ARTICLE
Journal of Cellular Biochemistry 106:186–192 (2009)

Journal of Cellular Biochemistry

FIAT Inhibition Increases Osteoblast Activity By Modulating Atf4-Dependent Functions

Vionnie W.C. Yu,[1,2] Jad El-Hoss,[1,2] and René St-Arnaud[1,2*]

[1]*Genetics Unit, Shriners Hospital for Children, Montreal, Quebec, Canada H3G 1A6*

[2]*Department of Human Genetics, McGill University, Montreal, Quebec, Canada H3A 2T5*

ABSTRACT

The ATF4 transcription factor is a key regulator of osteoblast differentiation that controls osteocalcin gene transcription and type I collagen protein synthesis. We have characterized factor-inhibiting ATF4-mediated transcription (FIAT), a leucine zipper protein that dimerizes with ATF4 to form inactive dimers that cannot bind DNA. Overexpression of FIAT in osteoblasts of transgenic mice inhibited osteocalcin gene transcription and reduced osteoblastic activity, leading to osteopenia (Yu et al. [2005] J Cell Biol 169:591–601). We therefore hypothesized that inhibition of FIAT would enhance ATF4 activity, leading to increased osteocalcin transcription, type I collagen synthesis, and mineralization. We used small interfering RNAs (siRNA) to knockdown FIAT in pools of MC3T3-E1 cells stably transfected with 1.3 kb of the mouse osteocalcin gene promoter driving expression of luciferase. Stable expression of the FIAT siRNA sequence inhibited FIAT expression without significantly affecting the level of total or Ribosomal S6 Kinase-2-phosphorylated ATF4 protein. Occupancy of the osteocalcin proximal promoter by ATF4 was increased and transcription of the osteocalcin-promoter-dependent luciferase reporter showed earlier onset and increased levels. Similarly, endogenous osteocalcin gene expression was enhanced in primary osteoblasts transfected with the FIAT siRNA. FIAT knockdown cells also displayed higher expression of bone sialoprotein, increased type I collagen protein synthesis, and enhanced mineralization. These data suggest that inhibition of FIAT expression increases ATF4 activity and confirm the important role of FIAT in osteoblast function. J. Cell. Biochem. 106: 186–192, 2009.

KEY WORDS: FIAT; ATF4; OSTEOCALCIN GENE TRANSCRIPTION; OSTEOBLAST; BZIP TRANSCRIPTION FACTORS

The basic domain-leucine zipper (bZIP) transcription factor ATF4 is a major regulator of osteoblast biology. This role appears relevant in the molecular etiology of skeletal dysplasiae, as ATF4 is a downstream target of the ribosomal S6 kinase-2 (RSK2) inactivated in Coffin-Lowry Syndrome [Trivier et al., 1996; Yang et al., 2004]. It was further shown to mediate neurofibromin signaling in osteoblasts, suggesting a role in the development of the skeletal abnormalities of patients with neurofibromatosis type I [Elefteriou et al., 2006; Wu et al., 2006].

Upon activation through phosphorylation by RSK2 in osteoblasts, ATF4 regulates osteoblast-specific gene transcription and the synthesis of type I collagen [Yang and Karsenty, 2004; Yang et al., 2004]. ATF4-deficient mice are runted [Tanaka et al., 1998; Hettmann et al., 2000; Masuoka and Townes, 2002] and harbor low bone mass, reduced osteoblast activity, decreased type I collagen synthesis, and inhibited osteocalcin and bone sialoprotein (BSP) gene transcription [Yang et al., 2004]. The potent activation of the osteocalcin gene is crucial for osteoblast differentiation and involves cooperative interactions between TFIIA-γ, RUNX2, SATB2, and ATF4 at the proximal osteocalcin promoter [Xiao et al., 2005; Dobreva et al., 2006; Yu et al., 2008a].

Factor-inhibiting ATF4-mediated transcription (FIAT) is a novel leucine zipper protein whose name was coined for its interaction with ATF4 and subsequent blockage of ATF4-directed osteocalcin gene transcription [Yu et al., 2005]. FIAT is a 66-kDa nuclear protein that lacks a basic DNA-binding domain but contains three identifiable leucine zipper (ZIP) domains. A protein identical to FIAT (named γ-taxilin) was characterized on the basis of its extended C-terminal coiled-coil, and shown to interact with syntaxin family members and with the transcriptional coactivator, αNAC [Nogami et al., 2004; Yoshida et al., 2005]. It thus appears that FIAT/γ-taxilin can interact with a variety of partners. Sorting out the physiological relevance of all interactions represents the next challenge and will require further studies. To alleviate the text, the acronym FIAT, which adequately defines the function of the protein that is reported here, will be used throughout. FIAT heterodimerizes

Grant sponsor: NIH; Grant number: 1R01AR053287-01A1.

*Correspondence to: René St-Arnaud, Genetics Unit, Shriners Hospital for Children, 1529 Cedar Avenue, Montreal, Quebec, Canada H3G 1A6. E-mail: rst-arnaud@shriners.mcgill.ca

Received 3 September 2008; Accepted 17 October 2008 • DOI 10.1002/jcb.21995 • 2008 Wiley-Liss, Inc.
Published online 17 November 2008 in Wiley InterScience (www.interscience.wiley.com).

with ATF4 through its second zipper [Yu et al., 2008b] and thereby prohibits ATF4 from binding to its cognate DNA sequence [Yu et al., 2005, 2006]. Transgenic mice overexpressing FIAT under the control of the osteoblast-specific fragment of the α1(I) collagen promoter are osteopenic [Yu et al., 2005, 2006], with reduced bone mineral density, lowered trabecular volume, and impaired bone rigidity [Yu et al., 2005]. This low bone mass phenotype was shown to be caused by impaired osteoblast activity, without changes in osteoblast proliferation or apoptosis [Yu et al., 2005]. Thus, the osteoblast-specific FIAT transgenic mice mimic several aspects of the ATF4-deficient mice phenotype.

Since overexpression of FIAT in osteoblasts leads to reduced ATF4 activity [Yu et al., 2005, 2006], we hypothesized that the converse inhibition of FIAT would augment ATF4 function. In this study, we applied the RNA interference technology [Almeida and Allshire, 2005; Bayne and Allshire, 2005] to achieve stable RNA knockdown of FIAT in osteoblastic cells. The phenotype of cells with attenuated FIAT expression is consistent with a role for FIAT in regulating ATF4 activity in osteoblasts.

MATERIALS AND METHODS

CONSTRUCTION OF SIRNA AND SHORT HAIRPIN RNA (SHRNA) EXPRESSION VECTORS

The 21 nt double stranded siRNA including 3′UU overhangs was designed from the FIAT mRNA coding sequence at position 762 using the siRNA Target Finder web tool from the Ambion webpage (Ambion, Inc.; www.ambion.com). The siRNA was tested (blasted) against the mouse genomic RefSeq RNA database (www.ncbi.nlm.nih.gov/BLAST) to avoid repetitive sequences. The sequences were as followed: sense 5′-GAACGACGUAAAGAAGCAAUU-3′ and antisense 5′-UUGCUUCUUUACGUCGUUCUU-3′.

To construct the shRNA expression vector, a 55 nt sense and antisense DNA corresponding to the aforementioned FIAT sequences, a loop sequence (5′-TTCAAGAGA-3′), and flanking *Bam*HI and *Hin*dIII restriction sites were designed using the Ambion's pSilencer Expression Vectors Insert Design Tool webpage (www.ambion.com) and synthesized. The sense and antisense single-stranded DNAs were annealed and subsequently ligated to the p*Silencer* 3.1-H1 puro vector (Ambion), which contains the human H1 promoter that utilizes DNA polymerase III for transcription. Successful insertion was verified by *Bam*HI and *Hin*dIII restriction digests and sequencing of the insert fragment.

TRANSIENT AND STABLE TRANSFECTION EXPERIMENTS

An MC3T3-E1 preosteoblastic cell line with an integrated osteocalcin promoter-luciferase reporter allele [Xiao et al., 1997] was used for both transient and stable transfections, and was cultured in αMEM containing 10% fetal bovine serum and 200 μg/ml G418 unless otherwise stated. In transient transfection, cells were plated at 4×10^4 cells/well in a 24-well plate and transfected with 100 mM of the FIAT siRNA, or an inert siRNA sequence as negative control using the siPORT *Amine* Transfection Agent (Ambion) according to the manufacturer's instructions. Cells were lysed 24 h post-transfection and 20 μl of cell lysate was used to measure luciferase activity.

For stable transfection, cells were plated at 1.5×10^5 cells/well of a 6-well plate and were transfected with 2 μg of expression vectors transcribing the FIAT siRNA, an inert siRNA sequence as a negative control, or the empty vector using the GenePORTER Transfection Reagent (Genlantis) and the instructions provided by the manufacturer. At 48 h post-transfection, cells were trypsinized and re-plated at a 1:40 ratio for selection with 2 μg/ml of puromycin. Cell populations were established as pools of stably transfected clones and thus represent the most frequent integration event.

RNA KNOCKDOWN IN PRIMARY OSTEOBLAST CULTURES

Primary osteoblast cultures were prepared from newborn mice calvaria as previously described [Yu et al., 2005] and transiently transfected at 12 days post-confluency (dpc) with 150 mM of the FIAT siRNA or the negative control siRNA as described above. RNA was harvested 48 h post-transfection using the RNAqueous-4PCR kit following the manufacturer's instructions (Ambion).

REAL-TIME REVERSE TRANSCRIPTION-PCR (RT-QPCR)

Stable siRNA pools were plated at 5×10^4 cells/well in a 24-well plate and cultured in the presence of 50 μg/ml ascorbic acid. RNA was harvested at cell confluence, and at 3, 7, 14, and 21 dpc using the RNAqueous-4PCR kit, adhering to the manufacturer's instructions (Ambion). RNAs from the stable siRNA pools or the transiently transfected primary osteoblasts were reverse transcribed into cDNA using the High Capacity cDNA Archive kit as per the manufacturer's recommendations (Applied Biosystems). Real Time PCR amplification was performed on an Applied Biosystems 7500 instrument using specific TaqMan assays for the FIAT, Osteocalcin (OCN), Atf4, Runx2, Osterix (OSX), Type I collagen α_1 chain (COL1a1), BSP and Gapdh genes, and the TaqMan Universal PCR Master Mix (Applied Biosystems). Expression level of each mRNA was quantified by the ΔC_t method and normalized to Gapdh levels.

WESTERN BLOTTING

The purified anti-FIAT antibody (180 μg/ml) raised against FIAT residues 111–125 [Yu et al., 2005] was used to probe immunoblots of nuclear extracts from the various siRNA stable pools. The anti-ATF4 antibody was purchased from Santa Cruz Biotechnology, Inc., while the anti-phospho-ATF4 antisera was a generous gift of Dr. G. Karsenty (Columbia University, New York, NY). Anti-rabbit antibodies conjugated with horseradish peroxidase were used as secondary antibodies and detected by ECL Western Blotting Detection Reagents (GE Healthcare Bio-Sciences).

CHROMATIN IMMUNOPRECIPITATION

Stable siRNA pools were plated at 1.5×10^6 cells per 100 mm petri dish and cells were harvested at day 14 post-confluence. Chromatin Immunoprecipitation was performed as described in detail elsewhere [Akhouayri et al., 2005]. Five microliter of purified DNA was used as template for PCR with the following primers which amplified the promoter region of the osteocalcin gene: Forward, 5′-AGGCAGCTGCAATCACCA-3′; Reverse, 5′-GCACCCTGCAGCATCCA-3′. Quantification of the signal intensity was performed using the software Scion Image Alpha 4.0.3.2 (Scion Corporation, Frederick, MD) following the manufacturer's instructions.

ANALYSIS OF COLLAGEN SYNTHESIS

Collagen synthesis by osteoblasts was determined using the previously detailed modification of the technique described by [Yang et al., 2004; Yu et al., 2005].

ALIZARIN RED STAINING

For alizarin red staining, cells were grown for 5 weeks post-confluence with non-essential amino acids in the presence of 50 μg/ml ascorbic acid and 5 mM β-glycerol phosphate, then fixed with 4% PFA and stained with 0.5% alizarin red (Sigma) at pH 5.0. Dark red mineralized nodules were counted and the results expressed as number of nodules per mm^2.

STATISTICAL ANALYSIS

Statistical analysis used Student's *t*-test when two variables were compared or analysis of variance with post hoc test when more than two samples were included in the analysis.

RESULTS

Before achieving stable silencing of FIAT expression in osteoblasts, we first assessed the silencing ability of different FIAT siRNA sequences by transient transfection of MC3T3-E1 osteoblastic cells. The siRNA sequence directed at position 762 of the FIAT transcript was the most efficacious at depleting FIAT mRNA and protein expression (data not shown). Therefore, we chose to conduct the rest of the experiments with this FIAT siRNA.

To study the long term effect of FIAT silencing in osteoblasts, we stably transfected an MC3T3-E1 cell line containing an integrated osteocalcin promoter (−1.3 kb)-luciferase reporter allele [Xiao et al., 1997] with expression vectors for the FIAT siRNA, or a negative control siRNA sequence that does not correspond to any inventoried sequence in murine mRNA databases, or the empty vector. Populations of cells representing pools of clones stably transfected with each of the plasmids (empty vector, negative control siRNA, and FIAT siRNA) were established. Figure 1A shows the 88% reduction of FIAT mRNA expression in cells tranfected with FIAT siRNA in comparison to negative control siRNA or vector alone, when cells were harvested at day 3 post-confluence. Analysis of protein expression at the corresponding time point by Western blot also revealed diminished FIAT protein level from cells containing FIAT siRNA compared to cells with negative control siRNA or empty vector (Fig. 1B). RT-qPCR recorded a 67–88% reduction of FIAT mRNA level in FIAT siRNA cells at confluence, 3, 7, 14, and 21 dpc, with a corresponding decrease of protein level at those time points (data not shown). Together, these data show that FIAT can be efficiently inhibited at the RNA and protein levels using RNA interference, and that this effect is stable for at least 21 days post-cell confluence.

We first investigated whether down-regulating FIAT expression affects ATF4 binding to the osteocalcin gene promoter by using chromatin immunoprecipitation. Chromatin obtained from stable pools expressing FIAT siRNA, negative control siRNA, or the empty vector was precipitated with control or anti-ATF4 antibodies.

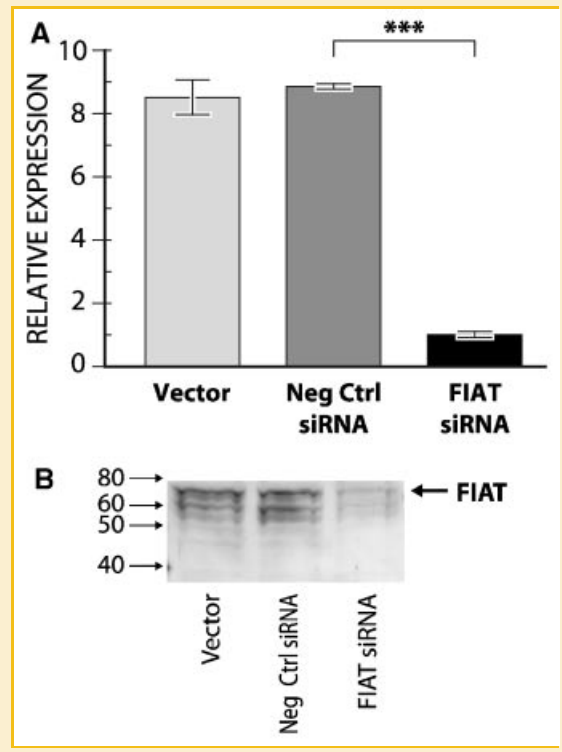

Fig. 1. Stable silencing of FIAT expression by siRNA. A: RT-qPCR showing that FIAT mRNA expression was reduced by 88% in MC3T3-E1 cells stably transfected with the FIAT siRNA, as compared to negative control (NegCtrl) siRNA or empty vector. B: Cells were harvested at the same time point (day 3 post-confluence) and extracts were probed with the anti-FIAT antibody to show decreased FIAT protein expression. The FIAT 66 kDa band is indicated by the arrow; the lower bands represent degradation fragments generated upon cell lysis. Molecular sizes in kDa are shown at the left of the gel. $^{***}P < 0.001$.

Precipitated chromatin was subsequently amplified by PCR using primers flanking the cognate ATF4-binding site on the osteocalcin promoter. Background levels of chromatin were immunoprecipitated in the absence of antibody or by an unrelated, control antibody directed against the yeast transcription factor GAL4 (Fig. 2, lanes 1–6). Anti-ATF4 antibodies enriched the immunoprecipitated osteocalcin promoter fragment (Fig. 2, lanes 7–9). Inhibition of FIAT in osteoblasts led to increased ATF4 binding and augmented the amount of chromatin specifically immunoprecipitated by the anti-ATF4 antibodies (Fig. 2, lane 9). When the signal intensity of the amplimers on the gel was quantified and the background signal (unrelated antibody) subtracted, relative values of 18.6, 15.6, and 57.6 were obtained for vector, negative control siRNA, and FIAT siRNA samples, respectively. Thus reducing the amount of FIAT in cells frees more ATF4 to bind its response element on the osteocalcin gene. These results are consistent with the model that FIAT represses ATF4 transcriptional activity by blocking the access of ATF4 to its binding site on the osteocalcin promoter [Yu et al., 2006].

Since FIAT prevents ATF4 from binding to its target sequence, reducing FIAT expression should lead to an augmentation of osteocalcin gene transcription due to enhanced ATF4 binding to DNA. We first tested this hypothesis using the stably integrated

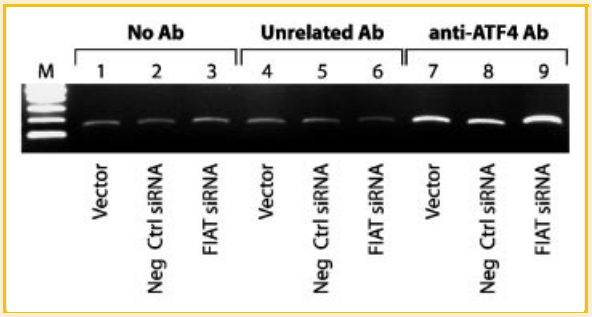

Fig. 2. FIAT inhibition enhances ATF4 binding to the osteocalcin promoter. Immunoprecipitation assays were performed with formaldehyde-crosslinked chromatin and protein A-sepharose beads (No Ab), an unrelated antibody (Ab), or antibodies against ATF4 (anti-ATF4 Ab). Ethidium bromide-stained agarose gels of PCR products obtained with primers flanking the ATF4 binding site are shown. The anti-ATF4 antibodies enriched the precipitated osteocalcin promoter fragment from stable pools expressing FIAT siRNA (lane 9), compared to pools expressing vector alone (lane 7) or negative control siRNA (lane 8). Lane 1–6 show background levels of chromatin immunoprecipitated by the beads and by an unrelated control antibody.

luciferase allele under the control of the proximal 1.3 kb osteocalcin promoter [Xiao et al., 1997]. In vector and negative control siRNA-transfected pools of cells, the expression of the reporter luciferase allele increased after several days in post-confluent cultures with medium containing ascorbic acid (Fig. 3A), as previously reported [Xiao et al., 1997]. Cells with stably silenced FIAT showed earlier onset of luciferase reporter gene expression and strongly induced expression at all times tested (Fig. 3A). These data confirm that FIAT is an important regulator of osteocalcin gene transcription. In parallel, we assessed the levels of total ATF4 protein (Fig. 3B) or RSK2-phosphorylated ATF4 protein (Fig. 3C). Some variation is apparent, mostly due to differences in loading (Fig. 3D). Of note however, is the observation that the dramatic stimulation of osteocalcin–luciferase gene transcription occurred without increases in the level of the serine 251, RSK2-phosphorylated ATF4 protein, which is the form that is involved in osteocalcin gene transcription [Yang et al., 2004] (Fig. 3C, lanes 3, 6, 9, 12, and 15). These results are consistent with a model in which FIAT suppression leads to relief of its inhibition of ATF4 activity without affecting the levels of ATF4 protein.

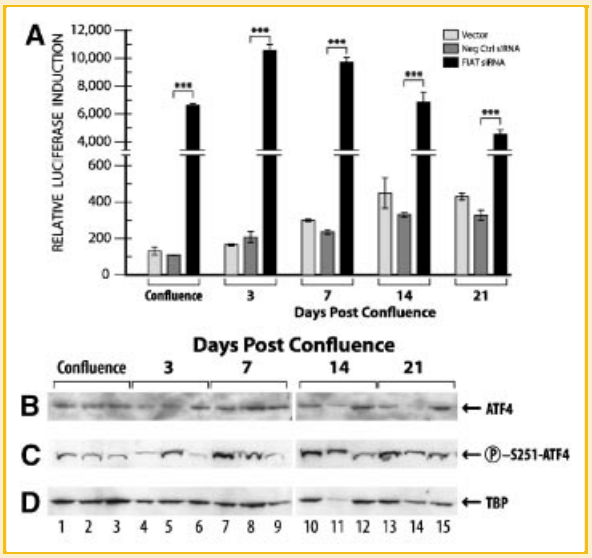

Fig. 3. FIAT inhibition increases proximal osteocalcin promoter activity. A: Cells extracts were prepared at the indicated time from pools of cells stably transfected with vector, a negative control siRNA (Neg Ctrl siRNA), or the FIAT siRNA and the expression of the osteocalcin–luciferase reporter allele was measured. Cells with stably silenced FIAT showed a striking induction of the reporter allele activity at all time points. $^{***}P < 0.001$. B–D: Immunoblots of the corresponding total ATF4 (B) and RSK2-phosphorylated ATF4 (C) proteins. TBP was probed as a loading control (D). P-S251-ATF4, ATF4 phosphorylated on serine residue 251 by RSK2. Lanes 1, 4, 7, 10, 13, vector-transfected cells; lanes 2, 5, 8, 11, 14, Neg Ctlr siRNA-expressing cells; lanes 3, 6, 9, 12, 15, FIAT siRNA-expressing cells.

We also measured the overall effect of FIAT knockdown on expression of osteoblast-related genes post-confluence. Transcript levels for Runx2, Osterix, and the α_1 chain of type I collagen were not significantly affected by the FIAT siRNA (Fig. 5A–C). FIAT RNA knockdown significantly augmented the expression of another ATF4 target gene, BSP [Elefteriou et al., 2006], at all time points tested (panel D).

ATF4 has been reported to regulate type I collagen synthesis in osteoblasts [Yang et al., 2004], conceivably through its role in controlling amino acids import [Harding et al., 2003; Chen et al., 2004]. We thus explored whether altering FIAT expression affects collagen production in MC3T3-E1 cells. The stable pools expressing

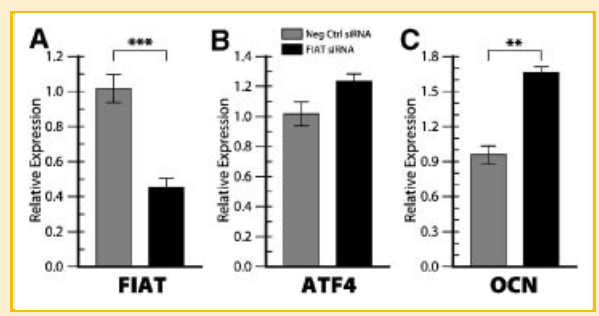

Fig. 4. FIAT knockdown increases endogenous osteocalcin gene expression in primary osteoblasts. Primary osteoblast cultures were prepared from newborn mice calvaria and transiently transfected at 12 days post-confluency with the FIAT siRNA or the negative control (Neg Ctrl) siRNA. RNA was harvested 48 h post-transfection and analysed for FIAT (A), ATF4 (B), or osteocalcin (OCN, panel C) expression by RT-qPCR. $^{**}P < 0.01$; $^{***}P < 0.001$.

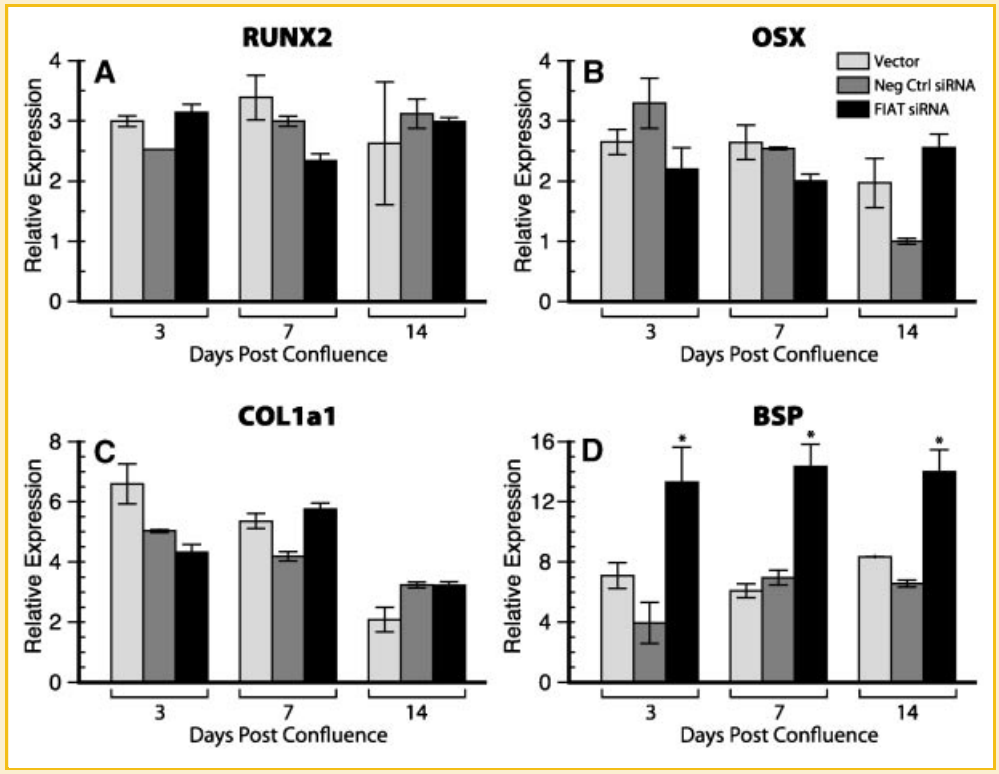

Fig. 5. Expression of endogenous osteoblast differentiation markers in FIAT knockdown cells. mRNA was isolated at the indicated time points and assayed for Runx2 (panel A), Osterix (OSX, panel B), the α_1 chain of type I collagen (COL1a1, panel C), or bone sialoprotein (BSP, panel D) using RT-qPCR. Neg Ctrl, negative control; $^*P < 0.05$.

FIAT siRNA, negative control siRNA, or containing the empty vector were grown for 3 or 14 dpc, labeled with [^{3}H]-proline in the presence or absence of non-essential amino acids, and harvested for collagen synthesis analysis. When cells were not supplied with amino acids, the FIAT knockdown pool produced more type I collagen than the control pools (Fig. 6B,C). In comparison, no noticeable differences were seen between the FIAT knockdown cells and the control cells when they were cultured in medium supplemented with non-essential amino acids (Fig. 6B,C, +NEAA). Therefore, FIAT silenced cells had increased type I collagen synthesis when they were stressed by amino acids deficiency, a situation where ATF4 activity is required [Harding et al., 2003; Yang et al., 2004]. The culture conditions (with or without non-essential amino acids) did not induce major changes in ATF4 protein expression (Fig. 6A; the reduced levels observed in lanes 10–12 are due to uneven sample loading as shown by TBP levels).

Finally, we determined whether osteoblast function is affected by FIAT silencing. Stable pools of cells expressing FIAT siRNA, negative control siRNA, or containing the empty vector were cultured with non-essential amino acids in the presence of 50 μg/ml ascorbic acid and 5 mM β-glycerol phosphate in order to induce matrix mineralization. Mineralized nodules were stained by alizarin red and counted at 5 weeks post-confluence. In comparison to the control cells, cells expressing the FIAT siRNA displayed increased mineralization and an increased number of nodules (Fig. 7). These results confirm that FIAT is a key regulator of osteoblast activity.

DISCUSSION

In this study, we achieved efficient knockdown of FIAT in MC3T3-E1 cells using RNA interference. Our analysis of the phenotype of the FIAT-attenuated osteoblastic cells are consistent with the model that FIAT modulates ATF4-dependent pathways.

FIAT is a leucine zipper nuclear protein that represses ATF4-mediated osteocalcin gene transcription in osteoblasts [Yu et al., 2005, 2006]. Transgenic mice overexpressing FIAT specifically in osteoblasts mimic several aspects of osteoblast-specific ATF4-deficient mice, including an osteopenic phenotype [Yu et al., 2005, 2006] with reduced osteoblastic activity [Yu et al., 2005]. Given this phenotypic resemblance and the functional interaction of FIAT and ATF4 impacting on osteocalcin gene transcription, we reasoned that FIAT regulates osteoblastic activity mainly through ATF4-dependent pathways. Indeed, chromatin immunoprecipitation experiments revealed that when FIAT expression was suppressed by RNA interference, increased amounts of ATF4 bound to the osteocalcin promoter (Fig. 2), leading to a higher transcriptional activity of the osteocalcin promoter-reporter (Fig. 3) and increased expression of the endogenous osteocalcin gene in primary cultures of calvarial osteoblasts (Fig. 4). Osteocalcin gene transcription depends on the complex interplay between several transcriptional regulators, including cooperative interactions between TFIIAγ, RUNX2, SATB2, and ATF4 [Xiao et al., 2005; Dobreva et al., 2006; Yu et al., 2008a]. No interaction has ever been reported between FIAT and TFIIAγ,

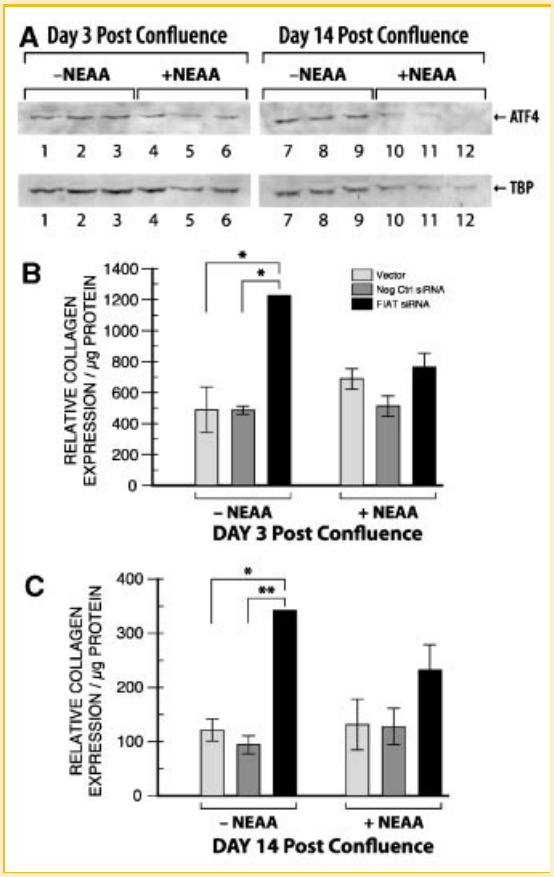

Fig. 6. Inhibition of FIAT enhances type I collagen synthesis. Pools of cells stably transfected with vector, a negative control siRNA (NegCtrl siRNA), or the FIAT siRNA were grown in the presence (+) or absence (−) of non-essential amino acids (NEAA) and [^{3}H]-proline-labeled. A: Cell extracts were probed with anti-ATF4 or anti-TBP antibodies to show protein expression. Lanes 1, 4, 7, 10, vector-transfected cells; lanes 2, 5, 8, 11, Neg Ctlr siRNA-expressing cells; lanes 3, 6, 9, 12, FIAT siRNA-expressing cells. B,C: Cell homogenates were digested to collagen with pepsin, precipitated, and resolved by SDS–PAGE for quantification of collagen synthesis. FIAT silenced cells produced more type I collagen in the absence of non-essential amino acids at both day 3 (B) and day 14 (C) post-confluence. $^*P < 0.05$; $^{**}P < 0.01$.

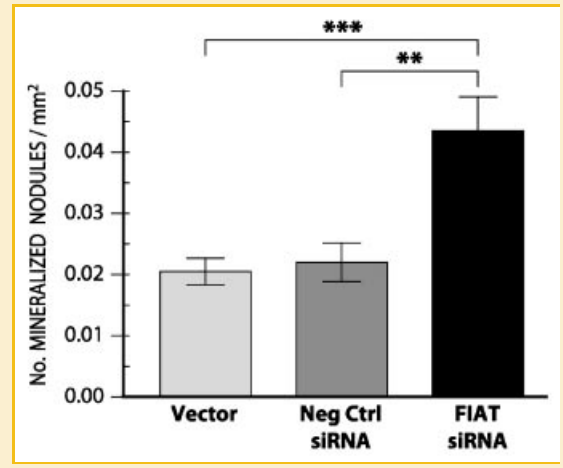

Fig. 7. Increased mineralization in FIAT siRNA expressing cells. Pools of cells stably transfected with vector, a negative control siRNA (NegCtrl siRNA), or the FIAT siRNA were grown in the presence of ascorbic acid and β-glycerophosphate. Cells were stained with alizarin red at 5 weeks post-confluence. Mineralized nodules were counted and results are expressed as number of nodules per square millimeter. Mineralization was enhanced in FIAT siRNA expressing cells relative to empty vector or negative control siRNA-expressing cells. $^{**}P < 0.01$; $^{***}P < 0.001$.

SATB2, or RUNX2, and it is not known whether FIAT can influence their activity. Our preferred interpretation of the gene expression monitoring data is that FIAT inhibition increases gene transcription by modulating ATF4 activity. Our observation that the expression of another ATF4 endogenous target gene, BSP [Elefteriou et al., 2006], was also increased in the context of FIAT inhibition in the established pools of FIAT knockdown cells (Fig. 5), supports this model.

ATF4 is an essential component in the pathways governing amino acids import and synthesis in response to oxidative stress or amino acid deprivation [Harding et al., 2003; Chen et al., 2004]. Yang et al. [2004] reported that type I collagen transcription was not affected in ATF4-deficient mice, but that the absence of ATF4 affected type I collagen protein synthesis, possibly due to a defect in amino acid import and/or synthesis. Thus knocking down FIAT, a regulator of ATF4 activity, could affect type I collagen protein levels without modifying its mRNA expression. Indeed, we observed that siRNA-mediated attenuation of FIAT expression did not change type I collagen mRNA levels (Fig. 5C), but caused a substantial increase of type I collagen protein synthesis in osteoblastic cells when they were not supplemented with non-essential amino acids (Fig. 6). When the cells were cultured in the presence of non-essential amino acids, there was no change in collagen production. This finding echoes the functional importance of ATF4 in amino acid import and synthesis. When cells are stressed with depleted amino acid resources, ATF4 activity is increased leading to stimulated expression of genes that mediate amino acids synthesis and transport [Harding et al., 2003]. This, in osteoblasts, results in an increase in type I collagen synthesis, the major secretory protein of the bone-forming cell. Our previous finding of no alteration in collagen production in FIAT-transgenic mice was most likely due to the relatively low level of FIAT transgene overexpression [Yu et al., 2005].

We previously reported that primary osteoblast cultures obtained from transgenic mice overexpressing FIAT exhibit attenuated mineralization when compared to wild-type cultures [Yu et al., 2005]. Reciprocally, we observed enhanced mineralization in cells that do not express FIAT following siRNA-mediated FIAT knockdown (Fig. 7). It is likely that increased collagen protein secretion by FIAT knockdown cells contributes to earlier matrix maturation. Additionally, changes in the expression levels of terminal osteoblast differentiation markers resulting from enhanced ATF4 activity in the absence of FIAT may promote osteoblast activity (Fig. 5). It also remains a possibility that FIAT normally interacts with additional bZIP transcriptional regulators of osteoblast activity, such as Fra-1 [Jochum et al., 2000; Eferl et al., 2004] or ΔFosB [Sabatakos et al., 2000], to inhibit their activity. Preliminary results using artificial promoter-reporter constructs suggest that FIAT may inhibit Fra-1-mediated transcription [St-Arnaud and Elchaarani, 2007], although

this remains to be validated using natural target promoters. Reduced FIAT expression achieved through RNA silencing would then lead to increases in Fra-1 activity, which would contribute to the enhanced mineralization that we observed in FIAT knockdown cells.

Our studies reinforce the role of FIAT in the modulation of ATF4 activity and in the regulation of osteoblast function. Moreover, by using RNA interference as an alternative approach for gene silencing, our results provide proof of concept for the osteoblast-specific inactivation of FIAT in mouse models.

ACKNOWLEDGMENTS

We thank Dr. Renny T. Franceschi (University of Michigan) for his generous gift of the MC3T3-E1 subclone stably transfected with the osteocalcin–luciferase reporter, and Dr. Gérard Karsenty (Columbia University) for providing antibodies against the phosphorylated form of ATF4. Mark Lepik prepared the figures. V.W.C. Yu was a Canada Bone Scholar funded by the CIHR Skeletal Health Training Grant.

REFERENCES

Akhouayri O, Quelo I, St-Arnaud R. 2005. Sequence-Specific DNA binding by the αNAC coactivator is required for potentiation of c-Jun-dependent transcription of the osteocalcin gene. Mol Cell Biol 25:3452–3460.

Almeida R, Allshire RC. 2005. RNA silencing and genome regulation. Trends Cell Biol 15:251–258.

Bayne EH, Allshire RC. 2005. RNA-directed transcriptional gene silencing in mammals. Trends Genet 21:370–373.

Chen H, Pan YX, Dudenhausen EE, Kilberg MS. 2004. Amino acid deprivation induces the transcription rate of the human asparagine synthetase gene through a timed program of expression and promoter binding of nutrient-responsive basic region/leucine zipper transcription factors as well as localized histone acetylation. J Biol Chem 279:50829–50839.

Dobreva G, Chahrour M, Dautzenberg M, Chirivella L, Kanzler B, Farinas I, Karsenty G, Grosschedl R. 2006. SATB2 is a multifunctional determinant of craniofacial patterning and osteoblast differentiation. Cell 125:971–986.

Eferl R, Hoebertz A, Schilling AF, Rath M, Karreth F, Kenner L, Amling M, Wagner EF. 2004. The Fos-related antigen Fra-1 is an activator of bone matrix formation. EMBO J 23:2789–2799.

Elefteriou F, Benson MD, Sowa H, Starbuck M, Liu X, Ron D, Parada LF, Karsenty G. 2006. ATF4 mediation of NF1 functions in osteoblast reveals a nutritional basis for congenital skeletal dysplasiae. Cell Metab 4:441–451.

Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, Calfon M, Sadri N, Yun C, Popko B, Paules R, Stojdl DF, Bell JC, Hettmann T, Leiden JM, Ron D. 2003. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. Mol Cell 11:619–633.

Hettmann T, Barton K, Leiden JM. 2000. Microphthalmia due to p53-mediated apoptosis of anterior lens epithelial cells in mice lacking the CREB-2 transcription factor. Dev Biol 222:110–123.

Jochum W, David JP, Elliott C, Wutz A, Plenk H, Jr., Matsuo K, Wagner EF. 2000. Increased bone formation and osteosclerosis in mice overexpressing the transcription factor Fra-1. Nat Med 6:980–984.

Masuoka HC, Townes TM. 2002. Targeted disruption of the activating transcription factor 4 gene results in severe fetal anemia in mice. Blood 99:736–745.

Nogami S, Satoh S, Tanaka-Nakadate S, Yoshida K, Nakano M, Terano A, Shirataki H. 2004. Identification and characterization of taxilin isoforms. Biochem Biophys Res Commun 319:936–943.

Sabatakos G, Sims NA, Chen J, Aoki K, Kelz MB, Amling M, Bouali Y, Mukhopadhyay K, Ford K, Nestler EJ, Baron R. 2000. Overexpression of DeltaFosB transcription factor(s) increases bone formation and inhibits adipogenesis. Nat Med 6:985–990.

St-Arnaud R, Elchaarani B. 2007. Identification of additional dimerization partners of FIAT, the factor inhibiting ATF4-mediated transcription. Ann NY Acad Sci 1116:208–215.

Tanaka T, Tsujimura T, Takeda K, Sugihara A, Maekawa A, Terada N, Yoshida N, Akira S. 1998. Targeted disruption of ATF4 discloses its essential role in the formation of eye lens fibres. Genes Cells 3:801–810.

Trivier E, De Cesare D, Jacquot S, Pannetier S, Zackai E, Young I, Mandel JL, Sassone-Corsi P, Hanauer A. 1996. Mutations in the kinase Rsk-2 associated with Coffin-Lowry syndrome. Nature 384:567–570.

Wu X, Estwick SA, Chen S, Yu M, Ming W, Nebesio TD, Li Y, Yuan J, Kapur R, Ingram D, Yoder MC, Yang FC. 2006. Neurofibromin plays a critical role in modulating osteoblast differentiation of mesenchymal stem/progenitor cells. Hum Mol Genet 15:2837–2845.

Xiao G, Cui Y, Ducy P, Karsenty G, Franceschi RT. 1997. Ascorbic acid-dependent activation of the osteocalcin promoter in MC3T3-E1 preosteoblasts: Requirement for collagen matrix synthesis and the presence of an intact OSE2 sequence. Mol Endocrinol 11:1103–1113.

Xiao G, Jiang D, Ge C, Zhao Z, Lai Y, Boules H, Phimphilai M, Yang X, Karsenty G, Franceschi RT. 2005. Cooperative interactions between activating transcription factor 4 and Runx2/Cbfa1 stimulate osteoblast-specific osteocalcin gene expression. J Biol Chem 280:30689–30696.

Yang X, Karsenty G. 2004. ATF4, the osteoblast accumulation of which is determined post-translationally, can induce osteoblast-specific gene expression in non-osteoblastic cells. J Biol Chem 279:47109–47114.

Yang X, Matsuda K, Bialek P, Jacquot S, Masuoka HC, Schinke T, Li L, Brancorsini S, Sassone-Corsi P, Townes TM, Hanauer A, Karsenty G. 2004. ATF4 Is a Substrate of RSK2 and an essential regulator of osteoblast biology; Implication for Coffin-Lowry Syndrome. Cell 117:387–398.

Yoshida K, Nogami S, Satoh S, Tanaka-Nakadate S, Hiraishi H, Terano A, Shirataki H. 2005. Interaction of the taxilin family with the nascent polypeptide-associated complex that is involved in the transcriptional and translational processes. Genes Cells 10:465–476.

Yu VW, Ambartsoumian G, Verlinden L, Moir JM, Prud'homme J, Gauthier C, Roughley PJ, St-Arnaud R. 2005. FIAT represses ATF4-mediated transcription to regulate bone mass in transgenic mice. J Cell Biol 169:591–601.

Yu VWC, Gauthier C, St-Arnaud R. 2006. Inhibition of ATF4 transcriptional activity by FIAT/γ-taxilin modulates bone mass accrual. Ann NY Acad Sci 1068:131–142.

Yu S, Jiang Y, Galson DL, Luo M, Lai Y, Lu Y, Ouyang HJ, Zhang J, Xiao G. 2008a. General transcription factor IIA-gamma increases osteoblast-specific osteocalcin gene expression via activating transcription factor 4 and runt-related transcription factor 2. J Biol Chem 283:5542–5553.

Yu VW, Gauthier C, St-Arnaud R. 2008b. FIAT represses bone matrix mineralization by interacting with ATF4 through its second leucine zipper. J Cell Biochem 105:859–865.