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Osteonectin-Null Mutation Compromises Osteoblast Formation, Maturation, and Survival

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Osteonectin, also known as SPARC (secreted protein acidic and rich in cysteine) or BM-40, is one of the most abundant noncollagenous proteins in bone. Analysis of osteonectin-null mice revealed that osteonectin is necessary for the maintenance of bone mass and normal remodeling, as osteonectin-null mice have decreased osteoblast number and bone formation rate. Cultures of bone marrow stromal cells and osteoblasts from control and osteonectin-null mice were used to determine the cellular basis for the mutant phenotype. We found that marrow stroma from osteonectin-null mice contains fewer osteoblastic precursors than that of control mice, and the osteonectin-null mutation did not affect the proliferation rate of stromal cells or osteoblasts. Whereas osteonectin-null cells could adopt an osteoblastic phenotype, a smaller proportion of these cells expressed markers of a fully differentiated osteoblast. Mutant cells exhibited decreased formation of mineralized nodules, as well as diminished expression of osteocalcin mRNA and response to PTH. Furthermore, osteonectin-null cells showed an increased tendency to form adipocytes, with enhanced expression of the adipocytic markers adipsin and CCAAT/enhancer binding protein δ. Osteonectin-null cells were also more susceptible to environmental stresses. These data indicate that osteonectin is important for osteoblast formation, maturation, and survival. (*Endocrinology* 144: 2588–2596, 2003)

THERE IS a reciprocal relationship between cells and their extracellular matrix (ECM). The composition of ECM regulates cell behavior, whereas cells often synthesize the matrix components in their local environment. The glycoprotein osteonectin or SPARC (secreted protein acidic and rich in cysteine, BM-40) is considered a matricellular protein, because it is a modular extracellular matrix protein that functions more as a regulator of cell behavior than as a structural part of the matrix (1). Numerous studies in nonskeletal systems have shown that osteonectin has a myriad of functions, including inhibition of cell proliferation, deadhesion, stimulation of metalloproteinase expression, and modulation of angiogenesis. In addition, it can bind to and inhibit the function of growth factors such as platelet-derived growth factor, TGFβ, and vascular endothelial growth factor (1). Osteonectin is a regulator of matrix assembly. Collagen fibrils in the skin of osteonectin-null mice are small and uniform in diameter, whereas fibrils in normal mice exhibit a variety of diameters (1). It is likely that defects in ECM assembly play a role in the development of cataracts and in the accelerated wound healing observed in osteonectin-null mice (2–4).

Osteonectin is expressed in areas of active remodeling. Intriguingly, osteonectin is one of the most abundant noncollagenous proteins in bone, which is a continuously remodeling tissue, but its function in bone is not understood (5). Initial data on the function of osteonectin in bone were obtained from analysis of the skeleton of osteonectin-null mice, which showed that osteonectin is important for normal remodeling and maintenance of bone mass (6). Osteonectin-null mice develop a low turnover osteopenia that becomes more severe as the animals age. The osteonectin-null mice have decreased matrix apposition rate and decreased osteoblast and osteoclast numbers and surface, causing decreased bone remodeling with a negative bone balance. Although both cortical and trabecular bone have decreased matrix apposition rates, the osteopenia affects primarily trabecular bone, which fulfills a more metabolic function and is increasingly susceptible to loss of mass in states of increased resorption. Cortical bone thickness is not altered by the osteonectin-null mutation; however, Fourier-transform infrared spectroscopy provides evidence that this tissue is also poorly remodeled and of decreased quality (7). Analysis of RNA from long bones and calvaria of control and osteonectin-null mice did not reveal significant changes in expression of a number of genes important for the osteoblastic phenotype, including α1(I) collagen, osteocalcin, osteopontin, and several metalloproteinases (6). Thus, the mechanisms responsible for the osteopenia observed in mutant mice remain undefined.

The negative bone balance observed in osteonectin-null mice implies that the decreased osteoblast number and matrix apposition rate are central defects associated with the mutation. Therefore, the present study was undertaken in an effort to understand how the osteonectin-null mutation causes decreased osteoblast numbers and decreased bone formation. Marrow stromal cells and osteoblastic cells derived from control and osteonectin-null mice were cultured *in vitro*, and the effects of the mutation on osteoblast proliferation, maturation, and survival were determined.

Abbreviations: C/CEBP, CCAAT-enhancer binding protein; CMV, cytomegalovirus; ECM, extracellular matrix; FBS, fetal bovine serum; WT, wild-type.

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Materials and Methods

Primary osteoblast and stromal cell cultures

Osteoblastic cells were isolated from parietal bones of neonatal mice by sequential collagenase digestion as previously described (8, 9). Both wild-type (WT) and osteonectin-null mice of mixed genetic background (129SV×C57BL/6), and WT and osteonectin-null mice that had been backcrossed four times into the C57BL/6 genetic background were used in these studies (3). All protocols were approved by the institutional animal care and use committee of Saint Francis Hospital and Medical Center. Cells were cultured in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with nonessential amino acids, 20 mM HEPES, 100 μg/ml ascorbic acid, and 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA). Subconfluent cultures were trypsinized and replated at 10,000 cells/cm^2, and these first passage cells were used for subsequent experiments. When the cells reached confluence, approximately 1 wk after plating, they were subsequently cultured in osteoblast differentiation medium (DMEM containing 10% FBS, 100 μg/ml ascorbic acid, 20 mM HEPES, and 5 mM β-glycerophosphate). Medium was changed twice a week for up to 3 wk post confluence (10). RNA was isolated from cells once a week, always 3 d after their last medium change.

Bone marrow stromal cells were isolated from the femurs of adult mice as described by Dobson (11). Cells were resuspended, counted, and plated in αMEM (Life Technologies, Inc.) containing 20 mM HEPES and 10% FBS. After 4 d of culture, half of the medium was replaced with fresh medium. After 7 d of culture, medium was changed and supplemented with 5 mM β-glycerophosphate, 100 nM dexamethasone, and an additional 50 μg/ml ascorbic acid (12). Medium was changed twice a week for up to 28 d. For analysis of colony-forming units, cells were plated at low density so that discrete colonies could be visualized (~10,000 nucleated cells/cm^2). For determination of osteoblastic phenotype, cells were plated at a higher density (1 million nucleated cells/cm^2) such that the culture would be confluent 7 d after plating.

Osteoblastic cell lines

Control and osteonectin-null osteoblast cell lines were created by transducing primary cultures of osteoblastic cells with replication incompetent retrovirus, packaged in PT67 cells, expressing the human papilloma virus 16 E6/E7 open reading frame under the control of the cytomegalovirus (CMV) promoter in the retroviral expression vector pLPNX (13). The E6/E7 gene products interfere with the function of tumor suppressors p53 and Rb, respectively, preventing cell cycle arrest without causing significant transformation. This strategy has been used to create numerous cell lines, including those derived from marrow stromal cells, schwannoma cells, ovarian and prostate epithelial cells, and myocardial fibroblasts (14–17). Pools of geneticin-resistant cells were propagated in αMEM containing 20 mM HEPES and 10% FBS. When the cells reached confluence, their culture medium was supplemented with 5 mM β-glycerophosphate. Medium was changed twice a week for up to 3 wk post confluence. Cell lines made with osteoblasts from mice of mixed genetic background were named mOb-I1 (mouse osteoblast-immortalized, mixed background), whereas lines made with osteoblasts from mice backcrossed four times into C57BL/6 were named mOb-I2 (mouse osteoblast-immortalized, backcrossed).

A retroviral expression vector for the overexpression of osteonectin was created by ligating cDNA corresponding to the coding region of mouse osteonectin in-frame with a Myc-hexahistidine tag (derived from pcDNA3.1MycHisA, Invitrogen, Carlsbad, CA) and subcloned into the retroviral expression vector pLPCX (Invitrogen). In the resulting construct, the CMV promoter drives the expression of mouse osteonectin, with a Myc-hexahistidine tag on the C terminus. Replication-defective retrovirus, packaged in 293gpg cells, was used to transduce control and osteonectin-null mOb-I1 and mOb-I2 cells (18). Pools of puromycin-resistant clones were cultured and analyzed.

Northern blot analysis

RNA was isolated with guanidine thiocyanate at acid pH, followed by phenol-chloroform extraction and ethanol precipitation (19). Equal amounts of RNA (5–10 μg) were denatured and subjected to electrophoresis through formaldehyde-agarose gels, and the RNA was blotted onto GeneScreen Plus as directed by the manufacturer (DuPont, Wilmington, DE). Restriction fragments containing portions of cDNA for osteonectin, osteopontin (provided by Marian Young, Bethesda, MD), α1(I) collagen (provided by Barbara Kream, Farmington, CT), CCAAT-enhancer binding protein (C/EBPδ) and -β (provided by Stephen McKnight, Baltimore, MD), adipsin, peroxisome proliferator-activated receptor-γ2, and a murine 18S rRNA (American Type Culture Collection, Manassas, VA), and a genomic DNA fragment for rat osteocalcin (provided by Jane Lian, Worcester, MA) were labeled with [α-^{32}P]deoxy-CTP (3000 Ci/mmol; DuPont) by random primed, second strand synthesis (Ready to Go, Pharmacia Biotech, Piscataway, NJ) (20–26). Hybridizations and posthybridization washes were carried out as previously described (9). Autoradiograms were analyzed by densitometry, and mRNA levels were normalized to those of 18S.

Western blot analysis

Samples of conditioned medium were precipitated with 3.3% trichloroacetic acid and were resuspended in reducing Laemmli sample buffer (27). Proteins were fractionated by PAGE on a 12% gel and transferred to Immobilon P membranes (Millipore Corp., Bedford, MA) (28). Membranes were blocked with 3% BSA and were exposed to a 1:5000 dilution of anti-Myc monoclonal antibody (Invitrogen), followed by exposure to horseradish peroxidase-conjugated goat antimouse IgG antiserum (Sigma-Aldrich Corp., St. Louis, MO). Membranes were developed using a horseradish peroxidase chemiluminescence reagent, and immunoreactive bands were visualized on Reflection x-ray film (DuPont).

Nuclear extracts were prepared from osteoblastic cells as described by Schreiber *et al.* (29), and 50-μg aliquots in reducing Laemmli sample buffer were subjected to PAGE. Western blotting was performed as described above, except that a 1:200 dilution of rabbit anti-FosB (sc-48, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used as primary antibody, and horseradish peroxidase-conjugated goat antirabbit IgG antiserum (Sigma-Aldrich Corp.) was used as secondary antibody.

[^{3}H]Thymidine incorporation

Stromal or osteoblastic cells were propagated as described. For each time point analyzed, always 3 d after the last change of medium, cells were transferred to serum-free medium and were pulsed with 5 μCi/ml [^{3}H]thymidine (89 Ci/mM; DuPont) for the last 2 h of culture. Cell layers were washed twice with PBS and twice with cold 5% trichloroacetic acid, and were resuspended in 0.2 N NaOH/0.3% sarcosyl. [^{3}H]Thymidine incorporation was determined by counting a neutralized aliquot of the cell lysate in the presence of scintillation fluid (30). The total protein content of the lysates was determined using the DC kit (Bio-Rad Laboratories, Inc., Hercules, CA), and data are expressed as disintegrations per minute per milligram of cell protein.

cAMP RIA

cAMP levels were determined in cultures that had been serum-deprived for 24 h and subsequently treated with 500 nM isobutylmethylxanthine (Sigma-Aldrich Corp.) for 10 min before the addition of PTH (0–100 nM) for 2 min. Cells were washed and extracted in 90% propanol in water (31). An aliquot of the extract was used to determine cAMP levels with a specific RIA kit, according to the manufacturer's instructions (Biomedical Technologies, Stoughton, MA), and an aliquot of the extract was used to determine total protein (DC assay, Bio-Rad Laboratories, Inc.). Data are expressed as picomoles of cAMP per milligram of cell protein.

Analysis of cell survival

Trypan blue exclusion was used to monitor cell survival under serum-free conditions. Cells were plated at 9,000/cm^2, and at confluence cultures were rinsed and cultured in serum-free medium (αMEM containing 0.1% BSA) for up to 48 h (32). At confluence, the cultures contained a similar number of cells (~28,000/cm^2). Viable cell number at confluence and after 24 and 48 h of serum deprivation was determined by hemocytometer.

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Cytochemistry

Alkaline phosphatase-positive cells were detected in cultures fixed with 3% formaldehyde/66% acetone in a citrate buffer using a leukocyte alkaline phosphatase kit (Sigma-Aldrich Corp.). Mineralization was detected in fixed cultures by staining with 2% Alizarin Red or by von Kossa staining. Intracellular fat was detected in cultures that were rinsed, air-dried for 1 h, and stained for 1 h with 0.5% Oil Red-O in 60% isopropanol (Sigma-Aldrich Corp.) (10).

Statistical analysis

Data are expressed as the mean ± SEM. Statistical differences were calculated by ANOVA.

Results

Previous histomorphometric analysis of osteonectin-null mice showed that mutant mice had decreased numbers of osteoclasts and osteoblasts (6). Marrow stromal cells derived from WT and osteonectin-null mice were cultured at low density to determine whether the decreased osteoblast number was due to a decrease in osteoblastic precursors. Cultures were fixed and stained for alkaline phosphatase activity after 10 d, such that colony-forming units with osteoblastic potential could be determined (33). Quantification of data obtained from 7- and 20-wk-old mice showed that marrow stromal cell preparations from osteonectin-null mice contained 10–20% fewer osteoblastic precursors than those from control mice, indicating a deficiency in the proliferation and/or survival of these cells (Fig. 1). [^{3}H]Thymidine incorporation experiments revealed that stromal cells from control and osteonectin-null mice did not have statistically significant differences in DNA labeling at 7 and 20 wk of age (Table 1).

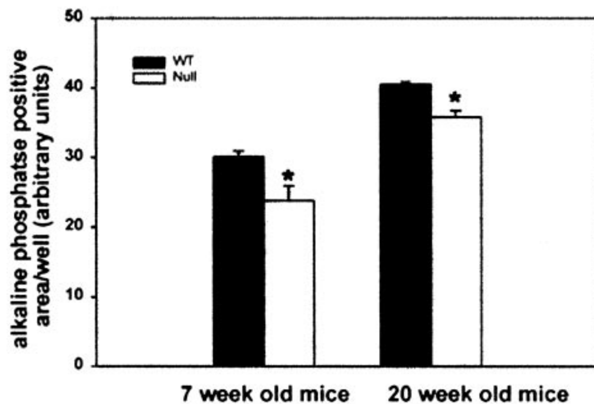

FIG. 1. Alkaline phosphatase-positive colony-forming units in marrow stroma from WT and osteonectin-null mice. Bone marrow stromal cells from 7- and 20-wk-old WT and osteonectin-null (Null) mice were cultured for 10 d in osteoblast differentiation medium and stained for alkaline phosphatase activity. The alkaline phosphatase-positive colony area in cultures of stromal cells was estimated from digitized images of the culture wells. Arbitrary units express alkaline phosphatase area per well, and six mice per group were used. *, Statistically different from age-matched control, $P \leq 0.01$.

One hallmark of an osteoblast is its ability to accumulate cAMP in response to stimulation with PTH (34). Therefore, differentiating stromal cell cultures were challenged with PTH, and cAMP accumulation was measured by RIA. WT and osteonectin-null cells exhibited a similar response to PTH at confluence and 1 wk post confluence; however, at 2 wk post confluence, the cAMP response in the mutant cells was blunted (Fig. 2). Together, the data acquired using stromal cells suggest that osteonectin-null stroma contains fewer osteoblastic precursors, and that osteonectin-null stromal cells can adopt an osteoblastic phenotype, although these cells may not be as functionally mature or robust as control cells.

This hypothesis was further examined in osteoblastic cells derived from neonatal mouse calvaria. Differentiating cultures of osteoblasts from osteonectin-null mice contained fewer mineralized nodules than those from controls (Fig. 3A). Northern blot analysis was used to determine the effect of the mutation on the expression of genes considered important for osteoblast differentiation (Fig. 3B). There was essentially no difference in α1(I) collagen or osteopontin mRNA between control and osteonectin-null cultures, but transcript levels for osteocalcin were decreased in the latter. In WT cells, osteocalcin mRNA increased throughout the time of culture, whereas in osteonectin-null cultures there was only a modest increase with time in culture. At 2 wk of culture, osteonectin-null cells had 73 ± 2% less osteocalcin mRNA than WT cells and 61 ± 5% less than WT at 3 wk of culture. These results suggest that a majority of osteonectin-null osteoblasts do not achieve a fully differentiated phenotype.

There is increasing evidence of plasticity between differentiated phenotypes in cells of the bone marrow, particularly between osteoblasts and adipocytes (35, 36). In osteoblast cultures from osteonectin-null mice, we noted increased numbers of cells containing oil red-staining lipid droplets, and this became more apparent as the cultures progressed (Fig. 4A). Northern blot analysis showed that the expression of genes important for adipocytic differentiation were increased in cultures of osteonectin-null osteoblasts. Whereas expression of peroxisome proliferator-activated receptor-γ2 mRNA, an early marker of adipocyte differentiation, was not affected by the osteonectin-null mutation, transcripts for adipsin, a late marker of adipocyte differentiation, were 1.7 ± 0.1-fold higher than WT at 2 wk and 3.0 ± 0.6-fold higher than WT at 3 wk in osteonectin-null cultures (Fig. 4B and data

TABLE 1. DNA synthesis in stromal cell cultures from WT and osteonectin-null mice

Time in culture (wk)	7-wk WT	7-wk Null	20-wk WT	20-wk Null
	[^{3}H]thymidine		dpm/mg	cell protein
1	2909 ± 254	2224 ± 113	1506 ± 47	1983 ± 223
2	915 ± 78	749 ± 42	488 ± 62	464 ± 70
3	993 ± 105	937 ± 115	456 ± 31	475 ± 40

Stromal cells were cultured in osteoblast differentiation medium for up to 3 wk and were pulsed with [^{3}H]thymidine for the last 2 h of culture. [^{3}H]Thymidine incorporation into DNA is expressed as dpm/mg cell protein, and data are shown as means ± SEM. Statistically significant differences ($P = 0.01$) were not detected; n = 12.

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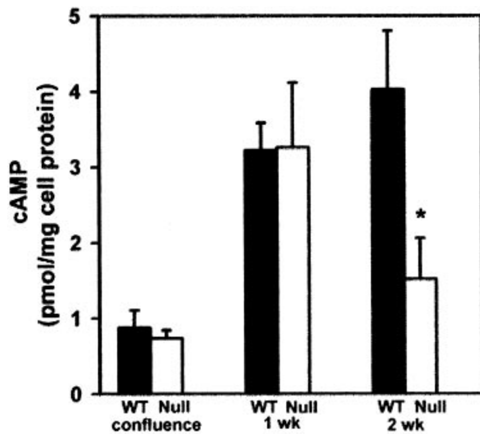

FIG. 2. Ability of WT and osteonectin-null stromal cell cultures to produce cAMP in response to 10 nM PTH. Bone marrow stromal cells from 24-wk-old mice were grown in osteoblast differentiation medium for up to 2 wk post confluence and then stimulated with PTH for 2 min. cAMP levels in cell lysates were assayed by RIA and normalized for total cell protein. Data are expressed as picomoles of cAMP per milligram of cell protein. *, Significantly different from control, $P \leq 0.01$ (n = 6 wells).

not shown). The C/EBP family of transcription factors plays an important role in both osteoblastic and adipocytic differentiation (10). Whereas the osteonectin-null mutation did not affect the expression of C/EBPβ mRNA, C/EBPδ transcripts were modestly, but consistently, increased in cultures of mutant cells undergoing *in vitro* differentiation (1.6 ± 0.1-fold *vs.* WT at 1 wk; 1.3 ± 0.1-fold *vs.* WT at 2 wk; 1.5 ± 0.1-fold *vs.* WT at 3 wk). These data indicate that osteoblastic cells from osteonectin-null mice are more highly disposed to form adipocytes than those from WT mice.

Osteonectin-null mice have fewer osteoblasts than controls, and [^{3}H]thymidine incorporation confirmed that, similar to stromal cells, the osteonectin-null mutation did not alter the proliferation rate of the osteoblastic cells under the culture conditions used (data not shown). Osteonectin-null osteoblasts may be more susceptible to stress-related death. Therefore, trypan blue exclusion was used to estimate cell survival under conditions of serum deprivation. This experiment showed that osteonectin-null cells did not survive stress as well as WT cells (Fig. 5).

We created immortalized cell lines from control and osteonectin-null osteoblastic cells to use as tools in the further study the function of osteonectin in bone. Initially, Alizarin Red and alkaline phosphatase staining were used to document that the cell lines retained the phenotype of their parental cells. Cells were cultured for up to 3 wk post confluence in osteoblast differentiation medium, and, as expected, osteonectin-null cultures contained fewer mineralized nodules (Fig. 6A). The ability to accumulate cAMP in response to PTH stimulation was used to assay the functional maturity of the mOb-I1 cell lines. Cells were cultured for 2 wk in osteoblast differentiation medium and challenged with increasing doses of PTH, and cAMP accumulation was determined. Compared with WT cells, the osteonectin-null osteoblastic cell line was less responsive to PTH, suggesting that these cells are not as differentiated as their WT counterparts (Fig. 6C). Furthermore, the expression profile of α1(I) collagen, osteocalcin, adipsin, and C/EBPδ in the cell lines was similar to that of the primary cultures (Fig. 6B and data not shown). For example, osteocalcin transcript levels were 71 ± 7% lower in osteonectin-null cells compared with WT at 4 wk and 55 ± 8% lower compared with WT at 5 wk. Adipsin transcript levels were 13.5 ± 3-fold higher in osteonectin-null cultures compared with controls at 4 wk and 10 ± 3-fold higher at 5 wk. As observed in the primary cultures, C/EBPδ mRNA levels were modestly, but consistently, increased in osteonectin-null cells (2.4 ± 0.9-fold *vs.* WT at 1 wk; 2.1 ± 0.3-fold *vs.* WT at 2 wk; 1.7 ± 0.05-fold *vs.* WT at 4 wk; 1.3 ± 0.03-fold *vs.* WT at 4 wk). Further, trypan blue exclusion showed that the osteonectin-null cell lines remained more susceptible to the stress of serum deprivation than WT cells (Fig. 7). Thus, the mOb-I1 and mObI-2 cell lines have phenotypes similar to the primary cells analyzed.

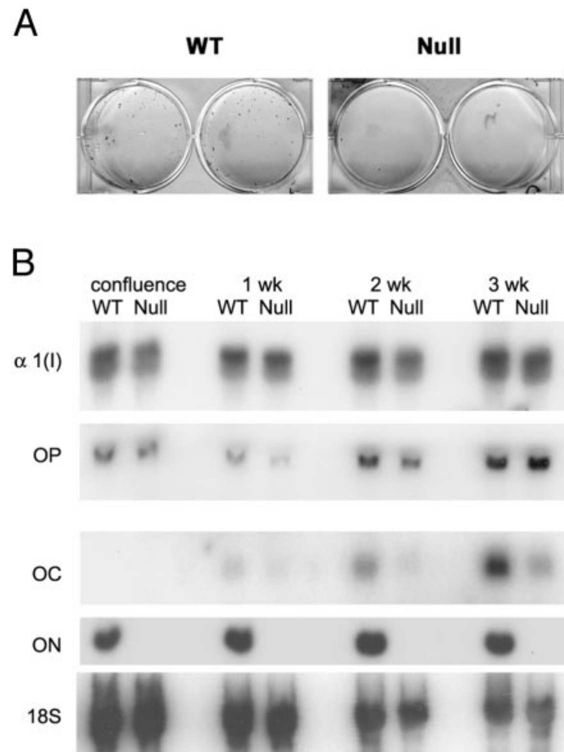

FIG. 3. Bone phenotype of primary cultures of osteoblasts from WT and osteonectin-null mice. A, Primary osteoblasts from neonatal mouse calvaria were cultured for 3 wk in osteoblast differentiation medium. Cells were fixed and subjected to von Kossa staining, which stains mineral black. Representative duplicate wells from cells of mixed genetic background are shown. B, Primary osteoblasts from neonatal mouse calvaria were cultured for up to 3 wk post confluence in osteoblast differentiation medium. Total RNA was isolated, subjected to Northern blot analysis, and probed with ^{32}P-labeled cDNA for α1(I) collagen [α1(I)], osteopontin (OP), osteocalcin (OC), and osteonectin (ON). Hybridization to cDNA for 18S RNA was used as a control for RNA loading. n = 3 replicate cultures. The data shown were obtained using cells from mice backcrossed into the C57/BL6 background, and similar data were obtained using cells from mice of mixed genetic background (not shown).

Recent studies revealed that over expression of ΔFosB, a Fos-related transcription factor arising from alternative splicing of FosB, stimulates osteoblastogenesis at the expense of adipogenesis (45). As osteonectin appears to have a similar

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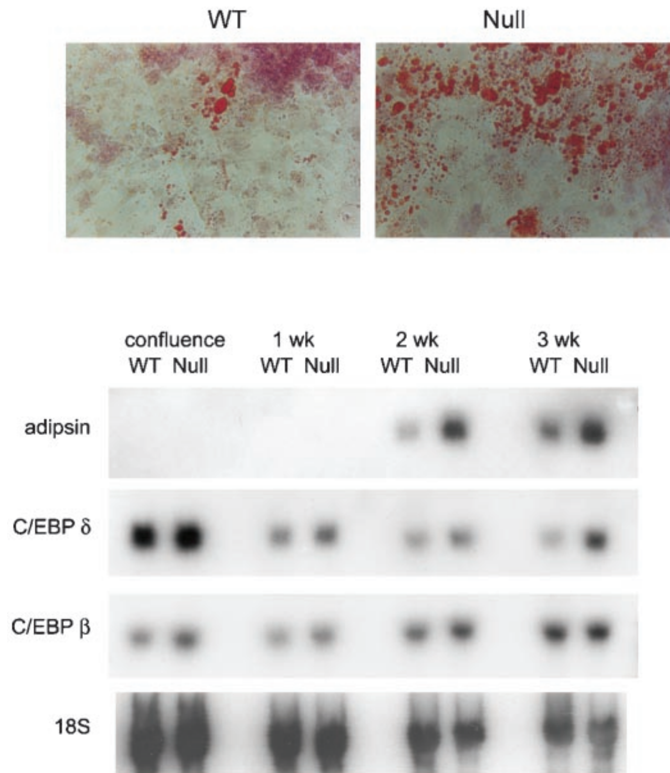

FIG. 4. Adipocytic phenotype of primary cultures of calvarial cells from WT and osteonectin-null mice. A, Oil Red staining of lipid droplets within primary cultures of control and osteonectin-null osteoblastic cells cultured for 2 wk in osteoblast differentiation medium. Representative 40-fold magnified fields are shown. Oil Red provides a bright *red* stain, whereas the hematoxylin counter stain is *purple*. B, Primary cells from neonatal mouse calvaria were cultured for up to 3 wk post confluence in osteoblast differentiation medium. Total RNA was isolated, subjected to Northern blot analysis, and probed with ^{32}P-labeled cDNA for adipsin, C/EBPβ, and C/EBPδ. Hybridization to cDNA for 18S RNA was used as a control for RNA loading. n = 3 replicate cultures. The data shown were obtained using cells from mice backcrossed onto the C57/BL6 background.

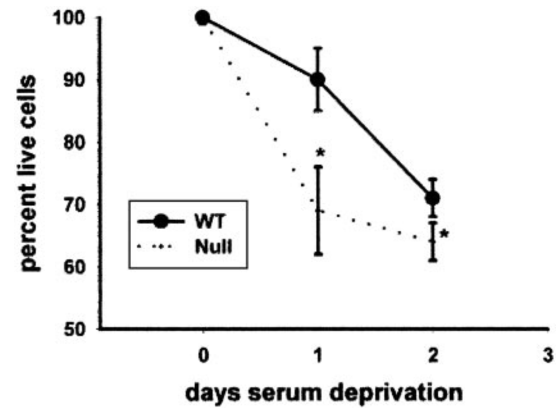

FIG. 5. Survival of primary cultures of WT and osteonectin-null osteoblasts subjected to the stress of serum deprivation. Cultures of osteoblastic cells at confluence (d 0) and 1 or 2 d after serum deprivation were trypsinized and counted. The number of surviving trypan blue-negative cells after 1 or 2 d of serum deprivation is expressed as a percentage of the cell number at confluence. *Solid line*, WT; *dotted line*, null. *, Statistically different from control, $P \leq 0.01$ (n = 6 wells).

function, we determined whether ΔFosB expression was altered in cultures of WT and osteonectin-null mOb-I2 osteoblastic cells by performing Western blot analysis of nuclear extracts from these cells (Fig. 8). ΔFosB expression increased as the cells under went *in vitro* differentiation, and its expression was slightly decreased in osteonectin-null cells at confluence and 1 wk of culture. Δ1ΔFosB and Δ2ΔFosB differ in length by 29 amino acids and result from the use of alternative translation start sites in the ΔFosB transcript. Levels of Δ1ΔFosB and Δ2ΔFosB, which could not be adequately resolved from each other under the electrophoresis conditions used, also increased as the cells differentiated; however, the expression of these isoforms was decreased in osteonectin-null cultures, most dramatically at 1 and 2 wk of culture. These data support the hypothesis that Δ1ΔFosB and Δ2ΔFosB play a role in mediating cell fate decisions in mesenchymal cells.

In a final set of experiments, replication incompetent retrovirus was used to deliver a recombinant, epitope-tagged osteonectin gene to the cell lines to determine whether the osteonectin-null mutation could be rescued. The expression of recombinant osteonectin was confirmed by Northern and Western blot analyses (Fig. 9A). In the Northern blot, the upper band represents transcript generated from the promoter within the viral long terminal repeat, whereas the lower band represents transcript generated from the CMV promoter. Cultures were grown for up to 3 wk post confluence in osteoblast differentiation medium, and staining for Alizarin Red-positive nodules showed that retroviral delivery of the osteonectin gene rescued the mineralization defect of the osteonectin-null cells (Fig. 9B). Interestingly, the expression of additional osteonectin in the control cells did not augment their osteoblastic phenotype, suggesting that these cells already exist in an osteonectin-replete environment. The recombinant osteonectin was also able to enhance the survival of the osteonectin-null cells under serum-free conditions (Fig. 9C). The data shown are for the mOb-I2 cell line, and similar effects were observed when the mOb-I1 cells were transduced with osteonectin-expressing retrovirus (data not shown).

Discussion

Previously, we found that osteonectin is important for normal remodeling and the maintenance of bone mass. Here, we demonstrate the cellular basis for this phenotype and provide data on potential molecular mechanisms. The skeletal defect in osteonectin-null mice results from decreased osteoblast and osteoclast numbers and decreased bone formation rate. As osteoclast development is highly dependent on osteoblast number and integrity, it stands to reason that decreased osteoclast number in osteonectin-null mice is due to decreased osteoblasts (37). The data presented here show that marrow stroma from osteonectin-null mice contains fewer osteoblastic precursor cells. As the osteonectin-null mutation did not appear to affect cell replication, it is likely that the cell survival defect significantly contributes to the decreased bone cell number seen *in vivo*.

Although osteonectin-null osteoblasts express transcripts for important bone matrix components, these cells do not

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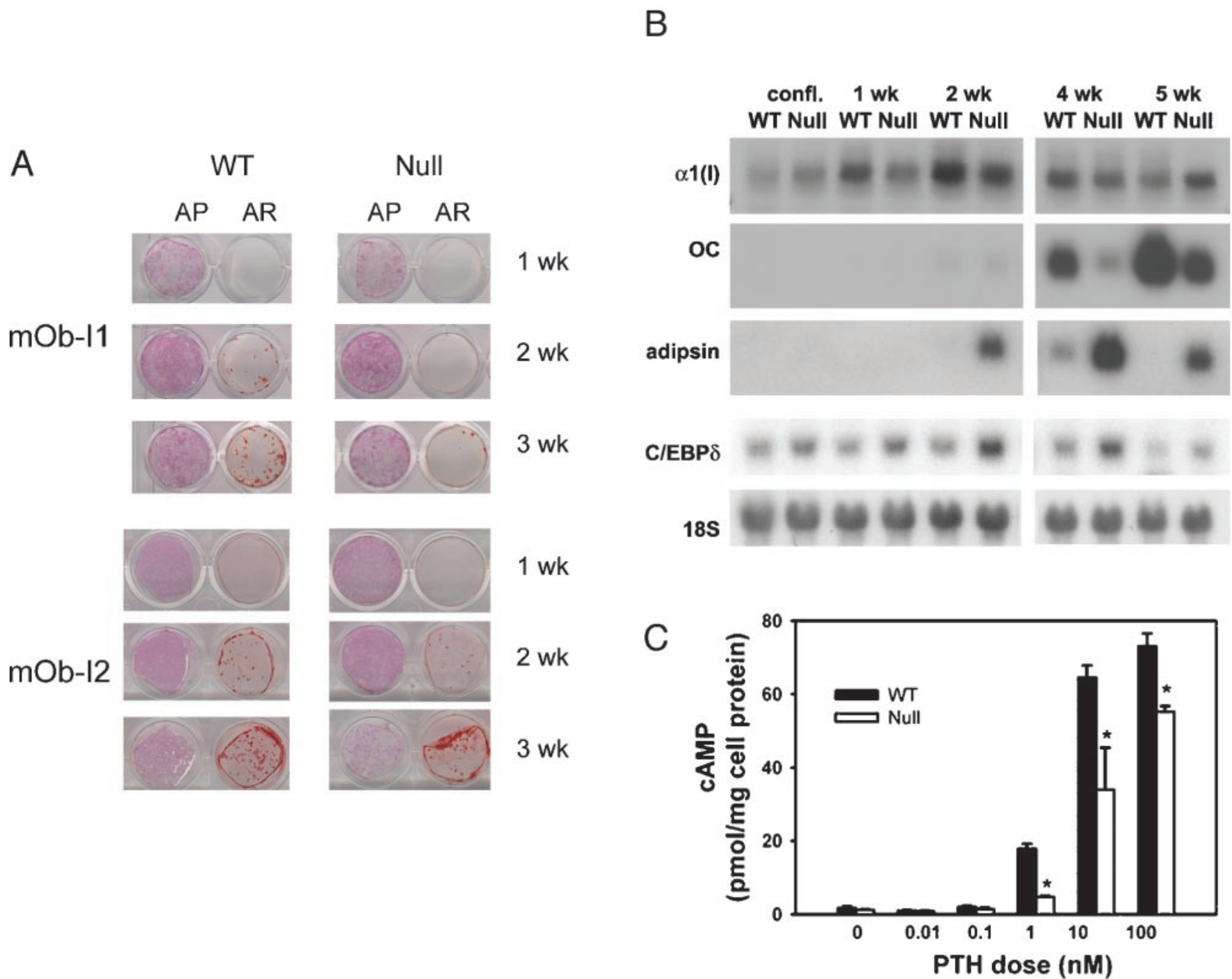

FIG. 6. Osteoblastic phenotype of osteoblastic cell lines made from WT and osteonectin-null mice. A, Alizarin Red (AR) and alkaline phosphatase (AP) staining of osteoblastic cell lines made from control and osteonectin-null mice cultured for up to 3 wk in osteoblast differentiation medium. mOB-I1, Immortalized mouse osteoblasts from mice of mixed genetic background; mOB-I2, immortalized mouse osteoblasts from mice backcrossed onto C57BL/6 background. B, WT and osteonectin-null osteoblast cell lines (mOb-I2) were cultured for up to 5 wk post confluence in osteoblast differentiation medium. Total RNA was isolated, subjected to Northern blot analysis, and probed with ^{32}P-labeled cDNA for $\alpha 1$(I) collagen [$\alpha 1$(I)], osteocalcin (OC), adipsin, and C/EBPδ. Hybridization to cDNA for 18S RNA was used as a control for RNA loading. n = 3 replicate cultures. C, Ability of WT and osteonectin-null osteoblast cell lines (mOb-I1) to produce cAMP in response to stimulation with doses of PTH up to 100 nM. Cells were grown in osteoblast differentiation medium for 2 wk post confluence and then stimulated with PTH for 2 min. cAMP levels in cell lysates were assayed by RIA and normalized for total cell protein. Data are expressed as picomoles of cAMP per milligram of cell protein. *, Significantly different from control, $P \leq 0.01$ (n = 6 wells).

achieve a fully mature phenotype, evidenced by decreased abundance of osteocalcin mRNA and decreased responsiveness to PTH. Our data suggest that osteonectin-null cells may be more likely to pursue adipocytic differentiation at the expense of the osteoblastic pathway. Although lipid-filled cells are occasionally seen in cultures of osteoblastic cells, such adipocytic cells were obvious in osteonectin-null osteoblast cultures. In cultures of WT cells, transcripts for the adipocytic marker adipsin are increased in the matrix mineralization phase of the culture (10). The expression of this adipocytic marker is more pronounced in osteoblastic cultures from osteonectin-null mice. Further, RNA for C/EBPδ, a transcription factor critical for adipogenesis, is increased in osteonectin-null osteoblastic cells, whereas the levels of Δ1/2ΔFosB, a transcription factor that stimulates osteoblastogenesis at the expense of adipogenesis, are decreased. Others have noted increased sc fat in osteonectin-null mice compared with controls (1). Our findings on the *trans*-differentiation of osteonectin-null osteoblastic cells to adipocytes are consistent with the idea that the presence of osteonectin within a matrix may provide an antiadipogenic signal. Both preadipocytes and preosteoblasts take differentiation cues from their extracellular matrix (5, 38). Given the pluripotential nature of marrow stromal cells, the presence of osteonectin in the bone matrix could provide signals that divert stromal cell differentiation toward the osteoblastic pathway at the expense of adipogenesis.

Several recent studies have suggested somewhat conflicting roles for C/EBPs in osteoblastic differentiation. C/EBPδ has been reported to enhance prostaglandin-induced tran-

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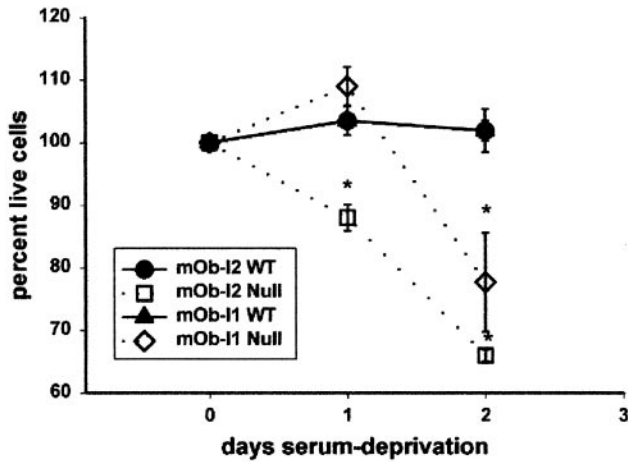

FIG. 7. Survival of WT and osteonectin-null osteoblast cell lines exposed to serum deprivation. Cultures of osteoblastic cell lines at confluence (d 0) and 1 or 2 d after serum deprivation were trypsinized and counted. The number of surviving trypan blue-negative cells after 1 or 2 d of serum deprivation are expressed as a percentage of the cell number at confluence. Note that the curves for mOb-I1 and mOb-I2 are superimposable. *, Statistically different from control, $P \leq 0.01$ (n = 6 wells).

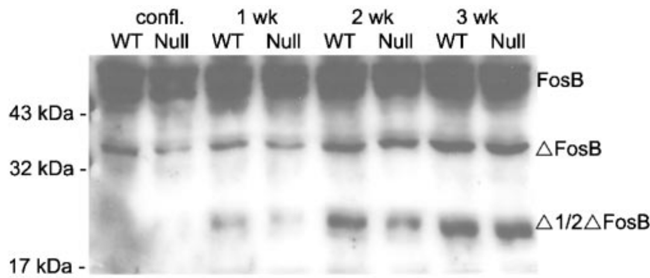

FIG. 8. Expression of FosB isoforms in osteoblastic cell lines made from WT and osteonectin-null mice. Western blot analysis was performed on nuclear extracts prepared from WT or osteonectin-null (Null) mObI-2 cells cultured for up to 3 wk post confluence in osteoblast differentiation medium. Western blots were probed with primary antibody against FosB that can detect all isoforms. The ΔFosB isoform results from alternative splicing, and the Δ1/2ΔFosB isoform results from alternative translation initiation within the ΔFosB transcript. n = 3 replicate cultures.

scription from the IGF-I promoter and to synergize with the Runt-related transcription factor Runx2 to stimulate osteocalcin gene transcription (39, 40). However, cortisol repression of the rat IGF-I promoter is mediated through a C/EBP-binding site, and cortisol induces the expression of C/EBPδ and -β in osteoblasts (10, 41). Cortisol stimulates adipogenic differentiation of stromal cells, and the inverse relationship between marrow fat and trabecular bone volume is well known (10, 42). Interestingly, transgenic mice overexpressing a dominant negative form of C/EBPβ in bone have both osteopenia and decreased adiposity (43). These data indicate that C/EBPs are important for both osteoblast and adipocyte differentiation, but their mechanisms of action are probably more complex than currently appreciated (44).

Increased levels of the Δ1/2ΔFosB isoforms of ΔFosB are associated with increased osteoblastic differentiation and decreased adipogenesis. Indeed, transgenic mice overexpressing ΔFosB develop osteosclerosis and have decreased fat in both bone marrow and extramedullary tissue (45). Osteonectin-null osteoblastic cells have decreased levels of Δ1/2ΔFosB compared with controls, and the mice have decreased adipose tissue. These data support the concept that

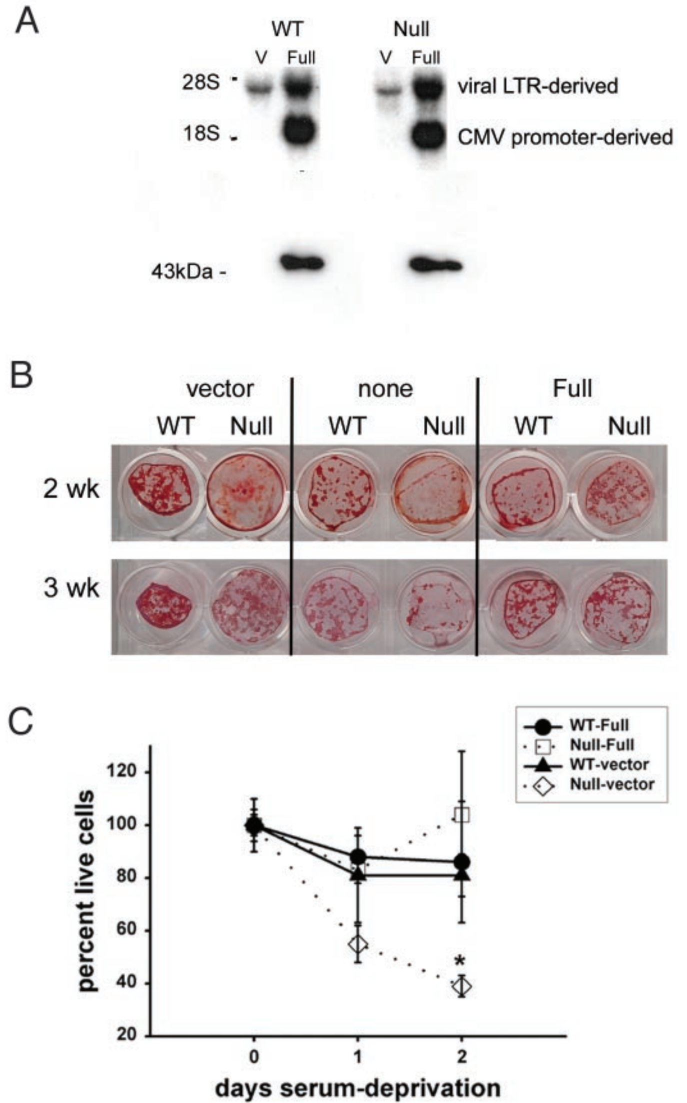

FIG. 9. Rescue of the mutant phenotype of osteonectin-null osteoblast cell lines using retroviral gene transfer of constitutively expressed, epitope-tagged mouse osteonectin. A, Northern (*top*) and Western (*bottom*) blot analyses documenting the expression of recombinant osteonectin RNA and protein, respectively. V, Cells transduced with empty vector; Full, cells transduced with vector expressing tagged, full-length osteonectin. The Northern blot was probed with ^{32}P-labeled cDNA corresponding to the Myc-histamine tag, and the Western blot was probed with primary antibody against the Myc portion of the epitope tag. B, Representative Alizarin Red staining of untransduced (none) osteoblastic cell lines or those transduced with vector alone (vector) or retrovirus carrying full-length osteonectin (Full). Cells were cultured for up to 3 wk in osteoblast differentiation medium. n = 3 replicate cultures. C, Survival of osteoblastic cell lines transduced with retrovirus expressing osteonectin. Cultures of osteoblastic cell lines at confluence (d 0) and 1 or 2 d after serum deprivation were trypsinized and counted. The numbers of surviving trypan blue-negative cells after 1 or 2 d of serum deprivation are expressed as a percentage of the cell number at confluence. *, Statistically different from control, $P \leq 0.05$ (n = 6 wells).

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levels of ΔFosB impact cell fate decisions in marrow and probably elsewhere, and that the Δ1/2ΔFosB isoforms, in particular, are active in this role. Although ΔFosB and its Δ1/2ΔFosB isoforms lack the classical C-terminal *trans*-activation domain of FosB, they can still act as activators or repressors of *trans*-activation and retain the ability to interact with Jun family members (45, 46). Our findings suggest that the composition of the extracellular matrix could regulate ΔFosB translation and provide a means for modulating osteoblastogenesis.

Osteonectin-null cells are more susceptible to environmental stresses, and retroviral delivery of a constitutively expressed osteonectin gene rescues this defect. C/EBPδ has been shown to induce growth arrest and apoptosis in mouse mammary tumor cells, and it is possible that increased C/EBPδ expression in osteonectin-null cells could play a role in their sensitivity to stress (47). We suggest that osteonectin provides a survival function for cells in times of stress. Indeed, osteonectin expression is induced in cells undergoing stress, as observed in culture shock, oxidative stress, and matrix remodeling (1, 48). The recent observation that osteonectin is markedly increased in adipocytes in early obesity may also be related to a stress response associated with the induction of the disease state (49). In addition, differentiating adipocytes acquire a relative resistance to apoptosis, and increased osteonectin expression may play a role in this phenomenon (50).

Osteonectin is a matricellular protein that has been assigned multiple functions that appear to vary with the cell system, culture conditions, or the fragment of the protein used (1). Our research confirms that the functions associated with osteonectin are very much dependent on the cell system investigated and the culture conditions used. For example, other investigators have shown that mesangial cells from osteonectin-null mice have decreased expression of type I collagen and TGFβ mRNA and protein (51). However, osteoblastic cells from control and osteonectin-null mice have similar levels of these transcripts (Delany, A. M., unpublished data). Other investigators showed increased proliferation in osteonectin-null mesenchymal cells; however, we did not observe this in our culture system, nor was this suggested by our *in vivo* data (6, 52). Further, others have shown that the addition of exogenous osteonectin to selected cell types induces the expression of the metalloproteinases 92-kDa gelatinase, collagenase, and stromelysin (53). Matrix metalloproteinases are important in bone remodeling, and in theory, one would expect osteonectin-null osteoblastic cells to have decreased expression of enzymes. However, altered expression of metalloproteinases collagenase-3 and gelatinase B was not detected in osteonectin-null osteoblasts (Delany, A. M., unpublished data).

We conclude that in the skeleton, osteonectin is necessary for normal osteoblast formation, maturation, and survival. The presence of osteonectin within the bone matrix provides an environment that supports osteoblastic differentiation at the expense of adipogenesis. In total, osteonectin is necessary for normal bone remodeling, maintenance of bone mass, and bone quality.

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