Vimentin Inhibits ATF4-mediated Osteocalcin Transcription and Osteoblast Differentiation*

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Activating transcription factor 4 (ATF4) is an osteoblast-enriched transcription factor that regulates osteocalcin expression and osteoblast terminal differentiation. To identify functional partners of ATF4, we applied ROS17/2.8 osteoblast nuclear extracts and purified recombinant His-ATF4 onto a Ni⁺ affinity matrix chromatography column. Vimentin was identified by liquid chromatography-mass spectrometry. Coimmunoprecipitation and pulldown assays revealed that vimentin interacted with ATF4 with its first leucine zipper domain. DNA cotransfection and gel retardation demonstrated that vimentin inhibited the transactivation activity of ATF4 on osteocalcin by preventing it to bind OSE1, the ATF4 binding site on the osteocalcin promoter. Northern hybridization revealed that vimentin was expressed at a high level in immature osteoblasts and a low level in fully differentiated osteoblasts. Down-regulation of vimentin by small interfering RNA induced endogenous osteocalcin transcription in immature osteoblasts. Conversely, ectopic overexpression of vimentin in osteoblasts inhibited osteoblast differentiation as shown by lower alkaline phosphatase activity, delayed mineralization, and decreased expression of osteoblast marker genes such as bone sialoprotein and osteocalcin. Together, our data uncover a novel mechanism whereby a cytoskeletal protein, vimentin, acts as a break on differentiation in immature osteoblasts by interacting with ATF4.

Osteoblasts, the bone-forming cells, originate from mesenchymal stem cells. The differentiation of mesenchymal stem cells to osteoblasts is regulated by several osteoblast-specific transcription factors, including Runx2 (1–4), osterix (5), and ATF4² (6, 7). ATF4 belongs to the CREB leucine zipper (LZ)-containing protein family and is known to regulate gene transcription by binding specifically to the ATF consensus core sequence, TGACGTCA (8). In osteoblasts, ATF4 binds to osteoblast-specific element 1 (OSE1), found in osteocalcin gene.

The abbreviations used are: ATF4, activating transcription factor 4; CREB, cAMP-responsive element-binding protein; EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, hemagglutinin; IF, intermediate filament; LC-MS, liquid chromatography-mass spectrometry; LZ, leucine zipper; NE, nuclear extract; Ni-NTA, nickel-nitrilotriacetic acid; OSE1, osteoblast-specific element 1; PBS, phosphate-buffered saline; PLZ, putative LZ domain.

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2 The importance of ATF4 in skeletal biology is demonstrated by the fact that deletion of Atf4 from the mouse genome results in a severe runt phenotype in addition to very low bone mass due to a failure of osteoblast terminal differentiation and synthesis of type I collagen, the main constituent of bone matrix proteins (7). ATF4 transcriptional activity can be enhanced by posttranslational modifications such as phosphorylation by RSK2 and suppressed by interacting with other proteins such as FIAT (7, 11).

Vimentin is a type III intermediate filament (IF) protein. IFs along with microtubules and actin microfilaments make up the dynamic cytoskeleton that maintains cell shape, enables intracellular transport, and supports cell division (12–14). Similar to other IF proteins, vimentin is expressed in a cell type- and developmental stage-specific manner. Vimentin is often expressed in undifferentiated and proliferative cells of mesenchymal origin and eventually replaced with cell type-specific IF subunits upon differentiation (15, 16). Studies using ras-transformed cells (17) and transgenic mouse models (18) have shown that vimentin regulates cell growth and differentiation. Recent studies using vimentin-deficient (Vim⁻/⁻) mice have revealed that loss of vimentin leads to failures in vascular adaptation resulting in pathological conditions, such as reduction of renal mass (19), malformation of glia cells (20), impairment of wound healing (21), reduced resistance of arteries to sheer stress (22), and disturbance of leukocytes homing to lymph nodes (23). However, the molecular mechanisms whereby vimentin regulates cell differentiation remain elusive.

Here, we show evidence that vimentin interacts directly with ATF4 and prevents its transcriptional activity, thereby inhibiting its function in osteoblast differentiation. The inverse correlation between the expression patterns of vimentin and osteocalcin, a downstream target of ATF4, during osteoblast differentiation suggests that a decline in vimentin expression may be a prerequisite to the process of osteoblast differentiation.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture medium and fetal bovine serum were purchased from Invitrogen. Restriction endonucleases and other DNA-modifying enzymes were purchased from either New England BioLabs or Promega. Radiolabeled nucleotides were obtained from PerkinElmer Life Sciences. Monoclonal anti-vimentin and antibodies against ATF4 (C20) were from Santa Cruz Biotechnology. All chemicals except for the indicated ones were from Sigma.
Cell Culture—COS1 monkey kidney cell and mouse osteoblastic MC3T3-E1 cell lines were cultured in Dulbecco’s modified Eagle’s medium and α-minimal essential medium, respectively. ROS17/2.8 rat osteoblastic cells were grown in Dulbecco’s modified Eagle’s medium/F-12 medium. All media were supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

His-tagged ATF4 and GST-Vimentin Protein Purification—Bacterially expressed His-tagged ATF4 protein was purified according to the method developed by Novagen. GST-vimentin protein was purified as described (24).

Pulldown Experiment—Nuclear extracts (NEs, 1 mg) isolated from ROS17/2.8 rat osteoblastic cells were incubated with or without His-ATF4 (10 μg) for 30 min at 4 °C and were loaded onto Ni2+-chelating Sepharose columns. After washing, proteins were eluted and resolved in 4–20% gradient SDS-PAGE and visualized by Coomassie Blue staining. Individual proteins of interest were excised and subjected to trypsin digestion and identified by liquid chromatography and mass spectrometry (LC-MS).

For mapping ATF4 binding domains of vimentin, 1 μg of purified GST-vimentin fusion protein, its variants, or GST (negative control) was incubated with glutathione-Sepharose beads in phosphate-buffered saline buffer (PBS, pH 7.4) at 4 °C with rotation for 1 h and washed three times with PBS buffer, pH 7.4. The beads were then incubated with His-ATF4 at 4 °C for 2 h followed by three washes with PBS, pH 7.4. Bound proteins were eluted by boiling for 5 min in 2× SDS sample buffer, separated by SDS-PAGE, and stained with Coomassie Brilliant Blue.

Similar methods were used to map vimentin binding domains of ATF4, except 1 μg of purified His-ATF4 or truncated variants was incubated with Ni-NTA-agarose beads in phosphate-buffered saline buffer (PBS, pH 7.4) at 4 °C with rotation for 1 h and then washed three times with PBS containing 15 mM imidazole. GST-vimentin was then added and incubated with rotation for 2 h at 4 °C after washing three times with PBS containing 50 mM imidazole.

NE Preparation—Nuclear extracts of osteoblastic cells were isolated essentially according to the methods described (10, 25).

Vimentin Subcellular Localization—MC3T3-E1 cells were transiently transfected with vectors expressing GFP-vimentin or GFP alone. Cells were then fixed 4 h after transfection in 2% paraformaldehyde for 20 min at room temperature, permeabilized with 0.5% Triton X-100 for 5 min, and stained with DAPI to visualize nuclei. Images were taken with an Olympus BX41 fluorescence microscope with filters for GFP and DAPI.

Immunoprecipitation—COS1 cells (85% confluent) in 10-cm plates were transfected with 8 μg of FLAG-ATF4, 6 μg of HA-vimentin, or both using Lipofectamine (Invitrogen). Cells were treated with 25 μM MG115 to stabilize ATF4 for 5 h before harvesting (6). Isolated NEs (500 μg) were immunoprecipitated with 5 μl of anti-FLAG M2 beads (Sigma) or anti-HA beads (Abcam) for 8 h at 4 °C. After washing three times with ice-cold Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.4), immunocomplexes and NEs (50 μl) as input controls were resolved by SDS-PAGE, transferred onto nitrocellulose membranes, and revealed by Western blotting using anti-FLAG M2 (Sigma) or anti-HA (Abcam) antibodies.

Plasmids—Mouse vimentin cDNA was amplified by reverse transcription-PCR using bone cDNA as template and cloned in pcDNA3.1 mammalian expression vector (Invitrogen), pEGFP-C1 expression vector (Clontech), or pGEX-4T1 bacterial expression vector (Amersham Biosciences). Three different truncation forms of mouse vimentin cDNA, named Vim1 (containing amino acids 1–155), Vim2 (containing amino acids 205–254), and Vim3 (containing amino acids 314–438), respectively, were PCR-amplified, flanked with cloning sites EcoRI and Sall, and inserted into mammalian expression vector pCMV-HA (Clontech) and bacterial expression vector pGEX-4T2. Mouse ATF4 cDNAs were subcloned into pCMV6CFLAG (Sigma) from a mammalian expression vector pCMV5/ATF4 (7). The integrity of all cDNA and production of the fusion protein were confirmed by DNA sequencing and Western blotting, respectively.

DNA Transfection and Luciferase Assay—COS1 cells were seeded at a density of 5 × 104/well in 24-well plates and transfected with 0.2 μg of the reporter plasmid (6OSE1-Luc or 3API-Luc), 0.05 μg of β-galactosidase, 0.2 μg of transcription factor plasmid (FLAG-ATF4 or FosB) with or without 0.2 μg of pcDNA3.1-vimentin using Lipofectamine. Cells were lysed 24 h later, and the luciferase activity was normalized to the β-galactosidase activity.

Short Hairpin RNAs and Transfection—Short hairpin RNAs targeting mouse Vim construct were cloned by ligating annealed oligonucleotides 5’-ACCTCAACAGGATC-CGGAGACAGGTttcaagagACCTGTCTCCGGTACTCGT-TTTT-3’ into a BbsI/BbsI-digested psiRNA-hH1neo G2 vector (InvivoGen, San Diego, CA). 2T3 cells (2 × 105 cells/well in 6-well plates) were transfected with 1.5 μg of the reporter plasmid, 1.5 μg of siRNA-control or siRNA-Vim, and 0.2 μg of β-galactosidase plasmid). Cells were lysed 48 h later, and the luciferase activity was normalized to the β-galactosidase activity. Total RNA was collected 48 h after transfection for Northern blot analysis.

Electrophoretic Mobility Shift Assays (EMSAs)—ROS17/2.8 NEs or His-ATF4 and increasing amounts of GST-vimentin, its deletion variants, or GST were incubated with 5 pmoI of a radiolabeled double-stranded OSE1 oligonucleotide (9) at room temperature for 10 min. EMSA was performed as described (10).

Establishment of Permanent Cell Lines—MC3T3-E1 cells (104 cells/10-cm dish) were transfected with 5 μg of pcDNA3.1 vector or pcDNA3.1-vimentin. Cells were trypsinized and replated in α-minimal essential medium containing 10% fetal bovine serum, and 400 μg/ml G418 was added 2 days after transfection followed by 2-week selection. The expression of vimentin was confirmed by Northern blot analysis using the bovine growth hormone poly(A)+ as a probe.

Northern Blot Analysis—Total RNA from different adult mouse tissues, MC3T3-E1 cells, primary calvarial osteoblasts, or bone marrow stromal cells was isolated using TRIzol (Invitrogen) according to the manufacturer’s protocols. Total RNA (5 μg) was resolved in 1% agarose gel, transferred onto nylon membranes. The membrane was cross-linked by UV light
and hybridized following standard protocols with the indicated cDNA probes described previously (6, 7) except for vimentin cDNA (nucleotides 792–1218), which was cloned in this study (see Plasmids above for cloning strategies).

Osteoblast Differentiation Assays—Confluent (d0) MC3T3-E1 in 12-well plates overexpressing vector (control) or vimentin were grown in G418-containing medium supplemented with 5 mM β-glycerophosphate and 100 μg/ml ascorbic acid for 2, 4, 6, 12, or 20 days. Alkaline phosphatase activity was measured, and mineralization of osteoblasts was assayed by von Kossa staining (Fig. 3, lower panel). Scale bar, 10 μm.

Statistics—Data are expressed as mean ± S.D. Statistical analysis was performed using an unpaired t test.

RESULTS AND DISCUSSION

Vimentin Interacts with ATF4—To identify ATF4-interacting proteins in osteoblasts, we used a biochemical approach with nuclear extracts from ROS17/2.8 rat osteoblasts and His-tagged ATF4. ATF4 was tagged with 6 histidines at its N terminus and expressed in bacteria to obtain large quantities of protein. Purified His-ATF4 was then incubated with nuclear extracts isolated from ROS17/2.8 rat osteoblasts, and the mixture was applied to a Ni-NTA column. After repeated washing, the ATF4-bound proteins were eluted, and elution fractions were resolved in a gradient SDS-PAGE. Slices containing a single protein band were subjected to tryptic digestion and LC-MS analysis. Among the specific ATF4-bound proteins identified in the LC-MS analysis, one 37 kDa band was revealed as vimentin, a type III intermediate filament protein (Fig. 1, A and B).

Vimentin is a cytoskeletal protein that has been well recognized as a protein localized in the cytoplasm and perinucleus, although it can be transported into the nucleus by DNA oligonucleotide mediation (26). The fact that it was pulled out from osteoblast nuclear extracts supported the nuclear localization of vimentin. To confirm this notion, we made GFP-vimentin fusion expression vector and introduced it into MC3T3-E1 cells. Our results showed that GFP-vimentin was present predominantly in the nucleus of a subset of GFP-positive cells, but GFP alone was distributed evenly in both nucleus and cytoplasm of all the GFP-positive cells (Fig. 1C).

Pulldown assays using either purified GST-vimentin or GST alone showed that ATF4 bound to GST-vimentin but not to the GST control (Fig. 2A). Conversely, vimentin bound to His-tagged ATF4 but not to Ni-NTA-agarose beads (Fig. 2B), confirming a direct interaction between vimentin and ATF4 in vitro. Reciprocal immunoprecipitation assays using nuclear extracts of COS1 cells transfected with HA-vimentin and/or FLAG-ATF4 expression vectors revealed that vimentin interacted with ATF4 in cells as well because FLAG-ATF4-bound agarose beads precipitated HA-vimentin, and conversely, HA-vimentin precipitated FLAG-ATF4 (Fig. 2C). These results indicate that the interaction between vimentin and ATF4 is direct and specific in vitro and in cells.

ATF4 has been predicted to form heterodimers preferentially through LZ domains (27), and it contains two LZ domains, namely N-LZ and C-LZ. The basic amino acid-rich DNA binding domain (b) is located close to C-LZ (Fig. 2D). Interestingly, we found three leucine-rich repeats within vimentin by visual examination of its primary structure and named them putative LZs (PLZ1 to PLZ3, Fig. 2E). To define which PLZ(s) mediated the interaction between vimentin and ATF4, we created three truncated variants of vimentin, Vim1, Vim2, and Vim3, which contained PLZ1, PLZ2, or PLZ3, respectively. We also used three truncated variants of ATF4 described previously (7) that covered amino acids 1 to 151 (1–151, containing N-LZ), 110 to 221 (110–221), and 186 to 349 (186–349, containing C-LZ), respectively, in the following experiments. Pulldown assays demonstrated that full-length vimentin or its truncated variant Vim1 bound strongly to full-length ATF4 (Fig. 2F). Full-length ATF4 and the two ATF4 truncated variants, 1–151 and 186–349, interacted with full-length vimentin (Fig. 2G). From these results, we inferred that Vim1 binds the C-LZ domain of ATF4.
Together, these results establish a novel interaction between a nuclear transcription factor, ATF4, and a cytoskeleton protein, vimentin.

Vimentin Represses the Transactivation Activity of ATF4—Osteocalcin expression is a hallmark of osteoblast terminal differentiation (28). ATF4 was originally identified as one of the two main regulators of osteocalcin gene transcription (2, 7, 9, 29). To understand the functional relevance of the vimentin-ATF4 interaction, we first tested whether vimentin affected the transcriptional function of ATF4. Consistent with previous findings (6), ATF4 activated the p6OSE1-Luc reporter containing six copies of OSE1 (9, 10) by about 50-fold in COS1 cells. This ATF4-dependent activation was inhibited by 70% when a vimentin expression vector was cotransfected (Fig. 3A). Similarly, vimentin inhibited ATF4-dependent activation of p160-Luc, a reporter construct containing a 160-bp native osteocalcin promoter, by 70% (Fig. 3B). The inhibition of ATF4-induced luciferase activity by vimentin was specific to ATF4 because vimentin did not affect the ability of FosB, another LZ-containing transcription factor, to activate a chimeric reporter gene containing three copies of AP1 binding sites, pAP1-Luc (Fig. 3C). These data indicate that vimentin selectively represses the transactivation activity of ATF4.

Vimentin Inhibits ATF4 Binding to Its Cognate DNA—Two important observations prompted us to test whether vimentin inhibited ATF4-mediated transactivation of osteocalcin by interfering with the binding of ATF4 to its cognate DNA element. First, vimentin bound to the ATF4 C-LZ, the motif in proximity to the basic DNA binding domain (Fig. 1, D and G), which may create a steric barrier for ATF4 to access the DNA. Second, vimentin can be detected in the nucleus of many cell types, including osteoblasts (Fig. 1C) and epithelial cells (30, 31). ATF4 binds OSE1 in the osteocalcin promoter in vitro and in vivo as demonstrated by EMSA and by the use of transgenic mice (6, 7, 9, 10). Using a similar assay, we found that GST-vimentin fusion protein but not GST alone inhibited endogenous ATF4 binding to OSE1 in a dose-dependent manner (Fig. 3E). Given the mapping data that indicated that the Vim1 was sufficient to mediate the interaction between vimentin and ATF4 (Fig. 2F), we tested whether Vim1 affected the transactivation activity of ATF4. DNA cotransfection using a Vim1

| FIGURE 2. Vimentin (Vim) interacts with ATF4. A, pulldown assay showing that GST-vimentin binds directly to ATF4. B, pulldown assay using His-ATF4 and Ni-NTA resin column showing that ATF4 binds directly to vimentin. C, reciprocal coimmunoprecipitation. NEs of COS cells transfected with HA-vimentin and/or Flag-ATF4 were immunoprecipitated with anti-HA or anti-FLAG antibody and visualized with anti-HA or anti-FLAG antibodies (top two panels). Total protein (10%) of each transfection served as loading control (bottom panel). D, schematic illustration of ATF4 primary structure showing two LZ domains at N and C termini. Letter b represents basic amino acid-rich DNA binding domain. E, schematic presentation of vimentin primary structure showing three PLZs of vimentin. F, PLZ1 of vimentin interacting with ATF4. Pulldown assay was carried out using purified GST-vimentin and its truncated variants GST-Vim1–3 and His-ATF4. Note that only full-length vimentin (Vim-fl) or its variant containing the first PLZ interacted with His-ATF4. G, LZs of ATF4 interacting with vimentin. Pulldown assay was performed using purified His-ATF4 and its three indicated deletional variants. Note that full-length ATF4 (Full length) and both of its LZ-containing variants, 1–151 and 186–349, bound vimentin. |
expression plasmid showed an inhibition of ATF4-dependent activation of 6OSE1-Luc reporter to an extent similar to that of full-length vimentin, whereas Vim2 or Vim3 had no effect (Fig. 3F). Consistently, Vim1 but not Vim2 or Vim3 inhibited His-ATF4 or endogenous ATF4 binding to OSE1 in a dose-dependent manner (Fig. 3, G and H). These data indicate that vimentin inhibits ATF4 transcriptional activity by affecting its binding to cognate DNA via the first LZ domain of vimentin.

Down-regulation of Vimentin Induces Endogenous Osteocalcin Transcription—To strengthen further our findings that vimentin inhibited the transcriptional activity of ATF4, we next tested whether knocking down endogenous vimentin in immature osteoblasts would conversely induce osteocalcin transcription. We thus constructed a vector expressing short hairpin RNA of vimentin (psiRNA-Vim) and introduced it into 2T3 mouse osteoblasts. Northern blot analysis demonstrated that endogenous vimentin mRNA was decreased in cells transfected with psiRNA-Vim. Supporting our hypothesis, endogenous osteocalcin mRNA was induced in cells depleted in vimentin by psiRNA-Vim (Fig. 4A). To confirm these expression data, we performed DNA cotransfection assays to determine whether vimentin depletion affected endogenous ATF4-dependent activation of luciferase activity driven by 6OSE1 (p6OSE1-Luc) or native 160-bp osteocalcin promoter (p160-Luc). Our results revealed that psiRNA-Vim but not the empty psiRNA vector enhanced ATF4-induced luciferase activity 2.5-fold in both 6OSE1-Luc and p160-Luc reporter constructs (Fig. 4, B and C). This induction in luciferase activity was specific to endogenous ATF4 because psiRNA-Vim did not significantly enhance the AP1-induced luciferase activity (Fig. 4D). Therefore, we conclude that vimentin acts as an inhibitor on endogenous osteocalcin transcription and thus as a break on the transactivation activity of ATF4.

Vimentin Delays Osteoblast Differentiation—To address whether the inhibitory action of vimentin on the transactivation activity of ATF4 had any biological relevance and specifically regulated osteoblast differentiation, we created MC3T3-E1 mouse osteoblastic cells that stably expressed vimentin. Stable expression of vimentin was confirmed by Northern blot analysis (Fig. 5A). The progression of osteoblast differentiation was monitored by alkaline phosphatase assay, von Kossa staining of mineralized nodules, and the temporal expression of immature osteoblastic differentiation markers, such as type I collagen (Col1a1), and differentiated osteoblast

**FIGURE 3.** Vimentin (Vim) inhibits ATF4-dependent transactivation of osteocalcin transcription by blocking its binding to OSE1. A–C, DNA cotransfections of COS1 cells with reporter construct containing six copies of ATF4 binding site OSE1, p6OSE1-Luc (A), a native osteocalcin promoter, p160-Luc (B), three copies of binding sites of AP1 family (C), and/or expression vectors of ATF4, FosB, and/or vimentin as indicated. Note that ATF4-induced but not FosB-induced luciferase activity was inhibited by more than 70% by vimentin. D, vimentin inhibiting the binding of ATF4 to OSE1. EMSA was performed using purified His-ATF4 and radiolabeled OSE1 as a probe. Note that vimentin inhibited ATF4 binding to OSE1 dose-dependently. E, EMSA showing that vimentin inhibits endogenous ATF4 (middle bands are ATF4-OSE1 complexes (9)) from ROS17/2.8 osteoblastic cells binding to OSE1. F, DNA cotransfection in COS1 cells with p6OSE1-Luc and expression vectors of FLAG-ATF4 and/or HA-Vim1, -Vim2, or -Vim3. G and H, EMSA. Note that GST-Vim1 inhibited His-ATF4 (G) or endogenous ATF4 (H) binding to OSE1 in a dose-dependent manner. The luciferase activities were normalized for the β-galactosidase activity and are presented as fold activation relative to the luciferase levels of the reporter construct alone. Values are the mean ± S.D. of three independent experiments. *, p < 0.01; **, p < 0.001; N.S., not significant.
Vimentin Inhibits ATF4

Calvarial cells, and bone marrow stromal osteoprogenitors that have the potential to differentiate into osteoblasts (32). Northern blot analysis showed that vimentin was highly expressed in vertebral bones, and it was the most abundant message RNA detected among all of the tissues examined (Fig. 6A). Interestingly, we found that the level of vimentin mRNA in MC3T3-E1 cells and primary calvarial osteoblasts decreased over time during the course of differentiation: high at the initiation of differentiation (day 0, Fig. 6, B and C), and low at the end of differentiation (day 10, Fig. 6, B and C). Similarly, in alkaline phosphatase-positive mouse bone marrow stromal osteoprogenitors, vimentin mRNA was high at day 5 after differentiation induction, but low at day 20 after differentiation induction (Fig. 6D). Consistently, vimentin protein showed the same expression pattern as its mRNA in rat primary osteoblasts (Fig. 6E). Collectively, these results suggest that down-regulation of vimentin during osteoblast differentiation may be a required mechanism to relieve its inhibition of ATF4 transcriptional activity and to promote osteoblast terminal differentiation (Fig. 6F).

In summary, this study identified a novel interaction between vimentin and ATF4. To our knowledge, our work revealed for the first time a direct interaction between an intermediate filament protein and a transcription factor. The experimental data establish that through its interaction with ATF4, vimentin prevents the binding of ATF4 to its target DNA and thus leads to delayed osteoblast differentiation. Consistent with its inhibitory role, vimentin expression is progressively decreased at both mRNA and protein levels during the course of osteoblast differentiation, whereas the expression of osteocalcin, a transcriptional target gene of ATF4, progressively increases. This piece of evidence suggests that at an early stage of osteoblast differentiation, the transcriptional activity of ATF4 is repressed by vimentin, which explains why osteocalcin transcription does not occur in these cells. This hypothesis is supported by the observation that ATF4 binds to osteocalcin promoter at day 8 but not at day 0 of differentiation in primary osteoblasts (33). Therefore, removal of vimentin-mediated inhibition is a prerequisite for preosteoblasts to differentiate into terminal osteoblasts.

Our study has also defined that the LZ domains of vimentin mediate the interaction between vimentin and ATF4. The sequence and structural similarities between the leucine-rich repeats of vimentin and the LZ motif of several other LZ-containing nuclear regulators belonging to the CREB family, such as CREB, Fos, and Jun, have been described previously. Based on computational analysis, the authors predicted the existence of molecular interactions between vimentin and these nuclear regulators through their similar leucine-rich amphipathic helices (34). Our mapping data

FIGURE 4. Down-regulation of vimentin by siRNA enhances osteocalcin expression. A–C, DNA cotransfections of 2T3 osteoblastic cells with reporter p6OSE1-Luc (A), p160-Luc (B), AP1-Luc (C), and vectors of control (psiRNA) or vimentin (psiRNA-Vim). Note that vimentin siRNA induced activation of osteocalcin promoter by -2-fold. Values are the mean ± S.D. of three independent experiments. *, p < 0.01; N.S., not significant. D, Northern blot using total RNA from 2T3 osteoblasts transfected with control or siRNA against vimentin. Note that depletion of vimentin triggered osteocalcin expression. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) mRNA was used as a loading control.

Consistent with the mineralization results, two marker genes for mature osteoblast, osteocalcin and Bone sialoprotein (Bsp), were expressed at day 6 after differentiation induction and increased over time in control MC3T3-E1 cells carrying empty vector. However, in cells that overexpressed vimentin, the mRNA levels of osteocalcin, Bsp, and osterix were not readily detectable at all time points examined, including days 6, 12, and 20 after differentiation induction, whereas the expression of Atf4, Col1a1, and Runx2 was not overly changed, especially if one takes into account of loading controls (Fig. 5D). From these results, we conclude that vimentin inhibits (i) endogenous osteocalcin transcription and (ii) osteoblast differentiation. The observation that vimentin inhibited the expression of mature osteoblast marker genes more dramatically than that of immature osteoblast marker genes was strikingly similar to what was seen in the Atf4−/− mice (7). This similarity strengthens our hypothesis that vimentin may execute its function in osteoblasts by affecting the function of ATF4.

Vimentin Expression Pattern in the Osteoblastic Lineage—To understand the physiological role of vimentin during osteoblast differentiation, we examined the expression pattern of vimentin in various mouse tissues, MC3T3-E1 osteoblasts, primary markers, such as osteocalcin and bone sialoprotein (Bsp). In addition, the expression of Runx2 and osterix, another two important osteoblast differentiation factors, was also examined. MC3T3-E1 cells carrying empty vector responded to osteogenic induction as indicated by increasing alkaline phosphatase activity over time. This response was inhibited in cells overexpressing vimentin 2 and 4 days upon induction (Fig. 5B). As expected, 6 days after differentiation induction, mineralized nodules started to form in MC3T3-E1 cells that carried control empty vector. In contrast, no mineralized nodules were observed in day 6 cultures of MC3T3-E1 cells that overexpressed vimentin. The onset of mineralized nodule formation in vimentin-expressing cells was only detected after 12 days of differentiation, and the number and size of mineralized nodules were still severely less after 20 days of differentiation compared with control cells (Fig. 5C).

Consistent with the mineralization results, two marker genes for mature osteoblast, osteocalcin and Bsp, were expressed at day 6 after differentiation induction and increased over time in control MC3T3-E1 cells carrying empty vector. However, in cells that overexpressed vimentin, the mRNA expression of osteocalcin, Bsp, and osterix were not readily detectable at all time points examined, including days 6, 12, and 20 after differentiation induction, whereas the expression of Atf4, Col1a1, and Runx2 was not overly changed, especially if one takes into account of loading controls (Fig. 5D). From these results, we conclude that vimentin inhibits (i) endogenous osteocalcin transcription and (ii) osteoblast differentiation. The observation that vimentin inhibited the expression of mature osteoblast marker genes more dramatically than that of immature osteoblast marker genes was strikingly similar to what was seen in the Atf4−/− mice (7). This similarity strengthens our hypothesis that vimentin may execute its function in osteoblasts by affecting the function of ATF4.

Vimentin Expression Pattern in the Osteoblastic Lineage—To understand the physiological role of vimentin during osteoblast differentiation, we examined the expression pattern of vimentin in various mouse tissues, MC3T3-E1 osteoblasts, primary
have now provided experimental evidence supporting these predictions, although only one of such putative LZ motifs in vimentin was shown to be responsible for this interaction. The biological significance of this association is supported by evidence demonstrating that the PLZ1 of vimentin behaves identically to the full-length protein to inhibit ATF4 binding and activation of the osteocalcin promoter. Therefore, this provides insights into the possibility that small molecules could be screened for their ability to block the binding of vimentin to ATF4 and to stimulate osteoblast terminal differentiation and function.

It is not understood at present whether the interaction between vimentin and ATF4 takes place in the nucleus or the cytoplasm. Several observations strongly suggest that vimentin, despite its well known cytoplasmic location, interacts with ATF4 within the nucleus. First, vimentin inhibits ATF4 to bind its cognate DNA. Second, vimentin was pulled out from ROS28/1.7 osteoblast nuclear extracts and recognized by an anti-vimentin antibody in Western blotting in the nuclear extracts of primary calvarial osteoblasts. Third, GST-vimentin was detected in the nucleus of MC3T3-E1 osteoblasts (Fig. 1C).

Supporting this, vimentin was observed independently in the nucleus of nonosteoblastic cells (31) and osteoblasts (30). Interestingly, vimentin can be modified by phosphorylation, leading to the generation of a 50-kDa truncated vimentin (35), the same molecular mass as vimentin observed in rat osteoblasts (Fig. 6E). It is unknown whether ATF4 binds to monomeric or polymeric forms of vimentin, which of these forms is present within the nucleus, and whether such interactions dictate the inhibitory and nuclear function of vimentin. Further studies are needed to elucidate these mechanisms.
Although less dramatically, vimentin overexpression also caused a decrease in the expression of some marker genes for immature osteoblasts, particularly osterix and Runx2, two important osteoblast differentiation factors (2–5). This result is interesting because it suggests that vimentin may also inhibit the function of osterix and Runx2 in the regulation of osteoblast differentiation. However, whether this effect is via a direct interaction between vimentin and Runx2 or osterix through domains other than the leucine-rich repeats, or via other indirect actions remains to be determined.

Based on our data, we speculate that the disappearance of vimentin in osteoblasts allows the progression of immature osteoblasts to fully differentiated and functional osteoblasts. One may wonder why there is such a need for several inhibitors of differentiation in the osteoblasts. One plausible explanation is that such inhibitors are important for the prevention of premature differentiation of osteoblasts, allowing for a sufficient pool of preosteoblasts with the proliferative potential to be maintained at the site of bone formation, in response to extracellular cues. Interestingly, some extracellular cues, such as parathyroid hormone (36) and transforming growth factor (37), regulate de novo synthesis of vimentin in osteoblasts. However, it is unknown whether this regulation is temporal or dependent on differentiation progression. Further studies are needed to identify molecules involved in the regulation of vimentin expression in osteoblasts.

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