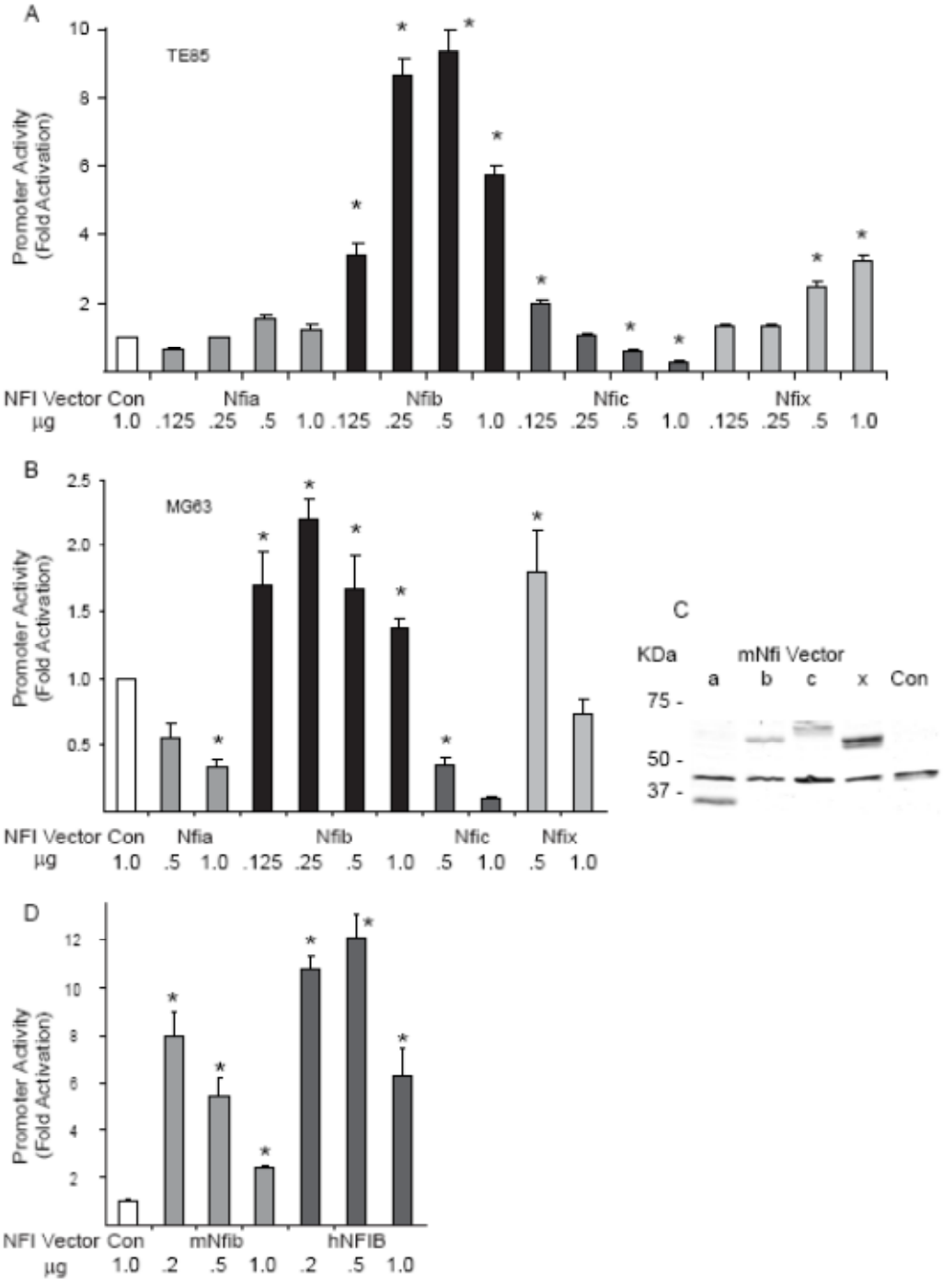
A
TE85
Promoter Activity (Fold Activation)
NFI Vector Con Nfia Nfib Nfic Nfix
µg 1.0 .125 .25 .5 1.0 .125 .25 .5 1.0 .125 .25 .5 1.0 .125 .25 .5 1.0
B
MG63
Promoter Activity (Fold Activation)
NFI Vector Con Nfia Nfib Nfic Nfix
µg 1.0 .5 1.0 .125 .25 .5 1.0 .5 1.0 .5 1.0
C
KDa
mNfi Vector
a b c x Con
75 -
50 -
37 -
D
Promoter Activity (Fold Activation)
NFI Vector Con mNfib hNFIB
µg 1.0 .2 .5 1.0 .2 .5 1.0

Figure 3. Effects of *Nfi* family transcription factors on *IGFBP5* promoter activity. Cells were cotransfected with 0.5 μg 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 ± 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 20μg 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 ($p<0.01$).

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 4A, $p<0.01$). NFIB protein expression was also reduced by 76% at 72 hours post transfection (Figure 4B). 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 4A). NFIC and NFIX mRNA levels in MG63 cells did not significantly change when NFIB expression was inhibited by siRNA (Figure 4A). 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 4C) 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 4C). 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 cotransfected 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 4D). *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.

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 5A). 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 5B). 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 5B). 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 5A) 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 5D and Figure 5E). 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.

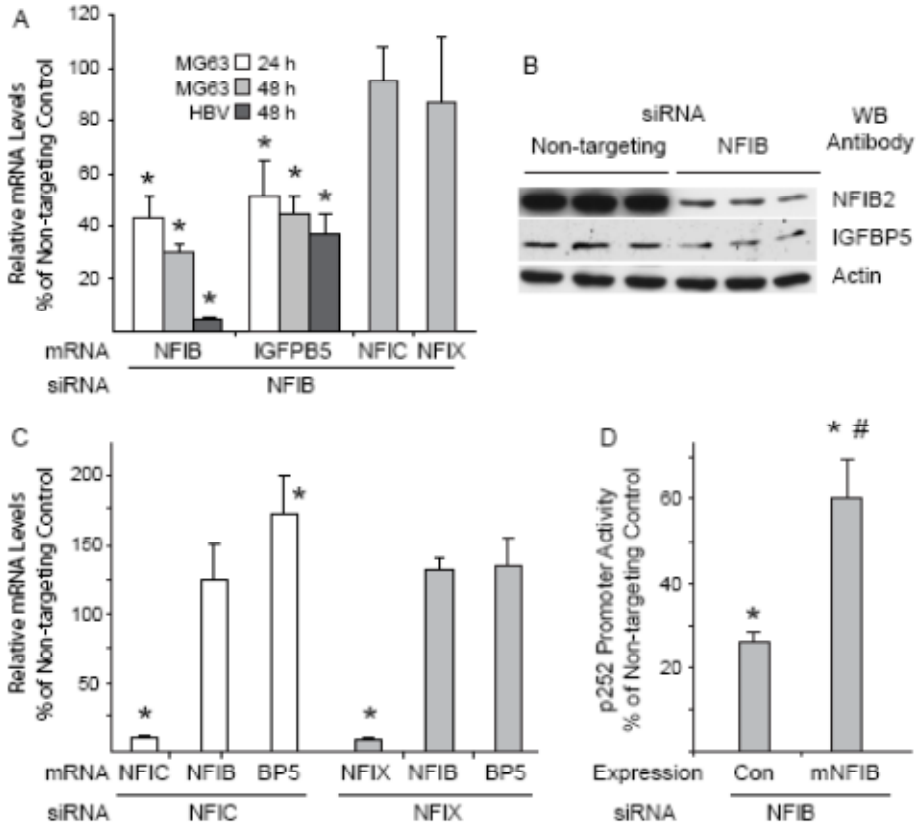
A
Relative mRNA Levels
% of Non-targeting Control
MG63 24 h
MG63 48 h
HBV 48 h
mRNA
siRNA
NFIB
IGFPB5
NFIC
NFIX
NFIB
B
siRNA
Non-targeting
NFIB
WB
Antibody
NFIB2
IGFBP5
Actin
C
Relative mRNA Levels
% of Non-targeting Control
mRNA
siRNA
NFIC
NFIB
BP5
NFIX
NFIB
BP5
NFIC
NFIX
D
p252 Promoter Activity
% of Non-targeting Control
Expression
siRNA
Con
mNFIB
NFIB

Figure 4. 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, $p < 0.01$.

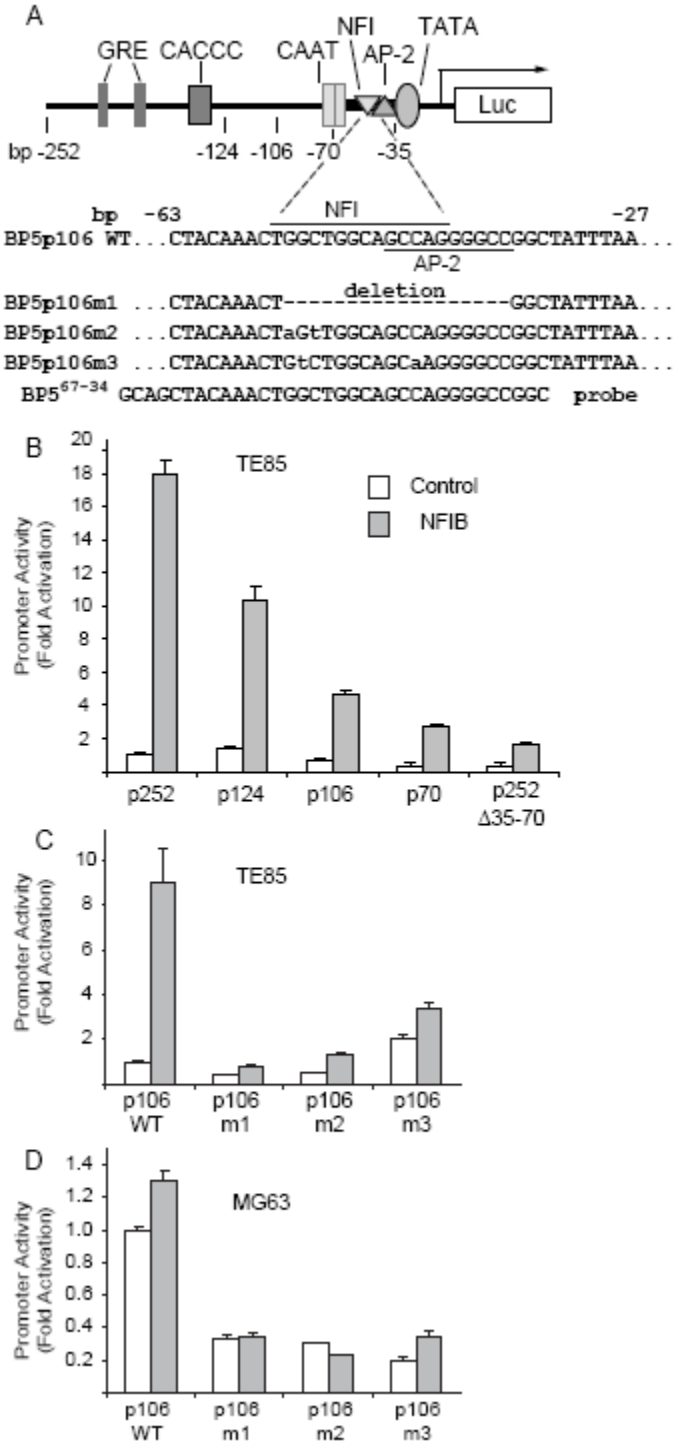
A
GRE
CACCC
CAAT
NFI
AP-2
TATA
Luc
bp -252
-124
-106
-70
-35
bp -63
NFI
-27
BP5p106 WT...CTACAAACTGGCTGGCAGCCAGGGGCCGGCTATTTAA...
AP-2
deletion
BP5p106m1 ...CTACAAACT----------GGCTATTTAA...
BP5p106m2 ...CTACAAACTaGtTGGCAGCCAGGGGCCGGCTATTTAA...
BP5p106m3 ...CTACAAACTGtCTGGCAGCaAGGGGCCGGCTATTTAA...
BP5 67-34 GCAGCTACAAACTGGCTGGCAGCCAGGGGCCGGC probe
B
TE85
Control
NFIB
Promoter Activity (Fold Activation)
p252
p124
p106
p70
p252 Δ35-70
C
TE85
p106 WT
p106 m1
p106 m2
p106 m3
D
MG63

Figure 5. 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 μg pCH control or pCHB Nfib expression plasmid plus 0.5 μg 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 μg of wt, m1, m2 and m3 BP5p106-luc constructs and 0.25μg of pCH control or Nfib HA tag expression vector. Altered nucleotides in m2 and m3 are indicated in lower case.

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 6A, 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 6B), 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 7 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 8). 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 CT 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.

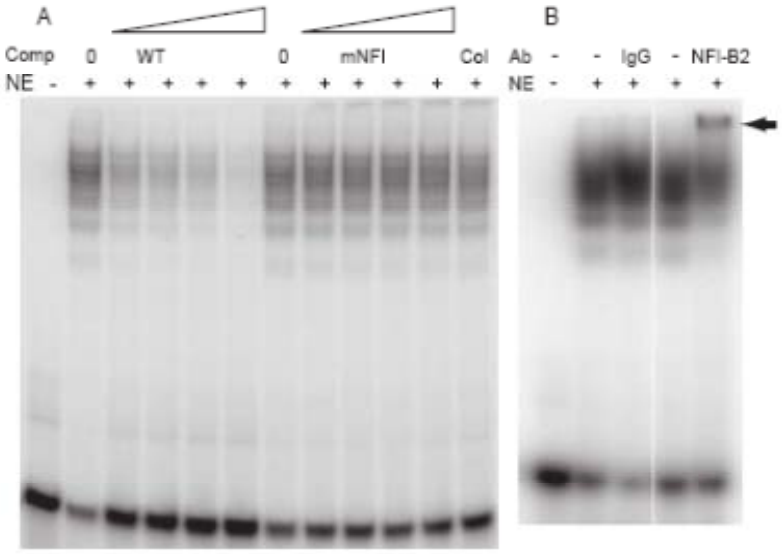
A
Comp
0
WT
0
mNFI
Col
NE
B
Ab
IgG
NFI-B2
NE

Figure 6. 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 μg 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 α 2 (I) collagen (Col) unlabeled competitors (Comp) were added to the reaction. B) Equal amounts (5 μg) 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 -
Ab -
Con -
Con HA
Nfib IgG
Nfia HA
Nfib HA
Nfic HA
Nfix HA

Figure 7. 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 μg 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.

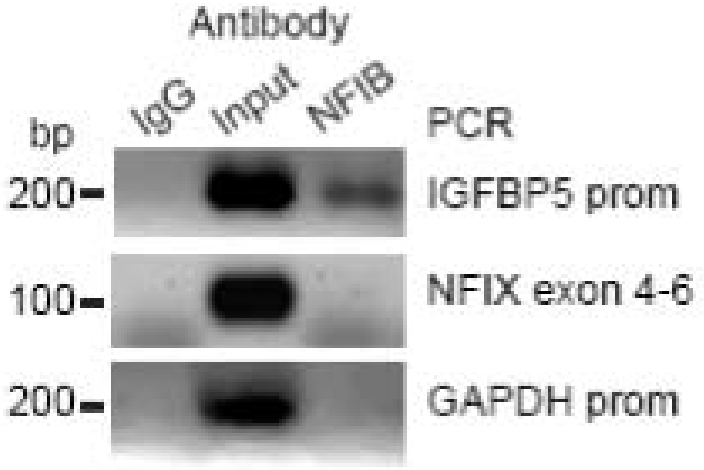
Antibody
IgG
Input
NFIB
bp
PCR
200
IGFBP5 prom
100
NFIX exon 4-6
200
GAPDH prom

Figure 8. NFI proteins bind *in vivo* to the *IGFBP5* proximal promoter. Formaldehyde-fixed MG63 chromatin (6 μg) was precipitated with 3μg 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 NFB2 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.

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 (1) 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 othercells.

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 besignificant to modulation of osteoblast proliferation and differentiation.

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CHAPTER THREE

REGULATION OF MURINE OSTEOBLAST DIFFERENTIATION BY NUCLEAR FACTOR I

Abstract

Bone formation is a complex process that requires a wide array of molecules in order to properly occur. Mesenchymal stem cells, which can be differentiated into adipocytes, myoblasts, fibroblasts and chondrocytes, can also give raise to osteoblasts under the activation of osteoblastic factors. Previous *in vitro* studies have shown that the transcription factor family NFI, regulates the mRNA expression of IGFBP5, stimulator of osteoblast proliferation and differentiation, in human osteoblasts. Moreover, the knockout *Nfix* model displays impaired endochondral ossification. We evaluated the role of the *Nfi* gene family in osteoblast differentiation using the MC3T3-E1 preosteoblast *in vitro* model for osteoblast differentiation. Nfix mRNA levels increased during MC3T3-E1 osteoblast differentiation induced by ascorbic acid and β-glycerophosphate. Stable knockdown of *Nfix* in MC3T3-EI cells was accomplished using lentiviral shRNA vectors. Cells stably transduced with a validated shRNA vector targeting Nfix or a non-targeting shRNA vector grown in differentiation media were observed to have delayed mineralization compared to control cells. mRNA transcript levels of osteocalcin (Ocn), bone sialoprotein (Bsp) and Runt-related transcription factor 2 (Runx-2) were significantly decreased in *Nfix* deficient calvarial osteoblasts. Promoter-luciferase reporter assays also showed that transient transfection of MC3T3-E1 cells with a plasmid

Nf1x expression vector significantly increased activation of the murine OCN promoter. Results suggest that Nfix has a critical role in osteoblast differentiation, perhaps through direct effects on expression of osteoblast specific genes such as osteocalcin.

Introduction

Osteogenesis

The bone formation process is a very complex one and requires several signaling pathways. Osteoblasts, arise from mesenchymal stem cells (MSCs) which can also give rise to, adipocytes, myoblasts, fibrous tissue forming cells, and chondrocytes (Krebsbach et al., 1999). The osteoblastogenesis process requires a wide array of signaling proteins including hormones, cytokines, growth factors, and extracellular matrix proteins. Appropriate stimulation of signaling pathways determines the commitment and fate of the MCSs into osteoblasts. Among the most relevant molecules controlling osteoblast differentiation are Runx2 (Cbfa1/AML3) and Osterix transcription factors, and extracellular regulators bone morphogenetic proteins (BMPs), Wnts and insulin like growth factors (IGFs) (McCarthy et al., 2000)

Runx2 and its downstream signaling molecule, osterix (OSX) function to activate osteogenic genes that are required for proper bone matrix synthesis (Ducy, 2000) .
The process of osteoblast differentiation involves three main characteristic stages that are characterized by the transcriptional regulation of osteoblastic genes at each particular stage: proliferation, extracellular matrix development and lastly mineralization. Some of the standard markers utilized to determine osteoblast lineage differentiation are Bsp and Ocn.

Nuclear Factor I

Previous experiments in human osteoblasts have shown that the NFI TF family is involved in regulating expression of IGFBP5, a protein that enhances IGF activities and bone formation.. Vertebrate NFI TFs comprise a family of closely related proteins, NFIA, NFIB, NFIC (originally identified as CTF-1), and NFIX. Gene knockout studies of NFI have shown that *Nfia* $^{-/-}$ mice display mainly neuroanatomical defects (Shu et al., 2003), *Nfib*$^{-/-}$ mice show lung and brain defects (Steele-Perkins et al., 2005), *Nfic* knockout caused primarily a disruption of tooth root and underlying mandibular bone development (Steele-Perkins et al., 2003). *Nfix*$^{-/-}$ mice were first reported to have brain malformation and severe kyphosis, due to impaired endochondral ossification. Transcriptional regulation by the four NFI gene members varies depending on cell type and gene promoter. NFIA inhibited Cbfa1/Runx2 promoter activity specifically in mouse fibroblast cell lines, and by its absence in osteoblasts was proposed to allow osteoblast specific expression (Zambotti et al., 2002) endochondral ossification and decreased mineralization in the vertebrae and long bones (Driller et al., 2007).

The purpose of this study is to determine the effects of *Nfix* gene knockdown on MC3T3-E1 induced differentiation.

Materials and Methods

Cell Culture

MC3T3-E1 cells (a gift from B. Frenkel,University of Southern California (Luppen et al., 2003) were maintained in αMEM supplemented with 10% fetal bovine serum. To induce differentiation, the media was supplemented with 50 μg/ml ascorbic

acid and 10 mM β-glycerophosphate (osteogenic media). Media was changed every 2-3 days and cells were maintained in osteogenic media for 14-21 days to induce differentiation.

RNA Extraction and Quantitative RT-PCR

Three independent samples of total RNA per group were isolated from MC3T3-E1 cells beginning 1 day after 80 % confluent cells were changed to osteogenic media, using a Gentra Systems Versagene™ kit (Fisher Scientific). 300 ng RNA from each sample 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 murine Nfia, Nfib, Nfic, Nfix, OCN, BSP, Runx2, OSX and PPIA (Table 3). PCR primers and other oligodeoxynucleotides were from Integrated DNA Technologies Inc. Relative mRNA levels were determined using the $2^{-\Delta\Delta Ct}$ method, as described (Perez et al., 2009) and were normalized to endogenous PPIA mRNA levels. These values were expressed relative to *Nfia* mRNA levels in the first time point in each cell line or relative to non-targeting control in siRNA knockdown experiments.

Short Hairpin Lentiviral Transduction

A set of 5 lentiviral MISSION shRNA vectors targeting different sequences in mouse Nfix was obtained as viral particles from Sigma Chemical Co. (St. Louis, MO). The viral constructs produce shRNA that is processed to active siRNA within the target cells, and also express a Puromycin resistance gene. MC3T3-E1 cells were separately

transduced at an MOI of 10 with each Nfix targeting viral vector and a validated non-targeting shRNA lentiviral vector. Cells were selected 3 days in Puromycin, and expanded without puromycin, and replicates of each line were used for RNA extraction and assessment of Nfix mRNA levels by qRT-PCR. One of the lentiviral constructs (TRCN0000075349) that targets a sequence in Exon 3 reduced Nfix mRNA levels by 80% so these cells were grown further. The cells maintained strong Nfix mRNA knockdown after multiple passage and cryopreservation.

Luciferase Reporter Assay

MC3T3-E1 cells were transiently transfected at 50% confluency in 6 well plates with 0.5 μg of OCN promoter-Luc plasmid DNA and 0.125 to 1μg of each murine Nfi plasmid expression vector (Boonyaratanakornkit et al., 1999a; Chaudhry, Lyons, & Gronostajski, 1997; Mukhopadhyay et al., 2001) using TurboFectin 8.0 (OriGene Technologies). Total plasmid DNA was adjusted by addition of pCH empty expression vector. MC3T3-E1 culture medium was changed to 1mg/ml BSA in DMEM after 18 h, cells were extracted in reporter lysis buffer (Promega) after an additional 24 h, and luciferase activity was determined as described(Boonyaratanakornkit et al., 1999a). Luciferase activities were normalized to protein concentrations determined by bicinchoninic acid (BCA) assay (PierceBiotechnology) and are presented as fold of control. Sample groups consisted of 6 replicate wells of cells.

Alizarin Red-S Staining

Alizarin red staining was used to assay mineralized nodule formation at days 18 and 21. Briefly cells were fixed for 30 min in 100% methanol for 30 min at -20°C, washed with PBS three times, stained with 40 mM Alizarin Red-S (Sigma-Aldrich, St. Louis,MO) pH 4.2 for 15 min at room temperature, washed with distilled water five times, rinsed in 1X PBS for 10 min to reduce nonspecific staining, and dried overnight. The intensity of red staining and nodule formation was evaluated by optical density scanning at 510 nm using a Biotek Synergy 2 plate reader that determines absorbance in 2000 circular spots covering 90% of the 9.4 cm^2 well surface.

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 ± SEM.

Results

NFI Gene Expression During Differentiation of MC3T3E1 Cells

MC3T3-E1cells and C57BL/6 bone marrow stromal cells were changed at 80% confluent density (Day 0) to differentiation inducing media (αMEM with FBS, ascorbic acid and β-glycerol phosphate) and RNA was isolated on Day 1 – 24. Relative expression levels were determined by qRT-PCR using specific primers described in Table 3. Bar graphs show relative levels of each Nfi gene member mRNA relative to

Nfia (Figure 9). Line graphs show change over time relative to the value for each Nfi gene on Day 1. Relative levels were determined by the $2^{-\Delta\Delta Ct}$ method. Data is based on means of 2 qRT-PCR values so statistical testing is not performed.

To determine if the Nfi gene members were expressed during osteoblast differentiation, MC3T3-E1 and mBMSC were grown in differentiation media. Total RNA was collected at different time points. We found that mRNA levels of *Nfia* and *Nfib* in differentiated MC3T3-T1 cells did not significantly change during deferentiation. In contrast, *Nfic* and *Nfix* gene expression at later stages of osteoblastogenesis increased around 2 fold, compared to *Nfia* day 1 (Figure 9). Comparable results found in mBMSCs, but *Nfix* expression was remarkably increased (4 fold) compared to *Nfia* at day 1.

Table 3. PCR primers used for assessing mouse *Nfi* and osteoblast marker gene expression. qRT-PCR was performed with cDNAs synthesized from total RNA using random hexamer RT primers.

Gene	Forward	Reverse
Nf1a	GTAGAGCCAGGCTTGCCATC	GGACTTGCTCTGGGTCCTGTA
Nfib F	GCCACAGTGATCCTGCCAAG	CTGCTCGGTGGAGAAGACAG
Nfic F	CCGGCATGAGAAGGACTCTAC	AGATGCCTCCTTCTATGTCTTCC
Nfix F	CTTCCACCAGCAGCACCAAG	GGCTCGACTGCTGGATGATG
Cyclophilin A F	GCATACAGGTCCTGGCATCT	GCTCTCCTGAGCTACAGAAG
Osteocalcin	GCTACCTTGGAGCCTCAGTC	ATGCGTTTGTAGGCGGTCTT
BSP	AGGTGCAGAAGGAACCACAG	CGTCCTCATAAGCTCGGTAA
Runx2	CTCTGATCGCCTCAGTGATT	GGACTTGGTGCAGAGTTCAG

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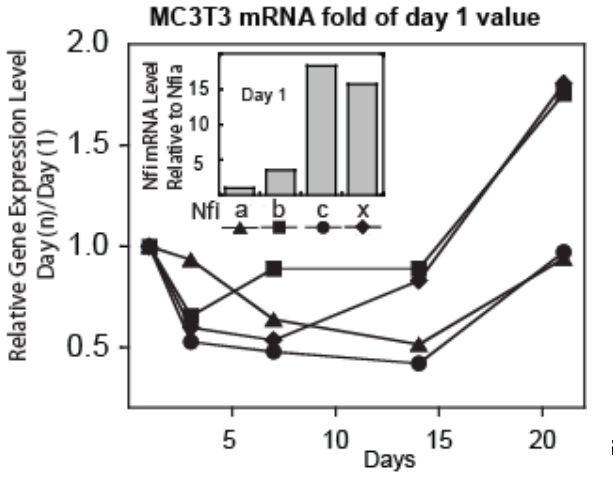

MC3T3 mRNA fold of day 1 value
Relative Gene Expression Level
Day (n)/Day (1)
2.0
1.5
1.0
0.5
5
10
15
20
Days
Nfi mRNA Level
Relative to Nfia
Day 1
15
10
5
Nfi a b c x

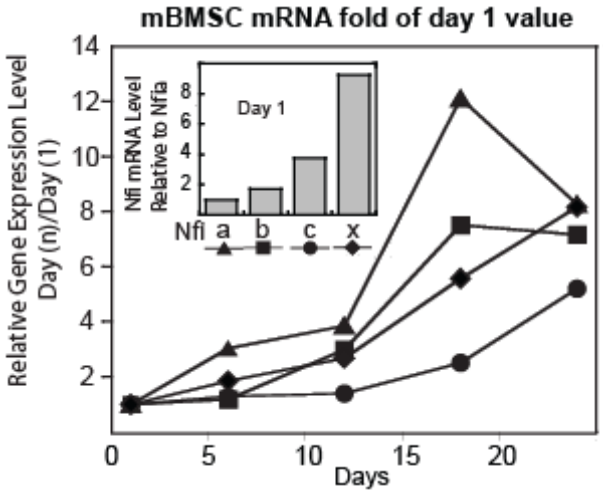

mBMSC mRNA fold of day 1 value
Relative Gene Expression Level
Day (n)/Day (1)
14
12
10
8
6
4
2
0
5
10
15
20
Days
Nfi mRNA Level
Relative to Nfia
Day 1
8
6
4
2
Nfi a b c x

Figure 9. NFI gene expression during differentiation of MC3T3E1 cells. MC3T3-E1cells and C57BL/6 bone marrow stromal cells were changed at 80% confluent density (Day 0) to differentiation inducing media (αMEM with FBS, ascorbic acid and β-glycerol phosphate) and RNA was isolated on Day 1 – 24. Relative expression levels were determined by qRT-PCR using specific primers described in Table 1. Bar graphs show relative levels of Nfi mRNA relative to Nfia. Line graphs show change over time relative to the value for each Nfi gene on Day 1. Relative levels were determined by the $2^{-\Delta\Delta Ct}$ method. Data is based on means of 2 qRT-PCR values so statistical testing is not performed.

Effects of Nfib and Nfix on IGFBP5 Expression in Mouse Osteoblasts

We examined the effects of *Nfi* proteins on *IGFBP5* promoter activity by transiently co-transfecting MC3T3-E1 cells with mouse Nfi expression vectors (pCH-B 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), which is highly conserved in mouse. Nfib was the most active in stimulating *IGFBP5* promoter activity at increasing concentrations (Figure 10A). In contrast, overexpression of Nfix was not active in stimulating *IGFBP5* transcription. These results correlate with our previous findings in human osteoblasts, which showed that Nfib was the strongest activator of IGFBP5 transcription among the NFI gene member family. Knockdown of Nfix in MC3T3-E1 increased *Igfbp5* mRNA levels (Figure 10B). Conversily, transient knockdown of Nfix caused a significant decrease in OCN mRNA levels. These results confirm our previous findings which demonstrated that Nfix negatively regulates *Igfbp5* transcription.

Effects of Nfix and Runx2 Expression on Mouse Osteocalcin Promoter Activity in MC3T3-E1 Cells

Based in our previous results showing that *Nfix* is the most abundant NFI gene member during murine osteoblast differentiation and that it affects osteocalcin mRNA levels, we decided to explore its role on the regulation of the OCN promoter. Given that there is a Runx-2 response element on the murine osteocalcin promoter, we decided to determine if overexpression of both *Nfix* and Runx-2 dose dependently activated it. As

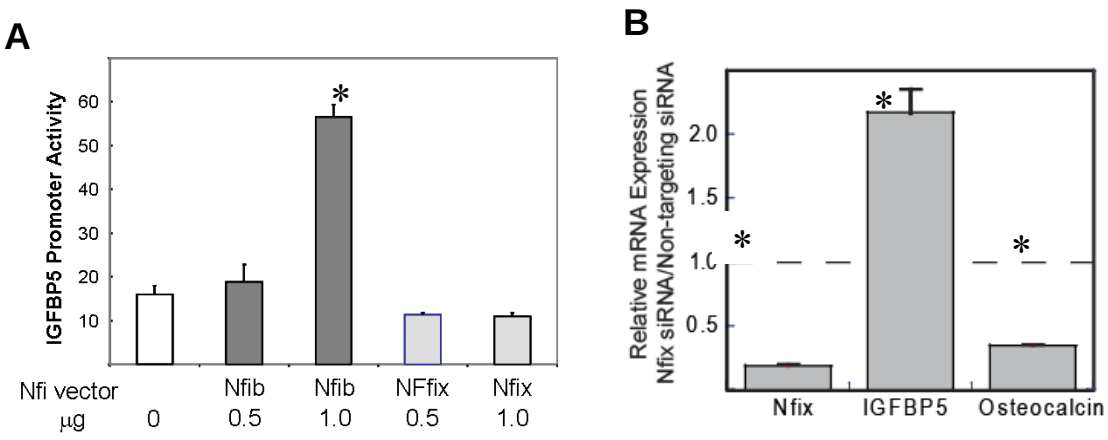

A
IGFBP5 Promoter Activity
60
50
40
30
20
10
*
Nfi vector
μg
0
Nfib
0.5
Nfib
1.0
NFfix
0.5
Nfix
1.0
B
Relative mRNA Expression
Nfix siRNA/Non-targeting siRNA
2.0
1.5
1.0
0.5
*
*
*
Nfix
IGFBP5
Osteocalcin

Figure 10. Effects of Nfib and Nfix on IGFBP5 expression in mouse osteoblasts A.Human IGFBP5 promoter luciferase reporter (reference) was cotransfected using TurboFectin 8.0 with Nfib or Nfix expression vectors in MC3T3 E1 cells. B. Short term Nfix expression knockdown with siRNA increases IGFBP-5 expression and inhibits Osteocalcin expression in MC3T3-E1 osteoblasts. Cells were transfected by electroporation with siRNA oligonucleotides targeting 4 sequences in the mouse Nfix mRNA sequence, or with non-targeting control siRNA . Cells were plated in 6 well plates at 50% confluent density and relative expression (Targeting/Non-targeting) was determine by qRT-PCR. Similar knockdown of Nfib did not affect IGFBP5 expression (data not shown). * significantly different from control vector.

shown in Figure 11, increasing amounts of transiently overexpressed Runx-2 protein dose dependently inhibited Nfix-mediated activation of the OCN promoter. These results suggest that interactions between Runx-2 and Nfix are perhaps required to modulate OCN transcription at earlier stages of osteoblast differentiation.

Long Term Knockdown of Mouse Nfix Expression in MC3T3-E1 Cells with Lentiviral shRNA Vector

In order to determine long term effects of *Nfix* knockdown during osteoblast differentiation, we transduced MC3T3-E1 cells with a shRNA lentiviral vector containing 5 sequences targeting murine *Nfix*. RNA was collected at different differentiation stages and the mRNA expression of several important osteoblast differentiation markers was analyzed. Relative mRNA expression of *Nfix* in *Nfix* knockdown cells remained constantly low through differentiation, compared to the non targeting control (Figure 12A). *Runx-2* expression was decreased in shNFIX targeting cells, compared to the control. Its expression remained mostly on plateau, expect at day 24 where it was the lowest level (Figure 12B). As expected, one of the osteoblast differentiation markers that was greatly affected was OCN (Figure 12C). OCN mRNA levels were dramatically decreased in *Nfix* deficient cells starting at the early time points and dropping its expression around -400 fold at later time points (day 12 and day 24). Another gene that was significantly downregulated by *Nfix* depletion was BSP. Levels of this gene were consistently low in all time points (Figure 12D).

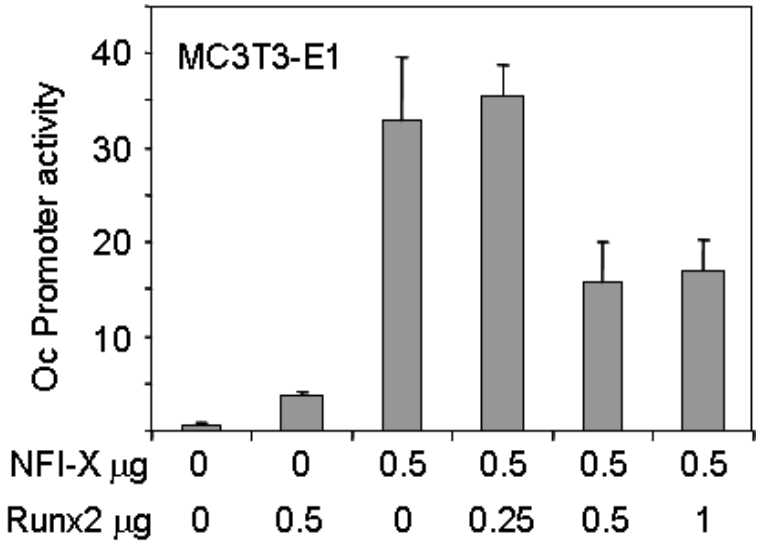

MC3T3-E1
Oc Promoter activity
40
30
20
10
NFI-X μg 0 0 0.5 0.5 0.5 0.5
Runx2 μg 0 0.5 0 0.25 0.5 1

Figure 11. Effects of NFI-X and Runx2 expression on Mouse Osteocalcin promoter activity in MC3T3-E1 cells. Mouse Nfix and human Runx2 expression plasmids were co-transfected with the p1316-Luc osteocalcin promoter reporter in sub-confluent cultures and promoter activity (Luciferase/cell protein) was determined at 48 h. Promoter activation in all groups was greater than control ($p < .05$). Activation was significantly greater with Nfix compared to Runx2 ($p < .05$).

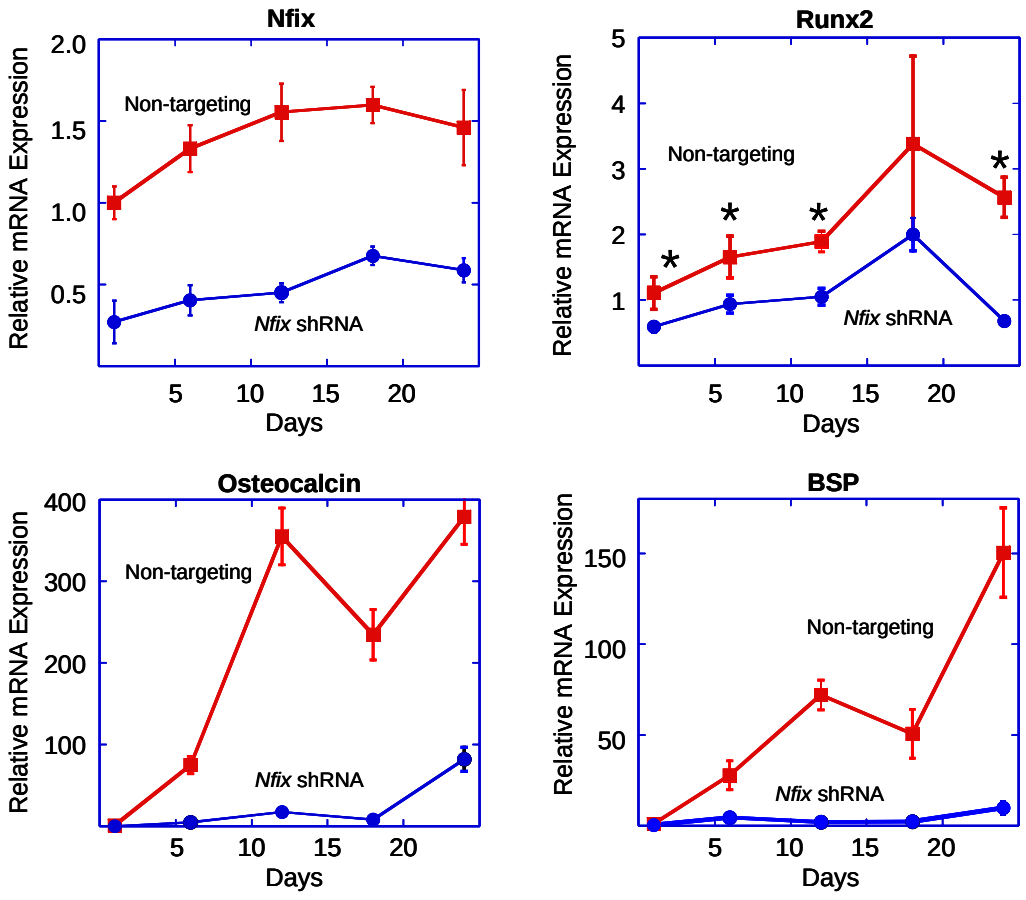
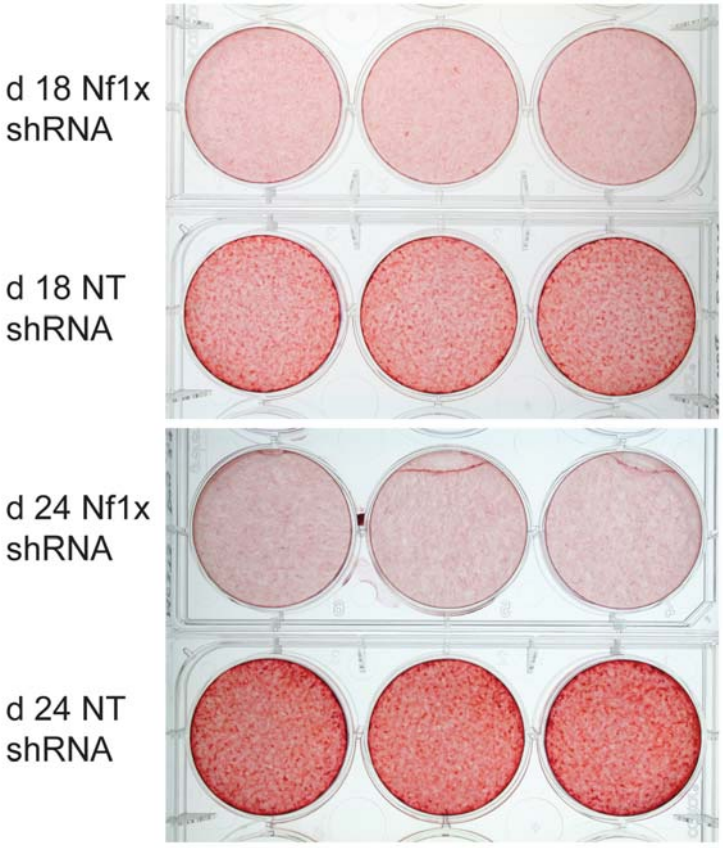

Nfix
Relative mRNA Expression
2.0
1.5
1.0
0.5
Non-targeting
Nfix shRNA
5
10
15
20
Days
Runx2
Relative mRNA Expression
5
4
3
2
1
Non-targeting
Nfix shRNA
*
*
*
5
10
15
20
Days
Osteocalcin
Relative mRNA Expression
400
300
200
100
Non-targeting
Nfix shRNA
5
10
15
20
Days
BSP
Relative mRNA Expression
150
100
50
Non-targeting
Nfix shRNA
5
10
15
20
Days

Figure 12. Long term knockdown of mouse Nfix expression in MC3T3-E1 cells with lentiviral shRNA vector. RNA was extracted at 1 – 24 days and relative mRNA expression was determined by qRT-PCR (N=6), shown as multiples of the Day 1 Non-targeting group value for each gene (using the $2^{-\Delta\Delta Ct}$ method). All points for non-targeting groups are significantly different from Nfix targeting except in the Runx graph, where significance is indicated by *. Significance was determined by T-test on ΔΔCt data.

Long Term Knockdown of Nfix Expression in MC3T3-E1 Cells with Lentiviral shRNA Vector- Effects on Mineralization

Following the same experimental procedure shown in Figure 12, we made parallel cultures that were fixed and stained at 18 and 24 days with Alizarin Red to assess mineralization. There was a large difference in mineralized staining intensity evident between non-targeting and Nfix-targeting groups (Figure 13). Quantitation of staining by plate reader scanning at 510 nm indicated that mineralization was inhibited by 50% in Nfix deficient cells at 18 days and inhibited 70% at 24 days. These data support the conclusion that *Nfix* is necessary for mouse osteoblast differentiation.


d 18 Nf1x shRNA
d 18 NT shRNA
d 24 Nf1x shRNA
d 24 NT shRNA

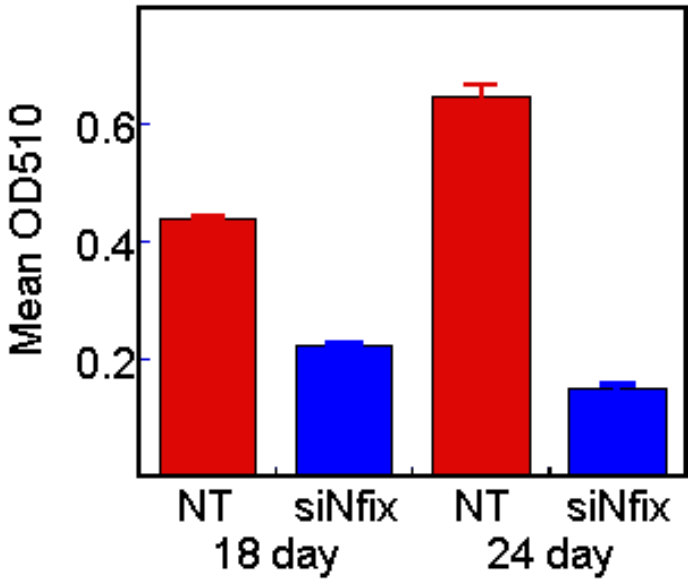

Mean OD510
0.6
0.4
0.2
NT
siNfix
NT
siNfix
18 day
24 day

Figure 13. Long term knockdown of Nfix expression in MC3T3-E1 cells with lentiviral shRNA vector- Effects on mineralization.Cultures transduced as in Figure 12 were fixed in methanol at 18 and 24 days, and mineralization was visualized by alizarin red staining. Stained wells were scanned at 510 nm using a Biotek Synergy 2 plate reader that determines optical density at in 2000 circular spots covering 90% of the 9.4 cm^2 well surface. NT: non-targeting. Data are mean OD510 ± sem of 3 wells. * significant difference between non-targeting and siNfi targeting.

Discussion

In previous studies we demonstrated that the NFI gene family has a significant role of in the regulation of the IGFBP5 transcription in human osteoblast cells (Perez-Casellas et al., 2009). Those studies suggested a possible regulatory role for this TF family during the process of osteoblast differentiation. In the present study we demonstrated that all four murine *Nfi* genes are expressed in MC3T3-E1 cells and mBMSCs at different stages of osteoblast differentiation. Among these *Nfi* genes, *Nfic* and *Nfix* were consistently the most abundant throughout differentiation compared to *Nfia*, which had the lowest relative mRNA expression. Transient overexpression of Murine Nfib increased *Igfbp5* promoter activity in MC3T3-E1 pre osteoblast cells. However, Nfix modestly increased *Igfbp5* promoter activity. These results complemented our past studies where we showed similar effects of human NFIX on regulation of *Igfbp5* promoter activity. Results of Nfix expression on promoter activity were corroborated by siRNA mediated gene knockdown experiments in which knockdown of endogenous *Nfix* expression increased *Igfbp5* mRNA. Relative mRNA expression levels of *Ocn* were also decreased . Based on this finding we decided to determine the role of Nfix and Runx-2 on murine Ocn promoter activity. We found that increasing amounts of Runx-2 affected Nfix- mediated activation of Ocn transcription. This repressive role of Runx2 when coexpressed with Nfix, can be in part a mechanism for regulating Ocn expression during early stages of osteoblast differentiation. Long term knockdown of Nfix expression in differentiating MC3T3-E1 cells tremendously affected mineral deposition and delayed mineralization. We later examined several osteoblast differentiation markers to determine if any of them was a target of Nfix. We found that Runx-2, BSP and Ocn mRNA were

decreased in *Nfix* depleted cells. Of these markers, Ocn was the gene that was affected the most with up to -400 fold decreas in expression. These results confirmed our previous findings that indicated that Nfix positively regulates Ocn transcription. We still need to determine if these effects are done directly by Nfix or if there are any other intermediate molecules involved in this process.

In summary, we report novel evidence for *Igfbp5* and *Ocn* transcriptional regulation by Nfix proteins in murine cell line MC3T3-E1. Nfix knockdown in differentiated MC3T3-E1 cells delayed the mineralization process. *Runx-2*, *Bsp* and *Ocn* are potential genes directly targeted by Nfix during osteoblast differentiation. These results suggest Nfix be a significant to regulator of osteoblast differentiation.

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CHAPTER FOUR

DISCUSSION

The Insulin-like Growth Factor (IGF) system is a major target of GC inhibition in bone. We found that GCs inhibit expression of IGF binding protein-5 (IGFBP5) which binds IGFs and stimulates osteoblast activity by IGF dependent and independent mechanisms. The work in this dissertation identifies the NFI gene family as an important regulator of IGFBP-5 transcription primarily in human, as well as murine osteoblasts.

In the first part of this dissertation we 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 mostly decreased promoter activity at increasing concentrations. 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. 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. 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 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 members bind to the palindromic sequence TGGC/A$(N)_5$GCCA or with lower affinity to the half-palindrome. They bind their recognition sequence as homo- or heterodimers. Heterodimer formation between NFIB and other NFI family members may produce different effects depending on the associated NFI proteins. 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 (Gronostajski et al., 1985).

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 present study provides more direct evidence that the NFI site does function in transcriptional regulation. Interactions between NFI and AP-2 interact on direct regulation of *IGFBP5* promoter activity remains to be determined. 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.

Recent studies and our current findings 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 (Driller et al., 2007), although the growth retardation was reduced in another study by feeding a soft diet (Campbell et al., 2008). *Nfic* deficiency in mice inhibited odontoblast differentiation and tooth root development (Steele-Perkins et al., 2003). 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 (Zambotti et al., 2002).

In the second part of this dissertation we examined a possible regulatory of this TF family during the process of osteoblast differentiation. In the present study we

demonstrated that all four murine *Nfi* genes are expressed in MC3T3-E1 cells and mBMSCs at different stages of osteoblast differentiation. Among these *Nfi* genes, *Nfic* and *Nfix* were consistently the most abundant throughout differentiation compared to *Nfia*, which had the lowest relative mRNA expression. Transient overexpression of Murine Nfib increased *Igfbp5* promoter activity in MC3T3-E1 pre osteoblast cells. However, Nfix modestly increased *Igfbp5* promoter activity. These results complemented our past studies. Results of Nfix expression on promoter activity were corroborated by siRNA mediated gene knockdown experiments in which knockdown of endogenous *Nfix* expression increased *Igfbp5* mRNA. Relative mRNA expression levels of *Ocn* was also decreased . Based on this finding we decided to determine the role of Nfix and Runx-2 on murine Ocn promoter. We found that increasing amounts of Runx-2 affected Nfix-mediated activation of the Ocn transcription. This repression role of Runx2 when coexpressed with Nfix, can be in part a regulatory mechanism of Ocn expression on early stages of osteoblast differentiation. Long term knockdown of Nfix in MC3T3-E1 differentiated cells tremendously affected mineral deposition and had delayed mineralization. We later examined several osteoblast differentiation markers to determine if any of them was a target of Nfix. We found that Runx-2, BSP and Ocn mRNA were decreased in *Nfix* depleted cells. Of these markers, Ocn was the gene that was affected the most with up to -400 fold inhibition of expression. These results confirmed our previous findings that indicated that Nfix positively regulates Ocn transcription. We still need to determine if these effects are done directly by Nfix or if there are any other intermediate molecules involved in this process.

In summary, we report novel evidence for *Igfbp5* and *Ocn* transcriptional regulation by Nfix proteins in murine cell line MC3T3-E1. Nfix knockdown in differentiated MC3T3-E1 cells delayed the mineralization process. *Runx-2*, *Bsp* and *Ocn* are potential genes directly targeted by Nfix during osteoblast differentiation. These results suggest Nfix be a significant to regulator of osteoblast differentiation.

Conclusions and Future Directions

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. We also demonstrated that murine *Nfix* knockdown affects the osteoblast mineralization, affecting BSP, OCN and Runx-2 relative mRNA levels. These results suggest that differential regulation of *IGFBP5* expression osteoblast differentiation by *NFI* gene family transcription factors may be significant to modulation of osteoblast proliferation and differentiation. Future studies should be performed to determine the possible mechanism of *Nfix* regulation of murine osteoblast differentiation utilizing the MC3T3-E1 cell line model. It would be of great importance to investigate if effects of OCN expression by *Nfix* are direct or through any intermediate molecules.

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