Col1a1 Promoter-targeted Expression of p20 CCAAT Enhancer-binding Protein β (C/EBPβ), a Truncated C/EBPβ Isoform, Causes Osteopenia in Transgenic Mice*

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CCAAT enhancer-binding protein (C/EBP) transcription factors regulate adipocyte differentiation, and recent evidence suggests that osteoblasts and adipocytes share a common pluripotent progenitor in bone marrow. However, little is known about the role of C/EBP transcription factors in the control of osteoblast differentiation or function. In this study, the function of C/EBP transcription factors was disrupted in osteoblast lineage cells by overexpressing a naturally occurring dominant negative C/EBP isoform. Expression of FLp20C/EBPβ was driven by a 3.6-kb Col1a1 promoter/first intron construct, and four transgenic (TG) mouse lines were established. Northern blotting and reverse transcription-PCR indicated that the transgene was targeted to bone, with lower levels of expression in lung, skin, and adipose tissue. TG mice from two lines showed reduced body weight compared with wild type littermates. All TG lines showed evidence of osteopenia, ranging from mild to severe, as evidenced by reduced trabecular bone volume. Severely affected lines also showed reduced cortical bone width. Dynamic histomorphometry demonstrated an associated decrease in mineral apposition and bone formation rates. Long bones and calvariae of TG mice showed reduced Col1a1 and osteocalcin mRNA levels and increased bone sialoprotein mRNA, consistent with an inhibition of terminal osteoblast differentiation. Ex vivo analysis of primary osteoblast differentiation showed similar effects on marker expression and reduced expression of the mature osteoblast marker Col2.3-green fluorescent protein, demonstrating a cell-autonomous effect of the transgene. These data suggested that C/EBP transcription factors may be important determinants of osteoblast function and bone mass.

CCAAT enhancer-binding proteins (C/EBPs)1 comprise a family of homologous transcription factors characterized by a carboxy-terminal leucine zipper dimerization domain and an adjacent highly conserved basic DNA binding domain (1, 2). C/EBPs are transcriptional regulators of gene expression, particularly those involved in energy metabolism (3) and immune or inflammatory responses (4–6). Over the past several years, C/EBPs have been shown to control cellular differentiation in several lineages, including hepatocytes (7), granulocytes (8), macrophages (9), and adipocytes (10). C/EBP regulation of adipogenesis represents the best characterized system to date. Collectively, the data from a number of pluripotent and pre-adipocytic cell lines support a model in which C/EBPβ and C/EBPδ activation represents the seminal event in the process, regulating commitment of progenitor cells to the adipocyte lineage (11). Subsequently, C/EBPδ and C/EBPβ induce expression of C/EBPα and peroxisome proliferator-activated receptor-γ, which in turn direct expression of terminal adipocyte marker genes (12–14). Work on C/EBP null mice has confirmed that C/EBPβ and C/EBPδ regulate commitment of progenitor cells to the adipocyte lineage, whereas loss of C/EBPα function interferes with terminal adipocyte differentiation and function (15).

A wealth of recent evidence suggests that the osteoblast and adipocyte lineages are closely related, diverging from a common pluripotent progenitor cell (16–18). However, a possible role for C/EBP transcription factors in osteoblast lineage progression has not been investigated. In the present study, we have used a loss-of-function strategy to test the hypothesis that C/EBP transcription factors play a role in osteoblast differentiation and/or function. Toward this end, a naturally occurring dominant negative C/EBPβ isoform (p20C/EBPβ, LIP) (19) has been targeted to cells of the stromal/osteoblast lineage using a 3.6-kb Col1a1 promoter fragment (pBOCol3.6-FLp20C/EBPβ). Mice expressing this transgene show evidence of osteopenia secondary to reduced bone formation, suggesting a role for C/EBP transcription factors in the regulation of osteogenesis.

EXPERIMENTAL PROCEDURES

DNA Constructs—The FLAGp20C/EBPβ (FLp20C/EBPβ) construct was prepared by PCR using the original cytofelovirus-LIP plasmid (kindly provided by Dr. S. McKnight) as template. The upstream primer was phosphorylated to allow unidirectional cloning and contained a non-complementary 5'-extension corresponding to the endogenous p20C/EBPβ Kozak sequence and the FLAG epitope. The downstream primer corresponded to the carboxyl-terminal leucine zipper domain and contained a stop codon. Following PCR amplification with Taq polymerase, a product of the predicted size (467 bp) was obtained and unidirectionally cloned into pCR3.1-Uni, a TA expression vector containing the cytofelovirus promoter for mammalian expression and a computed tomography; MOPS, 3-(N-morpholino)propanesulfonic acid; GFP, green fluorescent protein; BMD, bone mineral density; OC, osteocalcin; BSP, bone sialoprotein; WT, wild-type; TG, transgenic.

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§ The abbreviations used are: C/EBP, CCAAT enhancer-binding protein; DEXA, dual energy x-ray absorptiometry; microCT, microcomputed tomography; MOPS, 3-(N-morpholino)propanesulfonic acid; GFP, green fluorescent protein; BMD, bone mineral density; OC, osteocalcin; BSP, bone sialoprotein; WT, wild-type; TG, transgenic.

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8117
p20C/EBPα Expression Causes Osteopenia in Transgenic Mice

T7 RNA polymerase site for in vitro translation. The predicted Flp20C/EBPα expression was confirmed by sequencing.

To target expression of Flp20C/EBPα broadly throughout the osteoblast lineage, a set of IRES-Flp20C/EBPα containing constructs were prepared. Briefly, pCR3.1-Flp20C/EBPα was digested with HindIII and XbaI, and the resulting Flp20C/EBPα fragment was subcloned into an intermediate Clia vector that contains a polylinker and the bovine growth hormone polyadenylation sequence flanked by two Clia sites. Following Clia digestion, the Flp20C/EBPα-hovine growth hormone cassette (792 bp) was isolated and subcloned into pBCKSK+ (ClonIn5.2Xba), which contains a Clia site downstream of the 3.6 kb Col1α promoter and 1.6 kb Col1α first intron. Clones containing Flp20C/EBPα in the correct orientation were identified using asymmetric restriction cuts. The final construct is shown schematically in Fig. 2.

Cell Culture, Transfection, and Immunocytochemistry—Osteoblastic murine MC3T3-E1 cells and immortalized rat Py-1a MC3T3-E1 were plated at 5000 cells/cm² in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 50 μg/ml streptomycin in a humidified atmosphere of 5% CO2 at 37 °C. Cells were transiently transfected with Flp20C/EBPα (pCR3.1-Flp20C/EBPα), C/EBPα expression plasmids, and a 371 bp cyclo-oxygenase 2 promoter-luciferase reporter construct (20) using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. For immunostaining, transiently transfected cells were fixed in 2% formaldehyde, treated with Nonidet P-40, and blocked in buffer containing 5% nonfat milk. Cells were incubated with M2 anti-FLAG antibody (Sigma) overnight at 4 °C at a dilution of 1:2500. After washing with phosphate-buffered saline, cells were incubated with avidin-conjugated anti-rabbit IgG (Vector Labs, Burlingame, CA) for 45 min, horseradish peroxidase-conjugated streptavidin (Zymed Laboratories Inc., San Francisco, CA) for 45 min, and chromogen 3-amino-9-ethyl-carbazole (Sigma) for 20 min and counterstained with hematoxylin.

In Vitro Translation and Mobility Shift Analysis—10 pmol of each complementary single-stranded deoxyoligonucleotide corresponding to positions 171 to 142 of the interleukin-6 promoter was separately incubated with 5× kinase forward reaction buffer, (1)71-142ATP (3000 Ci/mmol; PerkinElmer Life Sciences) and T4 DNA kinase (Invitrogen) for 60 min at 60 °C for 75 min, and exposed to x-ray film at room temperature. A series of in vivo experiments was undertaken to confirm that Flp20C/EBPα exhibits appropriate nuclear localization, DNA binding, and dominant negative function. To ensure proper intracellular trafficking of the transgene product, transient transfection of the immortalized Py-1a rat osteoblast cell line was performed. These cells were chosen because of their high transfection efficiency. 48 h post-transfection, cells were fixed and Flp20C/EBPα was localized by immunocytochemistry. As shown in Fig. 1a, ~60% of the Py-1a cells stained positive for Flp20C/EBPα, with most of the transgene protein appropriately localized in an intranuclear staining pattern. Dynamic immunohistomorphometric measurements were made using the BioQuant system. The terminology and units used are those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research (21).

RESULTS

A series of in vitro experiments was undertaken to confirm that Flp20C/EBPα exhibits appropriate nuclear localization, DNA binding, and dominant negative function. To ensure proper intracellular trafficking of the transgene product, transient transfection of the immortalized Py-1a rat osteoblast cell line was performed. These cells were chosen because of their high transfection efficiency. 48 h post-transfection, cells were fixed and Flp20C/EBPα was localized by immunocytochemistry. As shown in Fig. 1a, ~60% of the Py-1a cells stained positive for Flp20C/EBPα, with most of the transgene protein appropriately localized in an intranuclear staining pattern. Dynamic immunohistomorphometric measurements were performed using the BioQuant system. The terminology and units used are those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research (21).
p20C/EBPβ Expression Causes Osteopenia in Transgenic Mice

Figure 1. Nuclear localization, DNA binding, and dominant negative function of FLAG-tagged p20C/EBPβ in vitro. a, transient transfection of FLp20C/EBPβ under control of the cytomegalovirus promoter was performed in rat Py-1a cells. Transgene protein was visualized by immunocytochemistry using the anti-FLAG M2 antibody. Examples of dark brown staining positive nuclei are indicated by arrowheads. b, mobility shift analysis showing binding of in vitro translated FLp20C/EBPβ to a C/EBP binding site. Binding was specifically competed by an oligonucleotide containing a C/EBP binding site and was supershifted by M2 anti-FLAG and anti-C/EBPβ antibodies. c, co-transfection study in MC3T3-E1 cells showing dominant negative inhibition of C/EBPβ-stimulated cyclo-oxygenase 2 promoter activity by FLp20C/EBPβ.

EBPβ binding was supershifted and/or reduced by the anti-FLAG M2 and anti-C/EBPβ antibodies.

The ability of FLp20C/EBPβ to function as a dominant negative transcription factor was determined by co-transfection of FLp20C/EBPβ and C/EBPβ in MC3T3-E1 cells. As shown in Fig. 1c, C/EBPβ overexpression caused a significant stimulation of the cyclo-oxygenase 2 promoter, an established C/EBPβ target (20). Co-expression of FLp20C/EBPβ caused a dose-related inhibition of C/EBPβ-stimulated promoter activity, demonstrating that FLAG-tagged p20C/EBPβ retains its dominant negative function.

Transgenic Mice—FLp20C/EBPβ was cloned into the pOBCol3.6 promoter construct containing the 1.6-kb Col1a1 first intron and the bovine growth hormone 3′-untranslated region and polyadenylation site (Fig. 2a). This construct was microinjected into CD-1 oocytes, and four TG lines were established. Relative to the WT littermates, lines 50–9 and 63 TG mice showed normal body mass, whereas lines 50–10 and 65 TG mice were ~40% smaller at 6 weeks of age (Fig. 2, b and c). Examination of line 50–10 mice late in embryogenesis (E18) revealed a decreased body weight, suggesting that the small size was a primary effect of transgene expression. The greatest difference in body weight was observed at 6 weeks of age. By 5 months, TG mice were only slightly smaller than their WT littermates (Fig. 2c).

Tissue Specificity of Transgene mRNA Expression—To determine the pattern of the FLp20C/EBPβ transgene expression in various tissues, RNA was extracted from calvaria, long bone, bone marrow, adipose tissue, brain, kidney, heart, lung, liver, and skin of 1-month-old TG mice (50–10) and their WT littermates. Expression of transgene mRNA was determined by Northern blotting with a C/EBPβ probe, taking advantage of its unique size to distinguish it from endogenous C/EBPβ mRNA. As shown in Fig. 3a, endogenous C/EBPβ was expressed in a variety of tissues, consistent with the ubiquitous expression pattern of this gene that has been reported previously (22). On the other hand, transgene mRNA expression was detected by Northern blot analysis only in calvariae and long bones of TG mice (Fig. 3a). Note that these tissues showed very high expression of endogenous COL1A1 mRNA relative to other tissues examined. Interestingly, the amount of steady-state transgene mRNA in line 50–10 was comparable with that of endogenous C/EBPβ mRNA. These results were corroborated by RT-PCR analysis of transgene mRNA using a unique 3′-FLAG primer and a 5′-primer that recognizes the first exon of the Col1a1 gene. By RT-PCR, transgene mRNA was detected at high levels in calvariae and long bones of TG mice (Fig. 3b). Lower levels of transgene mRNA expression were observed in tissues known to have low levels of endogenous COL1A1 expression, such as lung and skin (Fig. 3b; note that TG lanes 3–10 were exposed longer to demonstrate expression). These results are consistent with prior data on Col1a1-targeted transgene expression using chloramphenicol acetyltransferase and GFP reporters (23).
Osteopenia in Transgenic Mice—Bone mineral density and bone morphometry were examined in mice by the complementary approaches of DEXA, microCT, and histomorphometry. Although the data reported are on female mice, similar changes have been observed in males. DEXA was used to measure femoral BMD and total body fat content at various ages in female mice from lines 50–9, 50–10, and 63 (Fig. 4, top panel). Line 65 was not included in this analysis because of difficulty in obtaining older animals from this line. A significant reduction of BMD was observed in TG mice of lines 63 and 50–10 at ages ranging from 11 weeks to 10 months. No change in BMD was seen in line 50–9 TG mice during this time frame. Total body fat was not affected in TG mice at 11 weeks or 6 months of age. A significant decrease in total body fat was seen only in line 50–9 transgenic mice at 10 months of age (Fig. 4, bottom panel).

Trabecular bone volume in the distal femur was analyzed by microCT at 6 weeks of age (Fig. 5a–d). As shown in Fig. 5, a and b, line 50–10 showed a striking reduction in the amount of trabecular bone in the distal femur. Line 65 was even more severely affected, showing a trabecular bone volume of only 1.5% compared with 19.6% in WT and 5% in line 50–10 (Fig. 5d). Line 63 was moderately osteopenic, showing a trabecular bone volume of 10.3%. Corresponding decreases in trabecular number were observed in all three affected lines (Fig. 5d). As shown in Fig. 5, c and d, the slight decreases in trabecular bone volume and trabecular number for line 50–9 TG mice did not reach statistical significance. However, a follow-up study on an additional cohort of line 50–9 demonstrated a slight but significant decrease in TG trabecular bone volume compared with their WT littermates (WT 32.0 ± 1.8%, TG 23.9 ± 2.8%), suggesting that this line exhibits a mild trabecular osteopenia. Cortical thickness at the mid-diaphysis, measured by microCT, was reduced in lines 50–10 and 65, but not in line 50.9 (Fig. 5f; WT, 0.22 ± 0.02 mm; Line 50–9, 0.24 ± 0.03; Line 50–10, 0.15 ± 0.01; Line 65, 0.16 ± 0.01).

As a complementary approach to microCT, two lines were examined by static histomorphometry. Distal femurs from lines 50–9 and 50–10 were examined at 6 weeks of age. In accordance with the microCT data, line 50–10 showed severe osteopenia with a trabecular bone area (trabecular area/total tissue area) of 1.9% compared with 19.6% in WT femurs (Fig. 6, top panels; Table I). TG 50–10 mice also showed a significant decrease in the cortical width of the femur by histomorphometry (Table I). Consistent with the microCT findings, line 50–9 showed a tendency toward a reduced trabecular bone area that did not reach statistical significance. Tartrate-resistant acid phosphatase (TRAP) staining did not show a change in osteoclast number/unit bone surface. Dynamic histomorphometry was performed by sequential injection of calcine and xylene orange 10 days apart. As shown in Fig. 6 (lower panels), the interlabel distance was reduced in line 50–10 TG mice, indicating decreased mineral apposition and bone formation rates (Table II).

Expression of Transgenic and Osteoblast mRNA Markers in Bone—To determine whether osteoblast gene expression was affected in TG mice, we assessed the mRNA levels of the transgene as well as the osteoblast markers COL1A1, BSP, and OC in multiple TG lines by Northern blot analysis. We hypothesized that the level of transgene mRNA expression might account for differences in the degree of osteopenia observed between TG lines. As shown in Fig. 7, a and b, transgene mRNA levels in calvariae and long bones correlated with the extent of the osteopenia observed in the four TG lines, with the highest level of transgene expression seen in line 65. Transgene expression in line 50–10 was generally comparable with line 65, whereas line 50–9 showed the lowest expression, barely detectable by Northern blot analysis (Fig. 7b) but clearly demonstrable by RT-PCR (Fig. 7c). Although occasional variability was seen in individual mice, OC and COL1A mRNA levels were reproducibly decreased in the bones of TG mice, whereas BSP levels, in contrast, were increased (Fig. 7, a, b, and d). As expected, OC mRNA levels were lowest in bones from line 65 TG mice. Line 50–9, which exhibited only a mild trabecular osteopenia, exhibited qualitatively similar changes in the pattern of marker gene expression, although the changes were generally more modest than those seen in severely affected lines (Fig. 7, b and d). These alterations in the expression of bone markers were observed in both male and female mice of all TG lines (Fig. 7d).
Ex Vivo Primary Osteoblast Cultures—To determine whether the observed effects on osteoblast function represent a cell-autonomous effect of the transgene, we examined the differentiation of primary calvarial osteoblasts in vivo using line 50–10. These studies were carried out in a genetic background containing the pOB-Col2.3-GFP transgene, in which expression is restricted to mature osteoblasts, providing a real-time marker of terminal osteoblast differentiation. As shown in Fig. 8, A and B, GFP-positive nodules appeared in WT cultures by 14 days. Over time, these nodules increased in size and were always associated with regions undergoing mineralization, visible in the bright field images. Fig. 8A shows individual WT and TG nodules followed in real time. Compared with WT nodules, those in TG cultures demonstrated less intense expression of Col2.3-GFP, an effect that persisted from their first appearance at day 14 until the end of the experiment on day 28. Fig. 8B represents macroscopic fluorimaging of GFP in two WT and TG wells at each time point, demonstrating that GFP expression was uniformly reduced in all nodules. As shown in Fig. 8C, Northern blot analysis of mRNA expression in these cultures reflected the changes observed in vivo, with decreased OC and COL1A1 expression accompanied by increased expression of BSP.
DISCUSSION

In the present study we have demonstrated that TG mice with pOBCol3.6-targeted overexpression of p20C/EBPβ show evidence of osteopenia ranging from mild to severe. Reduced femoral trabecular bone volume and cortical thickness in these mice was associated with reduced expression of COL1A1 and OC mRNA and a decreased bone formation rate as determined by dynamic histomorphometry. Because osteocalcin number was not altered, it appears that these mice likely display a low turnover osteopenia secondary to reduced osteoblast differentiation and/or function.

Prior studies on patterns of Col1a1 transgene expression have demonstrated that pOBCol3.6 is broadly expressed temporally and spatially in osteoblast lineage cells. This promoter fragment drives expression of chloramphenicol acetyltransferase and GFP in mature osteoblasts located directly on the bone surface as well as in pre-osteoblasts localized in the peristeme at some distance from bone (24). In bone marrow stromal cell cultures, pOBCol3.6 expression is turned on with early markers of the osteoblast lineage such as alkaline phosphatase and endogenous COL1A1 mRNA and prior to more differentiated osteoblast markers such as BSP and OC (25, 26). In addition, pOBCol3.6 is expressed in other COL1A1-producing tissues such as lung and skin (23). At the outset of these studies, we considered the possibility that targeting p20C/EBPβ expression with pOBCol3.6 could result in an inhibition of adipogenesis. Osteoblasts and adipocytes share a common progenitor cell, and C/EBP transcription factors are known to be key regulators of adipocyte commitment and differentiation. Because pre-adipocytes are known to express COL1A1 (27), we reasoned that pOBCol3.6-targeted FLp20C/EBPβ might interfere with initiation of the adipogenic program by C/EBPβ and C/EBPδ. However, analysis of total body fat by DEXA revealed no differences in total body fat by DEXA (8 months: WT 12.3 ± 1.1%, n = 7; TG 14.7 ± 0.2%, n = 10). Therefore, although we have not ruled out possible subtle effects on body fat in older mice, it is clear that young p20C/EBPβ TG animals exhibit a pronounced bone phenotype in the absence of major alterations in total body fat. We have observed numerous adipocytes in the bone marrow of line 50–10 mice, which are severely osteopenic (Fig. 6, top panel), demonstrating that the reciprocity between trabecular bone and bone marrow adiposity observed in other models of osteopenia appears to be preserved in these mice.

The inability of the transgene to inhibit adipogenesis could relate to either the level of expression attained in pre-adipocytes, the transient nature of transgene expression in these cells, or both. COL1A1 expression is extinguished during adipocyte differentiation and is not expressed in mature adipocytes (27–30). Therefore, if dominant negative inhibition by p20C/EBPβ is incomplete in adipocyte progenitors, residual C/EBP activation could promote entry of these cells into the adipogenic program, causing extinction of transgene expression and unimpeded terminal adipocyte differentiation. Finally, although C/EBP function is indispensable for adipocyte differentiation in vitro, it should be noted that some differentiation of white fat did occur in C/EBPβ/C/EBPδ double knockout mice (15), suggesting the existence of alternative differentiation pathways in vivo.

Two TG lines, 50–10 and 65, showed reduced body size relative to their TG littermates. These lines showed evidence of a tooth phenotype grossly manifested by malocclusion, breakage, and overgrowth of the incisors. This appears to be associated with a dentin dysplasia phenotype that will be reported elsewhere. Mice exhibiting a tooth phenotype were provided with granulated food ad libitum to ensure adequate nutrition. Because significant differences in body mass were seen prior to weaning and in utero in lines 50–10 and 65, the observed decrease in body mass does not appear to result from a nutritional deficit secondary to the dental phenotype. Although a decrease in body mass would be predicted to yield a lower bone mineral density (31), the osteopenia in lines 50–10 and 65 is more severe than would be expected based on changes in body size. In particular, femoral trabecular bone was found to be nearly absent in these lines. Moreover, line 63 showed a reduction in femoral bone mineral density and trabecular bone volume in the absence of any change in body weight, indicating that the osteopenia is not dependent upon a decrease in body mass.

The severity of the osteopenia seen in this model appears to correlate with the level of transgene expression observed in bone. The lines with the highest level of transgene expression (50–10, 65) show the greatest reduction in both bone mass and osteoblast marker gene expression and also display reduced body weight and tooth abnormalities. Line 63 shows moderate osteopenia without evidence of dental defects or reduced body weight. Line 50–9, in which transgene expression is barely detectable as remarkable as those observed in severely affected lines. The hypothesis that the degree of osteopenia is related to transgene dosage is strengthened by our observation that crossing hemizygous line 50–9 TG mice has produced presumptive homozygous progeny characterized by reduced body weight, low BMD,
and a tooth phenotype (data not shown). We are attempting to confirm the genotype of these mice by two-dose genetic testing, because Southern blot analysis of gene dosage is somewhat equivocal, but breeding these severely affected mice has proven difficult. However, it should be noted that no hemizygous mice exhibiting such a phenotype have ever been observed in this line. Similarly, a cross between modestly affected hemizygous line 63 mice has also resulted in progeny with a severe skeletal and dental phenotype.

A number of studies have suggested important roles for members of the basic leucine zipper transcription factor superfamily in regulating bone cell differentiation and function. Wang et al. (32) report that c-fos null mice exhibit osteopetrosis because of a lack of osteoclasts. In contrast, mice that overexpress c-fos develop osteosarcomas (33). Recently, two models of basic leucine zipper overexpression have been reported that result in osteosclerosis due to increased bone formation. Overexpression of ΔFosB, a naturally occurring splice variant of FosB, or Fra-1, the product of the c-fos-related fosl1 gene, both caused an increase in bone mass resulting from enhanced osteoblast differentiation (34, 35). Interestingly, ΔFosB overexpression also caused a reduction in adipogenesis, which was not seen in the Fra-1-overexpressing mice. Because ΔFosB and Fra-1 are both truncated members of the AP-1 family that lack transcription activation domains, it is tempting to speculate that the actions of these proteins are due to dominant negative inhibition of AP-1 transcription factors. Similarly, the most straightforward interpretation of our studies is that osteopenia results from the loss or reduction of C/EBP signaling due to a classical dominant negative effect of the p20C/EBPβ transgene. This would indicate that endogenous C/EBP transcription factors play a positive role in regulating the differentiation or maintenance of the osteoblast phenotype. This interpretation is consistent with the findings of Fux et al. (36), who recently reported that C/EBPα could promote both the adipogenic and osteoblastic differentiation of pluripotent C2C12 cells. However, not all effects of truncated transcription factors that lack transactivation domains are attributable to dominant negative effects. For example, Fleischmann et al. (37) generated knock-in mice in which Fra-1 was substituted for c-fos under control of the endogenous c-fos promoter and found that Fra-1 rescued some c-fos functions, including osteoclasisogenesis, in a gene-dosage dependent manner. Other actions of c-fos, such as the induction of target genes in fibroblasts, were not restored by Fra-1. Other possible mechanisms in our model include gains-of-function resulting from temporal misexpression of p20C/EBPβ during osteoblast differentiation or effects on osteoblast proliferation or apoptosis. Several effects of C/EBP transcription factors on these processes have been reported that do not involve classical transcriptional mechanisms of action (38, 39) (for review, see Ref. 40). Recently, Pereira et al. (41) found that CHOP/DDIT3, another member of the C/EBP family, could promote the osteoblastic differentiation of ST-2 stromal cells. Although DDIT3, like p20C/EBPβ, can act as a dominant negative inhibitor of C/EBP function, it also has a number of unique functions, notably its induction by cellular stress and amino acid deprivation and its ability to mediate cell growth arrest and apoptosis.

Regardless of the molecular mechanisms involved, at the cellular level it appears that the p20C/EBPβ transgene interferes with terminal osteoblast differentiation or differentiated function. The increase in BSP expression, coupled with the reduction in OC mRNA levels, raises the possibility that there is a differentiation bottleneck and an accumulation of cells expressing a pro-osteoblastic phenotype. It is somewhat puzzling that the effects on COL1A1 mRNA expression are modest and not strongly correlated with the degree of ossified osteopenia in the various lines. However, it must be recognized that COL1A1 mRNA expression is not restricted to mature osteoblasts, and much of the observed expression in profoundly affected lines could be derived from cells whose development has been arrested at an earlier stage of the lineage. Such cells likely express BSP, accounting for the up-regulation of this marker, and could express high levels of COL1A1 mRNA without necessarily contributing to new bone matrix production. Primary calvarial osteoblast cultures derived from line 50–10 TG mice in a pOBCol2.3-GFP genetic background showed evidence of diminished GFP expression. In WT cultures, this marker is strongly expressed in mature osteoblasts located within mineralizing nodules. The failure of these cells to fully express GFP at control levels supports the interpretation that these cells do not undergo terminal differentiation or that a subset of terminal osteoblast marker genes may be down-regulated. Importantly, the expression of BSP was up-regulated in TG cultures, whereas OC mRNA was inhibited, reflecting the alterations of gene expression observed in vivo. Gutierrez et al. (42) report that bone-specific activation of OC transcription is dependent on a synergistic interaction between C/EBPs and Runx2 at the C/EBP binding site of the OC promoter.

In summary, these studies have suggested that C/EBP transcription factors may be involved in the regulation of both osteogenesis and adipogenesis in vivo. Further study of this model should enhance our understanding of the role of transcription factors in the regulation of osteoblast differentiation and function and in the control of bone mass.

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Col1a1 Promoter-targeted Expression of p20 CCAAT Enhancer-binding Protein β (C/EBP β), a Truncated C/EBPβ Isoform, Causes Osteopenia in Transgenic Mice
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