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Abstract

Forkhead box O1 (FOXO1) is upregulated during bone formation and in response to stimulation by bone morphogenetic proteins. Studies presented here examined the functional role of FOXO1 in a well defined culture system in which pre-osteoblastic cells undergo terminal differentiation in vitro. Mineralizing cultures of MC3T3-E1 cells were examined with or without FOXO1 knockdown by RNAi. Normal cells show the upregulation of FOXO1 and RUNX2 DNA binding activity, alkaline phosphatase activity, and mRNA levels of FOXO1, RUNX2, type 1 collagen, osteocalcin and MMP13 during formation of mineralizing nodules. In FOXO1 depleted cells each of these measurements was significantly reduced compared to values in control cells transfected with scrambled siRNA ($P < 0.05$). Depletion of FOXO1 also reduced the number of mineralized nodules formed. Moreover, chromatin immunoprecipitation assays revealed a direct interaction of FOXO1 with the RUNX2 promoter. Overexpression of FOXO1 reduced the MC3T3-E1 cell number and the number of PCNA positive cells with little effect on apoptosis. These findings indicate that FOXO1 plays an important role in promoting osteoblast differentiation and suppressing proliferation in differentiating cells.

Keywords

Bone differentiation, Forkhead, Mineralizing matrix, Osteoblast, RUNX2transcription factor

Disciplines

Amino Acids, Peptides, and Proteins | Dentistry | Osteopathic Medicine and Osteopathy

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FOXO1 Modulates Osteoblast Differentiation

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Abstract

Forkhead box O1 (FOXO1) is upregulated during bone formation and in response to stimulation by bone morphogenetic proteins. Studies presented here examined the functional role of FOXO1 in a well defined culture system in which pre-osteoblastic cells undergo terminal differentiation in vitro. Mineralizing cultures of MC3T3-E1 cells were examined with or without FOXO1 knockdown by RNAi. Normal cells show the upregulation of FOXO1 and RUNX2 DNA binding activity, alkaline phosphatase activity, and mRNA levels of FOXO1, RUNX2, type 1 collagen, osteocalcin and MMP13 during formation of mineralizing nodules. In FOXO1 depleted cells each of these measurements was significantly reduced compared to values in control cells transfected with scrambled siRNA ($P< 0.05$). Depletion of FOXO1 also reduced the number of mineralized nodules formed. Moreover, chromatin immunoprecipitation assays revealed a direct interaction of FOXO1 with the RUNX2 promoter. Overexpression of FOXO1 reduced MC3T3-E1 cell number and the number of PCNA positive cells with little effect on apoptosis. These findings indicate that FOXO1 plays an important role in promoting osteoblast differentiation and suppressing proliferation in differentiating cells.

Keywords

Bone; differentiation; forkhead; mineralizing matrix; osteoblast; RUNX2; transcription factor; ChIP; alkaline phosphatase; FOXO1; mineralizing culture

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Introduction

Osteoblasts are bone forming cells that are responsible for mineralization of the organic matrix [1]. The commitment of the differentiation of mesenchymal cells to osteoblasts is an important aspect of skeletal development and bone growth. Bone formation is a well organized process that involves recruitment of pluripotent mesenchymal cells, commitment, proliferation, growth arrest and osteoblast differentiation [2]. Osteoblast differentiation is controlled by external signals that induce a cascade that results in terminally differentiated osteoblasts [3]. Bone morphogenetic proteins are important members of the TGF-β superfamily which stimulate the migration and proliferation of mesenchymal progenitors, as well as commitment into the chondrogenic, osteogenic or adipogenic lineage. For example, low concentrations of BMP2 favour adipocyte differentiation and, in contrast, high concentrations of BMP2 favor chondrocyte and osteoblast differentiation [4, 5]. BMP2 works through the activation of SMADs which regulate the transcription of osteogenic genes such as Runt-related transcription factor 2 (RUNX2) [6]. RUNX2 determines the lineage of osteoblasts from multipotent mesenchymal cells, inducing osteoblast differentiation at early stages [7, 8]. RUNX2 has been shown in numerous studies to be crucial for osteoblast differentiation by regulating the expression of various osteoblastic genes such as type I collagen, osteopontin, and osteocalcin [8].

A potentially important transcription factor in regulating formation of a mineralizing matrix is forkhead-01 (FOXO1), a member of the forkhead-O family of transcription factors that include FOXO1, FOXO3, FOXO4 and FOXO6 [9]. FOXO1 and FOXO3 in particular have similar functions and are present in a wide range of cellular and developmental processes [9]. FOXO1 is activated in bone lining cells under conditions of inflammation and mediates cytokine induced apoptosis of osteoblastic cells [10]. A linkage between FOXO1 and osteoblast activity has been suggested indirectly by evidence that BMP-2 induces a two fold increase in FOXO1 mRNA levels in human mesenchymal stem cells, and that transfection of FOXO1 increased the reporter activity of an alkaline phosphatase promoter construct [11, 12]. More recently FOXO1 has been shown to play an important role in bone formation in vivo, although the mechanism through which this occurs has not been settled. In one publication, genetic deletion of FOXO1 suggested that FOXO1 promotes bone formation by modulating proliferation of osteoblastic precursors but not by affecting differentiation [13]. In another publication, deletion of FOXO1, FOXO3 and FOXO4 suggested that these factors promote bone formation in part by modulating differentiation of osteoblastic cells [14]. It is possible that deletion of FOXO1 alone led to compensatory changes in vivo, accounting for the difference in phenotype. To clarify the role of FOXO1 in differentiation, we undertook experiments to study the effects of FOXO1 knockdown on the formation of mineralizing cultures in vitro with particular attention to the impact on RUNX2.

Methods

Cell Culture

MC3T3-E1 cells (murine osteoblastic cells) were purchased from ATCC (Rockville, MD) and grown in α-MEM (HyClone, South Logan, UT) containing 10% fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA), 1% Penicillin/Streptomycin (Cellgro, Manassas, VA) and 1% non-essential amino acids (Cellgro) at 37° C in a humidified atmosphere of 5% CO_2 in air. To induce mineralization, the media was supplemented with 50 μg/ml of ascorbic acid (Fisher Biotech, Waltham, MA) and 10 mM of β-glycerolphosphate (Invitrogen, Carlsbad, CA) as described elsewhere [15]. In time course experiments day 0 was defined as the time just prior to incubation in mineralizing media and cells were incubated for up to 21 days in mineralizing media.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation assays were performed with the EZ ChIP™ system (Upstate Cell Signaling Solutions, Lake Placid, NY), according to manufacturer's directions, modified to include pre-clearing of lysates with 60 μl of a 50% slurry of protein G/salmon sperm DNA for 1hr at 4° C, and again overnight. The Runx2 promoter primer sequences used were Fwd: AACCTTCTGAATGCCAGGAA and Rev: GTGGGACTGCCTACCACTGT. FOXO1 and SP1 specific antibodies and control IgG were obtained from Santa Cruz Biotechnology, INC. (Santa Cruz). PCR was performed for 40 cycles at 30s at 95°C, 30s at 72°C and 30s at 55°C.

Transient Transfection

MC3T3-E1 cells were plated in 6 well plates and cultured for 2 days in mineralizing media. Transfection was carried out in cells approximately 70% confluent with the use 5 nM FOXO1 siRNA (target sequence: CCA GCT ATA AAT GGA CAT TTA) or scrambled siRNA (Qiagen, Valencia, CA) in media containing 0.35% FBS and HiPerFect Transfection Reagent (Qiagen) for 24 hours and then changed to standard mineralizing media. For mineralized nodules formation, MC3T3-E1 cells were transfected with FOXO1 siRNA or scrambled siRNA without previous exposure to mineralizing media and maintained in regular conditions for one week. They were then exposed to mineralizing media for another 2 weeks. In some experiments MC3T3-E1 cells were transfected with constructs containing the full length open reading frame of human FOXO1, FOXO1AAA that has mutated Akt phosphorylation sites so that it is constitutively active, pcDNA empty vector control, or pcDNA expressing green fluorescent protein. Plasmids were purchased from Addgene. MC3T3-E1 cells were plated in 4 chamber slides (10^4/chamber) and transfected with 1μg plasmid using Lipofectamine 2000 (Invitrogen). At the indicated time point cells were fixed in 4% paraformaldehyde and visualized for green fluorescent protein or nuclei. Transfection efficiency was greater than 80% (data not shown). Cells were stained with DAPI to determine cell number or incubated with antibody to PCNA purchased from (Santa Cruz Biotechnology (Santa Cruz, CA). The number of PCNA positive cells was determined following incubation with a biotinylated secondary antibody (Millipore, Billerica, MA) and streptavidin Alexa 546 (Invitrogen). In some experiments apoptosis was detected using a Dead End Fluorometric TUNEL kit (Promega, Madison, WI) following the manufacturer's instructions. Images were captured using Nikon Eclipse 90i Instrument (Nikon Instruments Inc., Melville, NY). Cells were counted using NIS-Elements Microscope Imaging Software (Nikon Instruments Inc.) in a blind fashion.

Lentivirus

Lentiviral constructs utilized the third generation, self-inactivating, replication incompetent lentiviral backbone vector pLVTHM, [16], a generous gift of the laboratory of Prof. Didier Trono (University of Geneva, Switzerland). The pLVTHM vector was modified for knockdown of FoxO1 by a short hairpin RNA (shRNA) designed to target the FoxO1 cDNA sequence CGGAGGATTGAACCAGTATAA. A second control shRNA without known mammalian gene target) was designed as a negative (non-targeting) control, TGACGACGAGCGCTCCTACAG. Vesicular stomatitis virus (VSV) pseudotyped lentiviral particles were generated by transfection of 293T cells with the lentiviral back-bone construct together with four helper plasmids encoding the viral genes Gag-Pol, Tat, Rev, and VSV-G [17]. Titers of fluorochrome-expressing lentiviruses were calculated as “293-transducing units” per ml (TU/ml) based on flow cytometry of infected 293 cells. MC3T3-E1 cells were maintained for 2 days in mineralizing media, and then infected at a multiplicity of infection (MOI) of 40 for 6 hours using hexadimethrine bromide after which cells continued incubation in mineralizing culture media. In some experiments cells were checked 96 hours after transduction for expression of green fluorescence protein (GFP) to

observe the efficiency of the transduction. Cells were maintained in mineralizing media for a total of 2 weeks.

Protein Extraction and Function

At specific time points cytoplasmic and nuclear proteins were extracted with the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockland, IL) and Halt Protease Inhibitor Cocktail (Pierce Biotechnology) following the manufacturer's instructions. Protein concentrations were determined by BCA (Pierce Biotechnology). The nuclear fraction was assayed for FOXO1 and RUNX2 DNA binding activity (Active Motif) following the manufacturer's instructions. The cytoplasmic fraction was tested for alkaline phosphatise activity using AP assay reagent A (GenHunter Corporation, Nashville, TN).

RNA Isolation and quantification by Real-time PCR

During selected time points total RNA was extracted and purified using QIAshredder spin columns (Qiagen, Valencia, CA) and the RNeasy Mini Kit (Qiagen), following the manufacturer's instructions. Reverse transcriptase reaction was performed with the use of MultiScribe Reverse Transcriptase Kit purchased from Applied Biosystems (Foster City, CA) and Real-time PCR was carried out with gene specific primers and normalized by the housekeeping gene RPL32 (Applied Biosystems). Osteocalcin mRNA levels were assessed with SYBR Green labeled primers with the sequence: 5′-GCAATAAGGTAGTGAACAGACTCC-3′ and 5′-AGCAGGGTTAAGCTCACACTG-3′ and was normalized to ß-actin with the sequence: 5′-GCTCTTTTCCAGCCTTCCTT-3′ and 5′-AGGTC TTTACGGATGTCAACG-3. An Osteogenesis Focused DNA Microarray (Oligo GEArray), purchased from SABiosciences (Frederick, MD) was carried out to characterize gene expression.

Mineralized Nodule Formation

After 21 days in culture cells were fixed with 10% phosphate-buffered formalin (Electron Microscopy Sciences, Hatfield, PA) and stained with alizarin red [18, 19]. The nodule number and size were analyzed by Image Pro Plus software (MediaCybernetics, Bethesda, MD). Alizarin red staining was eluted and the acid soluble calcium measured at OD 415 nm.

Statistical Analysis

Statistical analysis was assessed by One Way ANOVA with Scheffe's post-hoc for comparisons between multiple groups and students t-test test for comparisons between two groups, with significance set at $P < 0.05$. The data are presented as the fold maximum and expressed as mean ± standard error (SEMs). In all assays, three independent experiments were carried out with the exception of the chromatin immunoprecipitation assay that was performed twice independently with consistent results. For microarrays three independent sets of cells were stimulated, RNA was combined and three sets of microarrays were carried out separately.

Results

To explore the role of FOXO1 in osteoblast differentiation, FOXO1 DNA binding and FOXO1 mRNA were examined in MC3T3-E1 cells cultured under mineralizing conditions (Fig. 1A and B). FOXO1 mRNA levels were not significantly higher on day 7 but were increased 2.2 fold ($P < 0.05$) after 14 days when compared to day zero. FOXO1 DNA binding activity to an oligonucleotide consensus sequence, measured by ELISA, increased 1.7 fold on day 7 and 2.5 fold on day 14 ($P < 0.05$) when compared to day zero. To functionally examine the impact of FOXO1, RNAi experiments were carried out. Pilot

studies were conducted to determine both the length of time and degree of knockdown that could be achieved, and a dose of 5 nM of siRNA was selected (data not shown). FOXO1-specific siRNA when compared to scrambled siRNA reduced DNA binding by 50% (P <0.05) after 14 days. The positive control, excess unlabelled probe, reduced FOXO1 DNA binding by 62% (P <0.05), while non-specific probe had no effect (P >0.05). FOXO1 siRNA reduced FOXO1 mRNA levels by 48% on day 14 (P <0.05) (Fig.1B). Thus, FOXO1 siRNA had a significant effect for a relatively long period of time consistent with other reports that siRNA can cause extended knockdown of target genes including mineralizing cultures of MC3T3 cells [19, 20].

Since RUNX2 plays an important role in osteoblast differentiation the effect of FOXO1 knock down on RUNX2 DNA binding activity and mRNA levels were examined. MC3T3-E1 cells were transfected with FOXO1 siRNA or scrambled siRNA and nuclear protein and total RNA was obtained. RUNX2 DNA binding activity was approximately 2 fold increased at 14 days when compared to day zero (P <0.05), which decreased approximately 50% with FOXO1 knockdown (P <0.05) (Fig.2A). When the mRNA level of RUNX2 was accessed, an increase of 2.5 fold was found on day 7 (P <0.05) and an increase of 7 fold (P <0.05) was obtained on day 14 when compared to day zero. When FOXO1 was knocked down, RUNX2 expression decreased 55% (P <0.05) on day 14 when compared to scrambled siRNA (Fig. 2B).

To determine whether FOXO1 targets the RUNX2 gene directly during differentiation, the endogenous RUNX2 promoter was probed in chromatin immunoprecipitation (ChIP) assays in differentiating MC3T3-E1 cells. Promoter occupation by the transcription factor Sp1 was used as a reference point based on a recent report that it associates with the RUNX2 promoter during the early, proliferative phase of these cultures [21]. Sp1 was present on the RUNX2 promoter in the committed pre-osteoblast stage prior to differentiation (day 0), but not at later time points (days 7 and 14). In contrast FOXO1 association with the RUNX2 promoter was not detected at the zero time point. However, there was interaction on days 7 and 14 consistent with an association during differentiation. These findings reveal important dynamics in RUNX2 targeting by FOXO1.

Alkaline phosphatase activity and mRNA levels of type I collagen, osteocalcin and MMP13 were examined since they are known to be highly expressed in differentiated osteoblasts. Alkaline phosphatase activity showed an increase of 2 fold on day 7 (P <0.05) and 3.5 fold on day 14 (P <0.05) when compared to day zero. The effect of FOXO1 knockdown on day 7 was not significant but FOXO1 siRNA reduced alkaline phosphatase activity by 53% (P <0.05) on day 14 compared to scrambled siRNA (Fig.3A). At the mRNA level, type I collagen exhibited at 3.5 fold increase on day 14 (P <0.05) when compared to day zero and was reduced by 40% (P <0.05) on day 14 by FOXO1 siRNA when compared to scrambled siRNA (Fig. 3B). Osteocalcin mRNA levels increased 50 fold (P <0.05) on day 14 and the knockdown of FOXO1 reduced osteocalcin mRNA levels on day 14 by 40% (P <0.05) (Fig. 3C). MMP13 mRNA levels increased ~50 fold (P <0.05) on day 14 and were reduced by 44% (P <0.05) in cultures incubated with FOXO1 siRNA when compared to scrambled siRNA (Fig. 3D).

mRNA levels of a number of genes that reflect osteoblast differentiation were measured in a focused osteogenesis microarray. MC3T3-E1 cells were cultured in mineralizing media for 14 days and compared to cells collected at day zero to determine changes in osteogenic gene expression in mineralizing cultures. To examine the role of FOXO1 in osteoblast differentiation, day 14 cultures that had been transfected with FOXO1 siRNA were compared to those transfected with scrambled siRNA (Table 1). The results showed that 80% of the genes present in the array were up-regulated in the mineralized cultures when

compared to day zero and it included genes that control osteoblast differentiation such as transcription factors RUNX2, SMAD5 and SMAD7 and bone morphogenetic proteins BMP2, BMP5 and BMP8a, as well as genes that reflect differentiated osteoblast function such as extracellular matrix proteins MMP13, MMP2, MMP9, Col4a4, Col4a3, Col1a1 and Col9a1. Induction of many of these genes was impaired in FOXO1-depleted cells, including RUNX2, SMAD5, SMAD7, BMP2, BMP5, BMP8a, MMP13, MMP9, Col4a4, Col4a3, Col1a1 and Col9a1, which states the significance of FOXO1 in the regulation of osteogenic gene expression during osteoblast differentiation. A broad range of osteogenic genes is dependent on FOXO1, and FOXO1 acts directly at the major control point represented by RUNX2 induction.

MC3T3-E1 cells have the capacity to form mineralized nodules upon stimulation with ascorbic acid and β-glycerolphosphate [22, 23]. MC3T3-E1 cells were transfected with FOXO1 siRNA or scrambled siRNA and treated in mineralizing media as described above. Mineralized nodules were assessed after 21 days in culture by staining with alizarin red. The stain was eluted and the acid soluble calcium bound dye was quantified (Fig. 4A). Nodules were formed as expected by the mineralization group. Scrambled siRNA had no effect on the formation of a calcified matrix. In contrast, FOXO1 siRNA reduced mineralized matrix formation by approximately 50% when compared to scrambled siRNA (P <0.05). In parallel, the number of nodules was counted; reflecting differentiation of precursors to mature osteoblastic cells (Fig. 3B), the total nodule area was measured (Fig. 3C) and the area per nodule was accessed to reflect the impact on growth of nodules (Fig. 3D). Results indicate that FOXO1 knockdown reduced the number of nodules by approximately 50% and the total area of mineralized nodules was also reduced by approximately 50% (P <0.05). However, no difference was found in the average area per nodule with FOXO1 knock down (P >0.05).

A second approach was taken to confirm the sustained knock down of FOXO1 after 14 days using stable transduction with lentiviral constructs that expressed FOXO1 shRNA or scrambled shRNA. MC3T3-E1 cells were transduced with lentiviral vector and incubated in mineralizing media. GFP was detected only in transduced cells and showed transduction efficiency close to 100% (Fig. 5A). mRNA was examined for real-time PCR to assess the expression levels of FOXO1 knock down as well as the impact of FOXO1 knockdown on RUNX2 and osteocalcin mRNA levels (Fig. 5B-D). FOXO1 mRNA levels increased 1.7 fold (P <0.05) after 14 days in mineralizing media and were 52% decreased (P <0.05) with FOXO1 shRNA (Fig.5B). When RUNX2 was tested an increase of 3 fold was obtained after 14 days of culture in mineralizing media (P <0.05) and a reduction of 51% by FOXO1 shRNA when compared to scrambled shRNA (P <0.05) (Fig.5C). Osteocalcin showed an increase of 100 fold after 14 days of culture in mineralizing media and a reduction of 69% by FOXO1 shRNA when compared to scrambled shRNA (P <0.05) (Fig.5D).

Since FOXO1 has been reported to affect proliferation by inhibiting DNA synthesis we examined the effect of FOXO1 over-expression on MC3T3 cell number (Fig 6). Cells were transfected with a vector expressing GFP as a negative control or a plasmid expressing wild-type or mutated FOXO1 that was constitutively active, FOXO1AAA. Transfection with control GFP plasmid resulted in a decreased number of cells on days 5 and 7, most likely due to the effects of transfection per se. Cells with over-expression of GFP increased 140% over this time period while cells transfected with FOXO1 increased only 43% and with FOXO1AAA only 15%. Thus FOXO1 significantly reduced cell number. To distinguish whether changes in cell number were due to effects of FOXO1 on DNA synthesis versus apoptosis the number of PCNA positive and TUNEL positive cells was determined (Table 2). The number of PCNA positive cells were reduced approximately 90% when cells were

transfected with FOXO1 or FOXO1AAA compared to vector control (P <0.05). In contrast, FOXO1 overexpression had no significant effect on the number of TUNEL positive cells.

Discussion

MC3T3-E1 pre-osteoblastic cells undergo differentiation and formation of a mineralizing matrix. These cell cultures are frequently used to investigate the role of specific genes in osteoblast differentiation. In this study, knocking down FOXO1 with siRNA or by viral transduction with shRNA showed that FOXO1 plays an important role in promoting osteoblast differentiation. When FOXO1 was depleted, multiple markers of osteoblast differentiation were significantly reduced including mRNA levels of collagen 1, RUNX2, osteocalcin and MMP13. FOXO1 siRNA significantly reduced the up-regulation of alkaline phosphatase activity that occurs as a result of osteoblast differentiation. These results are unlikely to be due to global changes since FOXO1 knockdown did not affect that overall protein or total RNA levels per well (data not shown). Additional evidence that FOXO1 promotes osteoblast differentiation is the reduction in the number of mineralized nodules that formed when FOXO1 was targeted by siRNA. This biological parameter reflects terminal differentiation of osteoblasts from precursors.

FOXO1 knockdown sharply reduced RUNX2 mRNA levels, indicating that FOXO1 acts upstream of RUNX2 induction. The possibility that the biochemical mechanism of FOXO1 effects on osteoblast differentiation includes direct targeting of the RUNX2 gene was confirmed in ChIP assays, which indicate that FOXO1 targets the RUNX2 promoter directly, and with dynamics that correlate specifically with induction of differentiation. The reduction of RUNX2 mRNA levels was accompanied by a decrease in RUNX2 DNA binding activity, implying that direct targeting of downstream osteogenic genes by RUNX2 is impaired concordant with depletion of FOXO1. The results do not rule out the possibility that there are multiple mechanisms by which FOXO1 directly or indirectly modulates RUNX2 activity and expression of osteogenic genes, but they establish an early, direct, and essential role for FOX01 in osteoblast differentiation. This is underscored by the relatively large number of genes that were modulated by FOXO1 knockdown, which included transcription factors, enzymes associated with differentiated function, bone morphogenetic proteins, and extracellular matrix proteins.

FOXO1 has been shown to affect differentiation of adipocytes [24, 25]. Constitutively active FOXO1 inhibits the differentiation of pre-adipocytes into mature adipocytes, while a dominant-negative FOXO1 enhances adipocyte differentiation [24]. Interestingly, adipocytes and osteoblasts share similar precursors and tend to be differentially regulated so that inhibition of adipocyte differentiation is associated with promotion of osteoblast differentiation [26]. Thus, FOXO1 could preferentially modulate precursors toward an osteoblastic phenotype and away from an adipocyte pathway.

We found that upregulated expression of osteocalcin during osteoblast differentiation was significantly reduced when FOXO1 was targeted by siRNA or shRNA. This is consistent with a report that FOXO1 regulates osteocalcin expression and thereby affects energy regulation [27]. FOXO1 has previously been reported to contribute to apoptosis of bone-lining cells. Activation of a vigorous immune response in close proximity to bone leads to FOXO1-mediated apoptosis of bone cells [28], with a net negative effect on bone formation. Deletion of FOXO1, FOXO3 and FOXO4 has recently been reported to inhibit osteoblast differentiation, indirectly suggesting that FOXO1 has a positive effect on promoting differentiation (14). Our results provide direct support for this concept, showing that knockdown of FOXO1 interferes with differentiation of osteoblastic cells from committed precursors. Another publication has indicated that FOXO1 promotes bone formation by

protecting osteoblasts from oxidative stress and increases osteoblast proliferation (13). However, we found that FOXO1 overexpression in MC3T3-E1 cells reduced proliferation. It is possible that this apparent discrepancy is due to the approach used or that FOXO1 has different effects on proliferation depending on the degree of osteoblast differentiation. It should be noted that in most cell types FOXO1 inhibits proliferation [29]. In summary, the present study suggests that FOXO1 is an important transcription factor that interacts with the RUNX2 promoter and promotes osteoblast differentiation and differentiation-associated cessation of proliferation.

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References

1. Gerstenfeld L, Cullininane D, Barnes G, Graves D, Einhorn T. Fracture healing as a post-natal developmental process: molecular, spatial, adn temporal aspects of its regulation. J Cell Biochem. 2003; 88:873–84. [PubMed: 12616527]
2. Ai-Aql ZS, Alagl AS, Graves DT, Gerstenfeld LC, Einhorn TA. Molecular mechanisms controlling bone formation during fracture healing and distraction osteogenesis. J Dent Res. 2008; 87:107–18. [PubMed: 18218835]
3. Yamaguchi A, Komori T, Suda T. Regulation of osteoblast differentiation mediated by bone morphogenetic proteins, hedgehogs, and Cbfa1. Endocr Rev. 2000; 21:393–411. [PubMed: 10950158]
4. Senta H, Park H, Bergeron E, Drevelle O, Fong D, Leblanc E, Cabana F, Roux S, Grenier G, Faucheux N. Cell responses to bone morphogenetic proteins and peptides derived from them: biomedical applications and limitations. Cytokine Growth Factor Rev. 2009; 20:213–22. [PubMed: 19493693]
5. Wang EA, Israel DI, Kelly S, Luxenberg DP. Bone morphogenetic protein-2 causes commitment and differentiation in C3H10T1/2 and 3T3 cells. Growth Factors. 1993; 9:57–71. [PubMed: 8347351]
6. Jonason JH, Xiao G, Zhang M, Xing L, Chen D. Post-translational Regulation of Runx2 in Bone and Cartilage. J Dent Res. 2009; 88:693–703. [PubMed: 19734454]
7. Ji C, Casinghino S, Chang D, Chen Y, Javed A, Ito Y, Hiebert S, Lian J, Stein G, McCarthy T, Centrella M. CBFa(AML/PEBP2)-related elements in the TGF-beta type I receptor promoter and expression with osteoblast differentiation. J Cell Biochem. 1998; 69:353–63. [PubMed: 9581873]
8. Komori T. Regulation of bone development and extracellular matrix protein genes by RUNX2. Cell Tissue Res. 2010; 339:189–95. [PubMed: 19649655]
9. van der Vos KE, Coffer PJ. FOXO-binding partners: it takes two to tango. Oncogene. 2008; 27:2289–99. [PubMed: 18391971]
10. Behl Y, Siqueira M, Ortiz J, Li J, Desta T, Faibish D, Graves DT. Activation of the acquired immune response reduces coupled bone formation in response to a periodontal pathogen. J Immunol. 2008; 181:8711–8. [PubMed: 19050291]
11. Osyczka AM, Diefenderfer DL, Bhargave G, Leboy PS. Different effects of BMP-2 on marrow stromal cells from human and rat bone. Cells Tissues Organs. 2004; 176:109–19. [PubMed: 14745240]
12. Hatta M, Daitoku H, Matsuzaki H, Deyama Y, Yoshimura Y, Suzuki K, Matsumoto A, Fukamizu A. Regulation of alkaline phosphatase promoter activity by forkhead transcription factor FKHR. Int J Mol Med. 2002; 9:147–52. [PubMed: 11786925]
13. Rached MT, Kode A, Xu L, Yoshikawa Y, Paik JH, Depinho RA, Kousteni S. FoxO1 is a positive regulator of bone formation by favoring protein synthesis and resistance to oxidative stress in osteoblasts. Cell Metab. 2010; 11:147–60. [PubMed: 20142102]
14. Ambrogini E, Almeida M, Martin-Millan M, Paik JH, Depinho RA, Han L, Goellner J, Weinstein RS, Jilka RL, O'Brien CA, Manolagas SC. FoxO-mediated defense against oxidative stress in

osteoblasts is indispensable for skeletal homeostasis in mice. Cell Metab. 2010; 11:136–46. [PubMed: 20142101]

15. Hong D, Chen HX, Yu HQ, Liang Y, Wang C, Lian QQ, Deng HT, Ge RS. Morphological and proteomic analysis of early stage of osteoblast differentiation in osteoblastic progenitor cells. Exp Cell Res. 2010; 316:2291–300. [PubMed: 20483354]
16. Wiznerowicz M, Trono D. Conditional suppression of cellular genes: lentivirus vector-mediated drug-inducible RNA interference. J Virol. 2003; 77:8957–61. [PubMed: 12885912]
17. Mostoslavsky G, Kotton DN, Fabian AJ, Gray JT, Lee JS, Mulligan RC. Efficiency of transduction of highly purified murine hematopoietic stem cells by lentiviral and oncoretroviral vectors under conditions of minimal in vitro manipulation. Mol Ther. 2005; 11:932–40. [PubMed: 15922964]
18. Lee ER. Proteolytic enzymes in skeletal development: histochemical methods adapted to the study of matrix lysis during the transformation of a “cartilage model” into bone. Front Biosci. 2006; 11:2538–53. [PubMed: 16720332]
19. Kanazawa I, Yamaguchi T, Yano S, Yamauchi M, Yamamoto M, Sugimoto T. Adiponectin and AMP kinase activator stimulate proliferation, differentiation, and mineralization of osteoblastic MC3T3-E1 cells. BMC Cell Biol. 2007; 8:51. [PubMed: 18047638]
20. Raskopf E, Vogt A, Sauerbruch T, Schmitz V. siRNA targeting VEGF inhibits hepatocellular carcinoma growth and tumor angiogenesis in vivo. J Hepatol. 2008; 49:977–84. [PubMed: 18845354]
21. Zhang Y, Hassan MQ, Xie RL, Hawse JR, Spelsberg TC, Montecino M, Stein JL, Lian JB, van Wijnen AJ, Stein GS. Co-stimulation of the bone-related Runx2 P1 promoter in mesenchymal cells by SP1 and ETS transcription factors at polymorphic purine-rich DNA sequences (Y-repeats). J Biol Chem. 2009; 284:3125–35. [PubMed: 19017640]
22. Barbara A, Delannoy P, Denis BG, Marie PJ. Normal matrix mineralization induced by strontium ranelate in MC3T3-E1 osteogenic cells. Metabolism. 2004; 53:532–7. [PubMed: 15045704]
23. Sudo H, Kodama HA, Amagai Y, Yamamoto S, Kasai S. In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J Cell Biol. 1983; 96:191–8. [PubMed: 6826647]
24. Nakae J, Kitamura T, Kitamura Y, Bigss Wr, Arden K, Accili D. The forkhead transcription factor Foxo1 regulates adipocyte differentiation. Dev Cell. 2003; 4:119–29. [PubMed: 12530968]
25. Gerin I, Bommer GT, Lidell ME, Cederberg A, Enerback S, Macdougald OA. On the role of FOX transcription factors in adipocyte differentiation and insulin-stimulated glucose uptake. J Biol Chem. 2009; 284:10755–63. [PubMed: 19244248]
26. Takada I, Kouzmenko AP, Kato S. Wnt and PPARgamma signaling in osteoblastogenesis and adipogenesis. Nat Rev Rheumatol. 2009; 5:442–7. [PubMed: 19581903]
27. Rached MT, Kode A, Silva BC, Jung DY, Gray S, Ong H, Paik JH, DePinho RA, Kim JK, Karsenty G, Kousteni S. FoxO1 expression in osteoblasts regulates glucose homeostasis through regulation of osteocalcin in mice. J Clin Invest. 2010; 120:357–68. [PubMed: 20038793]
28. Behl Y, Krothapalli P, Desta T, Dipiazza A, Roy S, Graves DT. Diabetes-enhanced tumor necrosis factor-{alpha} production promotes apoptosis and the loss of retinal microvascular cells in type 1 and type 2 models of diabetic retinopathy. Am J Pathol. 2008; 172:1411–8. [PubMed: 18403591]
29. Huang H, Tindall DJ. Dynamic FoxO transcription factors. J Cell Sci. 2007; 120:2479–87. [PubMed: 17646672]

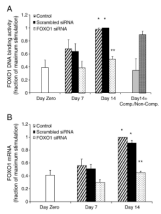

Figure 1. FOXO1 DNA binding activity and mRNA levels are increased in mineralizing cultures of osteoblastic cells and knocked down by FOXO1 siRNA

MC3T3-E1 cells were cultured under mineralizing conditions and transfected with either FOXO1 specific or scrambled siRNA. Nuclear protein or total RNA was isolated 0, 7 and 14 days later. (A) FOXO1 DNA binding activity was measured. A subset of the 14 day samples included excess competitive oligonucleotide (comp.) or excess non-competitive oligonucleotide (non-comp.). (B) FOXO1 mRNA levels were measured by real time PCR and normalized to ribosomal protein L32. The data presented are the means of three independent experiments ± SEM and are shown as the fraction of maximum stimulation. Asterisks indicate significantly increased values compared to day zero and double asterisks indicate significant reduction of FOXO1siRNA transfected cells compared to scrambled siRNA ($P < 0.05$).

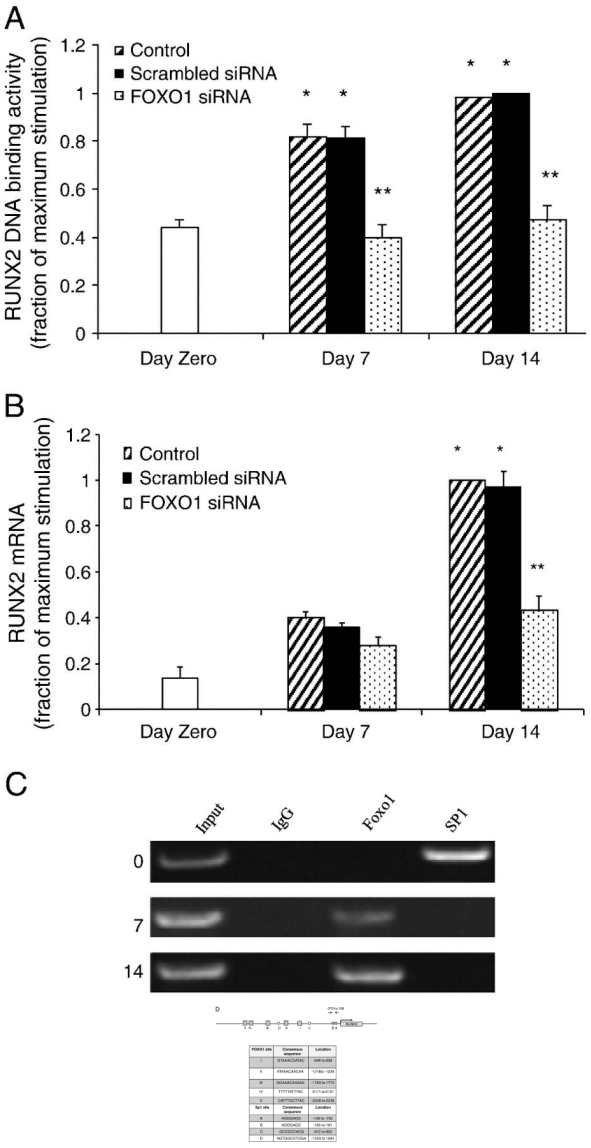

Figure 2. RUNX2 levels are reduced by FOXO1 knockdown and FOXO1 interacts with the RUNX2 promoter

MC3T3-E1 cells were cultured under mineralizing conditions and transfected with either FOXO1 specific or scrambled siRNA. After 0, 7 and 14 days nuclear protein and total RNA were extracted. (A) RUNX2 DNA binding was assessed. (B) RUNX2 mRNA levels were measured by real time PCR and normalized to ribosomal protein L32. For (A) and (B) the data presented are the mean of three independent experiments ± SEM and are shown as the fraction of maximum stimulation. Asterisks indicate significantly increased values compared to day zero and double asterisks indicate significant reduction of cells transfected with FOXO1 siRNA compared to scrambled siRNA ($P < 0.05$). (C) DNA was isolated from MC3T3-E1 cells after 0, 7, and 14 days of culture in mineralizing media. Nuclear lysates were examined by chromatin immunoprecipitation (ChIP) with antibodies to FOXO1 and Sp1 as indicated. Input DNA and DNA immunoprecipitated with non-specific IgG were included as positive and negative controls, respectively. The results are representative of two independent experiments. (D) Schematic representation of the 5′ untranslated region of murine RUNX2. The numbering system was adapted from reference 21. The promoter

sequence was analyzed using the PROMO website. (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) Squares represent the location of five predicted FOXO1 binding sites (I –V). The open circles (C,D) represent the location of SP1 binding sites predicted by PROMO. The shaded circles (A,B) represent additional potential SP1 sites (21). Arrows indicate the location of the primers used in the ChIP analysis.

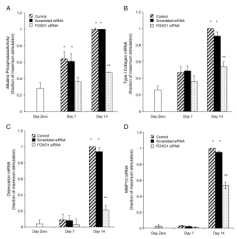

Figure 3. Knockdown of FOXO1 inhibits up-regulation of markers of osteoblast differentiation

MC3T3-E1 cells were maintained under mineralizing conditions and transfected with either FOXO1 specific or scrambled siRNA. After 0, 7 and 14 days cytoplasmic protein and total RNA were obtained. (A) Alkaline phosphatase activity was measured. (B-D) mRNA levels of Type 1 collagen, osteocalcin, and MMP13 were analyzed by real time PCR and normalized to ribosomal protein L32. The data presented are the mean of three independent experiments ± SEM and are shown as the fraction of maximum stimulation. Asterisks indicate significantly increased values compared to day zero and double asterisks indicate significant reduction compared to cells treated with scrambled siRNA ($P < 0.05$).

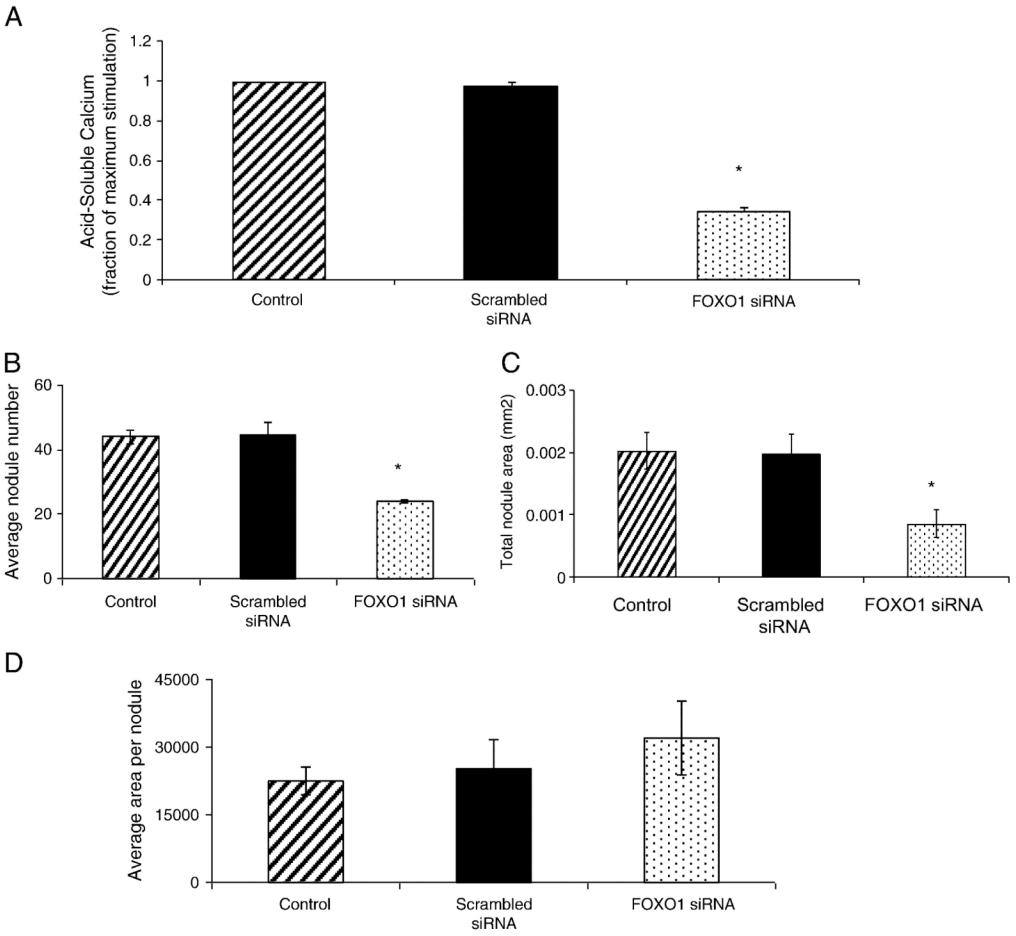

Figure 4. The formation of mineralized nodules is reduced by FOXO1 knockdown

(A-D) MC3T3-E1 cells were transfected with either FOXO1 specific or scrambled siRNA and cultured under mineralizing conditions. After 21 days cells were stained with Alizarin red. (A) Acid soluble-calcium was measured spectrophotometrically and is presented as the fraction of maximum stimulation. (B) The total number of nodules was counted. (C) The total nodule area was measured. (D) The average area per nodule was quantified. The data are the mean of three independent experiments ± SEM. Asterisks indicate significantly reduced values compared to cells treated with scrambled siRNA ($P < 0.05$).

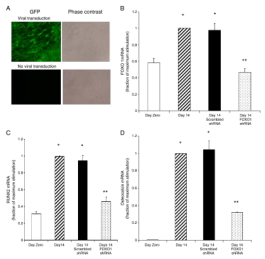

Figure 5. Lentiviral mediated FOXO1 shRNA reduces FOXO1, RUNX2 and osteocalcin mRNA levels

MC3T3-E1 cells were transduced with lentiviral constructs expressing GFP and FOXO1 shRNA or scrambled shRNA. After 14 days of culture in mineralizing media GFP was detected by immunofluorescence and total RNA isolated. The mRNA levels of FOXO1, RUNX2 and osteocalcin were measured by real time PCR and normalized to ribosomal protein L32. (A) GFP detection in transduced cells. (B) FOXO1 mRNA levels. (C) RUNX2 mRNA levels. (D) Osteocalcin mRNA levels. The data presented are the mean of three independent experiments ± SEM and are shown as the fraction of maximum stimulation. Asterisks indicate significantly increased values compared to day zero and double asterisks indicate significant reduction.

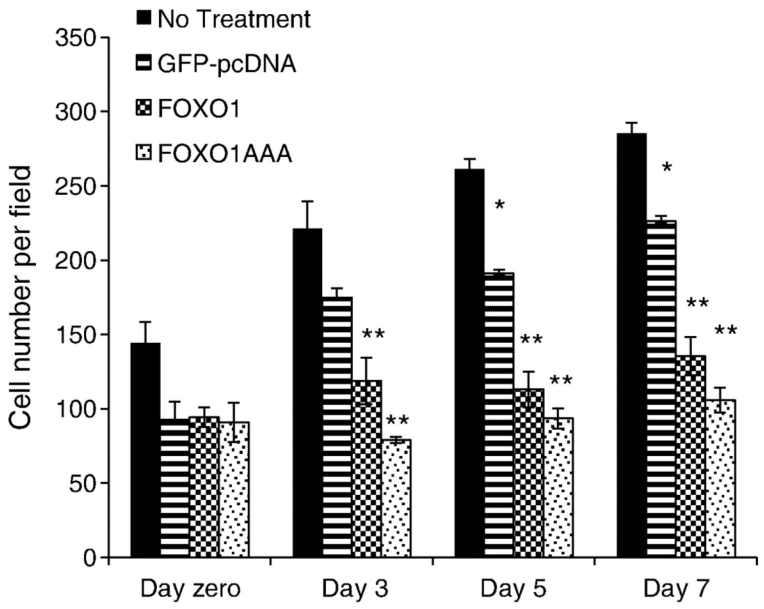

Figure 6. Overexpression of FOXO1 reduces proliferation

MC3T3-E1 cells were transfected with GFP-pcDNA, FOXO1 or FOXO1AAA and cultured from 0 to 7 days. Cells were stained with DAPI and the number of cells per field was counted with a fluorescence microscope. The data presented are the mean of three independent experiments ± SEM. Asterisks indicate significantly increased values compared to no treatment, double asterisks indicate significant reduction compared to cells treated with transfected with GFP-pcDNA ($P < 0.05$).

Table 1

Focused osteogenic microarray

	Fold Change Induced by Differentiation		Fold Change in FOXO1 vs. Scrambled siRNA in Differentiated Cultures	
Gene Name	**Mean**	**Median**	**Mean**	**Median**
Mmp13	**5.40**	**4.99**	**0.41**	**0.49**
Itgav	**3.88**	**3.92**	**0.39**	**0.45**
Ibsp	**3.78**	**3.75**	0.92	0.82
Fgf2	**3.77**	**4.10**	**0.41**	**0.42**
Col11a1	**3.59**	**2.99**	**0.48**	**0.51**
Gdf10	**3.57**	**3.44**	**0.52**	**0.58**
Col12a1	**3.27**	**3.28**	**0.35**	**0.35**
Dcn	**3.23**	**3.13**	0.62	0.63
Col18a1	**3.15**	**2.86**	**0.44**	**0.45**
Itga3	**3.13**	**3.02**	**0.42**	**0.46**
Phex	**3.10**	**3.08**	**0.41**	**0.49**
Fgf1	**3.09**	**3.17**	**0.41**	**0.41**
Msx1	**3.06**	**2.49**	**0.41**	**0.39**
Col4a4	**2.99**	**2.82**	**0.37**	**0.38**
Col19a1	**2.93**	**2.72**	**0.39**	**0.39**
Bmp6	**2.91**	**2.92**	**0.37**	**0.35**
Bmp8b	**2.90**	**2.95**	**0.34**	**0.33**
Mmp8	**2.89**	**3.14**	**0.41**	**0.44**
Bmp2	**2.89**	**2.78**	**0.32**	**0.34**
Ambn	**2.84**	**2.93**	**0.41**	**0.37**
Csf2	**2.83**	**2.96**	**0.43**	**0.45**
Bmpr1a	**2.82**	**2.73**	**0.37**	**0.39**
Cd36	**2.82**	**2.86**	**0.38**	**0.38**
Fgfr1	**2.81**	**2.74**	**0.42**	**0.37**
Comp	**2.81**	**2.61**	**0.40**	**0.40**
Col2a1	**2.80**	**2.44**	**0.53**	**0.54**
Col4a3	**2.80**	**2.89**	**0.42**	**0.43**
Bmp5	**2.76**	**2.86**	**0.35**	**0.37**
Col14a1	**2.75**	**2.84**	**0.38**	**0.36**
Dmp1	**2.74**	**2.41**	**0.53**	**0.46**
Calcr	**2.73**	**2.85**	**0.36**	**0.38**
Bmp7	**2.70**	**2.67**	**0.40**	**0.39**
Itgam	**2.69**	**2.42**	**0.40**	**0.41**
Fgf3	**2.69**	**2.71**	**0.43**	**0.37**
Bmp8a	**2.68**	**2.54**	**0.40**	**0.43**
Bmpr1b	**2.67**	**2.60**	**0.35**	**0.34**
Egf	**2.66**	**2.58**	**0.40**	**0.42**
Fgfr3	**2.64**	**2.64**	**0.42**	**0.42**

	Fold Change Induced by Differentiation		Fold Change in FOXO1 vs. Scrambled siRNA in Differentiated Cultures	
Gene Name	**Mean**	**Median**	**Mean**	**Median**
Akp2	**2.62**	**2.26**	**0.50**	**0.59**
Col1a1	**2.61**	**2.63**	**0.43**	**0.44**
Col9a1	**2.58**	**2.68**	**0.49**	**0.56**
Col4a6	**2.58**	**1.82**	**0.47**	**0.50**
Dspp	**2.57**	**1.92**	**0.58**	**0.53**
Runx2	**2.56**	**2.57**	**0.44**	**0.43**
Col10a1	**2.54**	**2.11**	**0.51**	**0.51**
Enam	**2.54**	**2.33**	**0.49**	**0.46**
Mmp2	**2.50**	**3.04**	0.71	0.80
Smad7	**2.49**	**2.53**	**0.40**	**0.40**
Bmp1	**2.45**	**2.44**	**0.46**	**0.46**
Itga2	**2.43**	**2.43**	**0.45**	**0.50**
Tgfbr3	**2.42**	**1.87**	**0.54**	**0.64**
Itga2b	**2.40**	**1.70**	0.65	0.75
Col15a1	**2.36**	**2.34**	**0.54**	**0.45**
Smad9	**2.35**	**2.26**	**0.43**	**0.39**
Mmp9	**2.31**	**2.21**	**0.54**	**0.54**
Tnf	**2.30**	**2.14**	0.52	0.62
Fgfr2	**2.30**	**2.24**	**0.45**	**0.42**
Sost	**2.29**	**2.30**	**0.44**	**0.45**
Ctsk	**2.27**	**2.43**	0.65	0.41
Col6a2	**2.26**	**2.19**	0.61	0.45
Smad5	**2.26**	**2.21**	**0.44**	**0.42**
Smad6	**2.26**	**2.25**	**0.43**	**0.45**
Anxa5	**2.24**	**2.82**	0.82	0.75
Tgfb1	**2.24**	**2.29**	**0.43**	**0.40**
Bmp3	**2.23**	**2.16**	**0.45**	**0.53**
Icam1	**2.22**	**2.15**	**0.44**	**0.45**
Col7a1	**2.22**	**2.64**	0.50	0.45
Tgfbr1	**2.21**	**2.16**	**0.42**	**0.42**
Flt1	**2.20**	**1.84**	0.68	0.53
Col4a2	**2.17**	**1.73**	0.60	0.53
Vegfa	**2.14**	**1.93**	**0.48**	**0.47**
Bmp4	**2.12**	**2.29**	**0.51**	**0.56**
Tuft1	**2.12**	**2.14**	**0.51**	**0.41**
Col3a1	**2.12**	**1.83**	1.13	0.60
Smad4	**2.06**	**1.78**	**0.46**	**0.52**
Smad1	**2.00**	**1.87**	**0.44**	**0.44**
Csf3	**1.99**	**1.91**	0.57	0.62
Cdh11	**1.92**	**1.74**	0.70	0.63
Smad3	**1.91**	**1.71**	**0.54**	**0.55**

	Fold Change Induced by Differentiation		Fold Change in FOXO1 vs. Scrambled siRNA in Differentiated Cultures	
Gene Name	**Mean**	**Median**	**Mean**	**Median**
Tfip11	**1.91**	**1.87**	**0.46**	**0.46**
Twist2	**1.91**	**1.81**	**0.43**	**0.41**
Col4a1	1.90	1.53	0.73	0.70
Col9a3	1.90	1.29	0.69	0.77
Bmpr2	**1.85**	**1.72**	0.61	0.58
Fn1	**1.85**	**2.02**	0.82	0.84
Serpinh1	1.85	1.15	0.72	0.88
Igf1	**1.81**	**1.84**	0.60	0.53
Ahsg	1.74	1.57	0.51	0.62
Mmp10	**1.73**	**1.72**	0.59	0.70
Tgfb2	**1.71**	**1.95**	**0.49**	**0.44**
Igf1r	1.69	1.77	**0.54**	**0.51**
Tgfb3	1.64	1.30	0.71	0.81
Twist1	1.60	1.44	0.66	0.68
Scarb1	1.55	1.06	0.69	0.84
Sox9	1.53	1.23	0.77	0.89
Vdr	1.53	1.36	**0.55**	**0.59**
Smad2	1.52	1.32	0.57	0.68
Vegfb	1.48	1.32	0.72	0.74
Col6a1	1.47	0.69	1.01	0.84
Col8a1	1.47	1.35	1.00	0.83
Itgb1	1.46	1.35	0.67	0.69
Vegfc	1.44	1.39	0.53	0.56
Col4a5	1.40	1.24	0.66	0.55
Pdgfa	1.38	1.55	0.93	0.72
Nfkb1	1.37	1.30	0.67	0.61
Col1a2	1.32	1.35	0.91	0.91
Mgp	1.30	1.37	0.84	0.73
Tgfbr2	1.29	1.18	0.67	0.74
Vcam1	1.25	1.04	0.78	0.92
Col5a1	1.09	1.11	0.91	0.85
Spp1	1.02	0.87	0.95	0.85
Bgn	0.90	0.95	1.70	1.07
Sparc	0.77	0.99	1.61	0.83

MC3T3-E1 cells were maintained under mineralizing conditions. In some cases cells were transfected with either FOXO1 specific or scrambled siRNA. At day zero and day 14 total RNA was isolated and an osteogenic focused microarray was carried out. The data presented are the mean and median of three microarrays carried out per assay condition and compared to the matching control. Values in bold illustrate changes where mean and median values demonstrated a minimum of 1.7 fold increase or 0.6 fold decrease (corresponding to 1.7 fold decrease). Thus the mean fold change met a minimum threshold and the median values ensure that not less than two out of the three microarrays independently met this threshold.

Table 2

Overexpression of FOXO1 reduces the number of PCNA positive cells but not TUNEL positive cells

	PCNA positive (percent)	TUNEL positive (percent)
No treatment	83 ± 3.2	0.33 ± 0.01
Vector control (pcDNA)	69.3 ± 8.6	0.9 ± 0.01
FOXO1	6.3 ± 1.3*	0.8 ± 0.01
FOXO1AAA	7.3 ± 3.4*	0.43 ± 0.01

MC3T3-E1 cells were transfected with control vector, FOXO1 or FOXO1AAA and cultured for 5 days. PCNA positive cells were measured by immunohistochemistry and apoptosis was assessed by the TUNEL Assay. Data are expressed as the percent positive cells and are the mean of three independent experiments ± SEM. Asterisk indicates significant reduction of cells compared to vector control ($P < 0.05$).