Misexpression of CCAAT/enhancer binding protein beta causes osteopenia

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Abstract

CCAAT/enhancer binding proteins (C/EBPs) are expressed by osteoblasts and adipocytes during differentiation. C/EBPβ is critical for adipogenesis; however, its role in osteoblastogenesis is unclear, and its function in the postnatal skeleton is not known. To study C/EBPβ in osteoblasts *in vivo*, we created transgenic mice expressing full length C/EBPβ under the control of a 3·8 kb fragment of the human osteocalcin promoter. Two transgenic lines were established in a friend leukemia virus strain B genetic background, and compared with wild type littermate controls. Both C/EBPβ transgenic lines exhibited osteopenia, with a 30% decrease in bone volume, due to a decrease in trabecular number. The number of osteoblasts and osteoclasts per bone perimeter was not changed. Bone marrow stromal cells from C/EBPβ transgenics showed reduced mineralization, and reduced alkaline phosphatase mRNA levels. Calvarial osteoblasts from C/EBPβ transgenics displayed reduced alkaline phosphatase activity. To determine the consequences of the *Cebpb* deletion *in vivo*, the phenotype of *Cebpb* null mice was compared with that of wild type controls of identical genetic composition. *Cebpb* null mice exhibited reduced weight, body fat, and bone mineral density, and decreased bone volume, due to a decrease in trabecular number. The number of osteoblasts and osteoclasts per bone perimeter was not changed. C/EBPβ downregulation by RNA interference in calvarial osteoblasts had no effect on osteoblast differentiation/function. The phenotype of the *Cebpb* inactivation may be secondary to systemic indirect effects, and to direct effects of C/EBPβ in osteoblasts. In conclusion, C/EBPβ plays a role in mesenchymal cell differentiation and its misexpression *in vivo* causes osteopenia.

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Introduction

CCAAT/enhancer binding proteins (C/EBPs) are a family of transcription factors that play a role in cell proliferation, differentiation, and apoptosis (Nerlov 2007). Six members of the C/EBP family have been characterized and are termed α, β, δ, γ, ε, and ζ, which is also known as C/EBP homologous protein (CHOP) (Ramji & Foka 2002). C/EBP proteins contain a highly conserved leucine-zipper DNA-binding domain (DBD) and a leucine dimerization domain that enables the formation of homo- and hetero-dimers, which bind to similar DNA sequence motifs. C/EBPs act as regulators of gene expression either by direct DNA binding or by interacting with other transcription factors, including peroxisome proliferator-activated receptor-γ2, retinoic acid receptor α, runt-related transcription factor-2 (Runx-2), and nuclear factor of activated T-cells (Clarke *et al.* 1997, Gutierrez *et al.* 2002, Yang & Chow 2003, Nerlov 2007). C/EBPs are expressed in multiple cell types, including osteoblasts and adipocytes, and C/EBPβ and δ are required for adipogenesis (Rosen *et al.* 2000, Pereira *et al.* 2002, Hata *et al.* 2005, Wiper-Bergeron *et al.* 2007).

C/EBPβ exists in three different isoforms, which are translated from different initiation sites within the same mRNA. All three isoforms share the same DNA-binding domain; however, differences in the length of the amino terminal end, determine different functions (Calkhoven *et al.* 2000). The full length isoform, termed as liver-enriched activating protein⋆ or LAP⋆, consists of 296 amino acids and acts as a transcriptional activator, it is commonly referred to as C/EBPβ. A second isoform, termed LAP, lacks the first 21 amino acids present in the amino terminal end of full length LAP⋆, and it is generated from a different translation start site. LAP and LAP⋆ are functionally identical. A third isoform, termed as liver-enriched inhibitory protein (LIP), consists of 144 amino acids and acts as a dominant negative inhibitor of both LAP⋆ and LAP, since it lacks transactivating activity but retains the ability to bind DNA and to dimerize with the other C/EBPβ isoforms (Fig. 1; Nerlov 2007). The function of C/EBPβ is regulated by acetylation and phosphorylation at specific residues (Tang *et al.* 2005, Cesena *et al.* 2007, Li *et al.* 2007). Acetylation at Lys-39 of murine C/EBPβ is necessary to activate the transcription of *C-fos* and *Cebpa*, whereas sequential phosphorylation is required for the acquisition of

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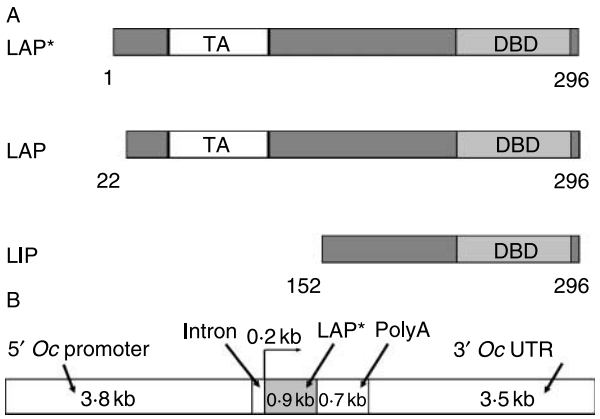

Figure 1 In panel A, a schematic representation of C/EBPβ isoforms demonstrating the amino acid used at the start of translation, the transactivation domain (TA) and the leucine-zipper DNA binding domain (DBD). In panel B, a schematic representation of the construct used for the creation of C/EBPβ transgenic mice; UTR is the untranslated region of the osteocalcin gene.

C/EBPβ DNA binding activity and transactivation of target genes, such as *Cebpa* and activating protein 2 during adipogenesis *in vitro* (Cesena *et al.* 2007, Li *et al.* 2007).

Cebpb null mutant mice are viable, possibly because of partial rescue from other members of the C/EBP family, or because C/EBPβ is dispensable during development. *Cebpb* null mice are affected by a lymphoproliferative disorder, imbalanced lymphocyte helper response, and impaired lipid metabolism in brown fat tissue leading to defective thermoregulation (Screpanti *et al.* 1995, Tanaka *et al.* 1997). *Cebpb* null mice exhibit impaired regenerative capacity of hepatocytes, impaired liver function (Greenbaum *et al.* 1998, Croniger *et al.* 2001), and decreased levels of systemic insulin-like growth factor (IGF) I (Staiger *et al.* 2008).

Mesenchymal cells are multipotent cells that can differentiate into cells of various lineages, including osteoblasts, adipocytes, and chondrocytes (Bianco & Gehron 2000). The fate of mesenchymal cells and their differentiation toward cells of the osteoblastic lineage is controlled by a network of intracellular and extracellular signals, and C/EBPβ regulates the commitment of cells of the osteoblastic and adipocytic lineage (Canalis *et al.* 2005, Hata *et al.* 2005, Winslow *et al.* 2006, Franceschi *et al.* 2007). Although the role of C/EBPβ in adipocyte differentiation is well known, its effects on osteoblastogenesis are less clear (Rosen *et al.* 2000). Previous studies from our laboratory have shown that cortisol inhibits osteoblastogenesis and induces the expression of adipocytic markers, which are associated with an increase in C/EBPβ expression in ST-2 stromal cells (Pereira *et al.* 2002). C/EBPβ represses Runx-2 transcription and osteoblastic differentiation of MC3T3 cells; however, C/EBPβ interacts with Runx-2 to induce osteocalcin expression (Gutierrez *et al.* 2002, Iyer *et al.* 2004, Hata *et al.* 2005, Wiper-Bergeron *et al.* 2007).

The intent of this study was to define the function of C/EBPβ in skeletal tissue *in vivo*. For this purpose, we created transgenic mice overexpressing C/EBPβ under the control of a 3·8 kb (kb) fragment of the osteoblast specific human osteocalcin promoter, and determined their body composition and skeletal phenotype. *Cebpb* null mice were also studied, and their body composition and skeletal phenotype determined.

Materials and methods

Generation of transgenic mice and cebpb null mice

After introduction of an optimal Kozak consensus sequence upstream of the translation initiation codon, an 890 bp (bp) fragment, coding for full length murine C/EBPβ or LAP★ (S L McKnight, Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX, USA), was cloned downstream of a 182 bp artificial intron and a 3·8 kb fragment of the human osteocalcin promoter (E Gardiner, University of Queensland, Brisbane, Australia), and upstream of polyadenylation sequences and a 3·5 kb fragment of the 3′ untranslated region and flanking DNA of the *Osteocalcin* gene (Fig. 1; Cao *et al.* 1991, Sims *et al.* 1997). Nucleotide sequence analysis confirmed the absence of mutations and correct orientation of the construct. Microinjection of linearized DNA into pronuclei of fertilized oocytes from FVB (for tropism to friend leukemia virus strain B (FVB)) inbred mice, and transfer of microinjected fertilized eggs into pseudopregnant FVB mice were carried out at the transgenic facility of the University of Connecticut Health Center (Farmington, CT, USA). Positive founders were identified by Southern blot analysis of tail DNA (Irwin 1989). Founder mice were bred to wild type FVB mice to generate transgenic lines, and their phenotype was examined.

To study the effects of the *Cebpb* deletion *in vivo*, *Cebpb* null heterozygous mice ($Cebpb^{+/-}$) (S Akira, Osaka University, Osaka, Japan) were re-derived in a C57BL/6 background, and backcrossed five times to obtain a uniform genetic background (Tanaka *et al.* 1995). *Cebpb* null homozygous mice ($Cebpb^{-/-}$) were obtained by intermating $Cebpb^{+/-}$ mice, and their phenotype was examined. All animal experiments were approved by the Animal Care and Use Committee of Saint Francis Hospital and Medical Center.

X-ray analysis, bone mineral density, and body composition

Radiography was either performed in C/EBPβ transgenic and wild type littermate controls or in $Cebpb^{-/-}$ mice and, age and sex, matched wild type controls of the same genetic composition. The mice were anesthetized with tribromoethanol (Sigma–Aldrich), and the X-rays were performed at an intensity of 30 kw for 20 s on a Faxitron X-ray system (model MX 20, Faxitron X-Ray Corp., Wheeling, IL, USA). Total bone mineral density (BMD) (g/cm^2) and total body fat (g) were measured on anesthetized mice using the PIXImus small animal DEXA system (GE Medical System/LUNAR,

Madison, WI, USA; Nagy *et al.* 2001). Calibrations were performed with a phantom of defined value, and quality assurance measurements were performed before each use. The coefficient of variation for total BMD is less than 1% ($n=9$).

Bone histomorphometric analysis

Static and dynamic histomorphometry was carried out on transgenic mice and wild type littermate controls and *Cebpb*$^{-/-}$ mice and wild type, sex and age, matched controls of the same genetic composition. Mice were injected with calcein, 20 mg/kg, and demeclocycline, 50 mg/kg, at an interval of 2 days for 1 and 1·5 month old animals and 7 days for 3-month old animals. The mice were killed by CO_2 inhalation 2 days after the demeclocycline injection. Longitudinal sections, 5 μm thick, were cut on a microtome (Microm, Richards-Allan Scientific, Kalamazoo, MI, USA) and stained with 0·1% toluidine blue or von Kossa. Static parameters of bone formation and resorption were measured in a defined area between 181 and 1080 μm from the growth plate, using an OsteoMeasure morphometry system (Osteometrics, Atlanta, GA, USA; Gazzerro *et al.* 2005). Osteoclasts were identified as multinucleated cells on the trabecular surface and their identity confirmed by the expression of tartrate resistant acid phosphatase determined by histochemical analysis of selected cells, using a commercial kit according to manufacturer's instructions (Sigma–Aldrich). For dynamic histomorphometry, mineralizing surface per bone surface and mineral apposition rate were measured on unstained sections under u.v. light, using a B-2A set long pass filter, and bone formation rate (BFR) was calculated. The terminology and units used are those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research (Parfitt *et al.* 1987).

Serum C-terminal cross-linked telopeptide of type I collagen

The bone remodeling marker cross-linked telopeptide of type I collagen (CTX) was measured in serum by ELISA using RatLaps ELISA kits (Nordic Bioscience Diagnostics, Herlev, Denmark), according to manufacturer's instructions.

Cell cultures

1-month old C/EBPβ heterozygous transgenic and wild type littermate controls from both sexes were killed by CO_2 asphyxiation, the femurs were aseptically removed and used to recover bone marrow stromal cells by centrifugation as described (Gazzerro *et al.* 2005). Cells were plated at a density of 5×10^5 cells/cm^2 and cultured in minimum essential medium (α-MEM, Invitrogen) containing 15% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA, USA) at 37 °C in a humidified 5% CO_2 incubator. Confluent cells were switched to α-MEM containing 10% FBS in the presence of 100 μg/ml ascorbic acid and 5 mM β-glycerophosphate and cultured up to 2 weeks to assess changes in mineralized nodule formation and gene expression.

Osteoblasts were isolated from parietal bones of 3- to 5-day old C/EBPβ heterozygous transgenic and wild type control littermates from both sexes, by sequential collagenase digestions, as previously described (McCarthy *et al.* 1990). Cells were cultured in DMEM (Invitrogen) supplemented with non-essential amino acids, 20 mM HEPES, 100 μg/ml ascorbic acid, and 10% FBS. Osteoblasts were cultured to 70% confluence and trypsinized; these first passage cells were used for subsequent experiments. Cells were cultured for up to 10 days after confluence, in the presence of 100 μg/ml ascorbic acid and 5 mM β-glycerophosphate (Sigma–Aldrich) to assess changes in gene expression, and treated with bone morphogenetic protein-2 (BMP-2, Wyeth Research, Collegeville, PA, USA) or control vehicle to determine alkaline phosphatase activity (APA).

RNA interference

To downregulate C/EBPβ expression *in vitro*, a 19-mer double stranded small interfering (si) RNA targeted to the murine C/EBPβ mRNA sequence was obtained commercially, and a scrambled 19-mer small interfering RNA (siRNA) with no homology to known mouse or rat sequences was used as a control (Applied Biosystems, Foster City, CA, USA; Elbashir *et al.* 2001, Sharp 2001). C/EBPβ or scrambled siRNA, both at 20 nM, were transfected into sub-confluent primary murine osteoblasts using siLentFect lipid reagent, in accordance with manufacturer's instructions (BioRad). Cells were allowed to recover for 24 h prior to the determination of APA. To ensure adequate C/EBPβ downregulation, and to measure mRNA levels of osteoblast markers, total RNA was extracted in parallel cell cultures 96 h after the transfection of siRNAs, and mRNA levels determined by real-time reverse transcription-PCR (RT-PCR).

Cytochemical assays and APA

To determine mineralized nodule formation, bone marrow stromal cells were fixed with 3·7% formaldehyde and stained with 2% Alizarin Red (Sigma–Aldrich; DAHL 1952). APA was determined in 0·5% Triton X-100 cell extractsby the hydrolysis of *p*-nitrophenyl phosphate to *p*-nitrophenolmeasured by spectroscopy at 405 nm after 10 min of incubation at room temperature according to the manufacturer's instructions (Sigma–Aldrich). Data are expressed as picomoles of *p*-nitrophenol released per min per mg of protein.The total protein content was determined in cell extracts by the DC protein assay, in accordance with manufacturer's instructions (Bio-Rad).

Table 1 Weight and body composition of 1 and 3 month old C/EBPβ transgenics and wild-type littermate control mice

	Weight	Total BMD	Total body fat
1-month old heterozygous			
Female			
Wild type	12·6±0·4	336±8	1·8±0·1
C/EBPβ transgenics	12·3±0·2	343±5	2·2±0·1
Male			
Wild type	19·5±0·6	365±5	2·8±0·1
C/EBPβ transgenics	18·7±0·9	354±7	2·2±0·4
1-month old homozygous			
Female			
Wild type	16·6±0·5	342±10	3·0±0·1
C/EBPβ transgenics	15·3±0·7	324±4	2·9±0·1
3-month old heterozygous			
Female			
Wild type	21·7±0·8	502±9	3·8±0·1
C/EBPβ transgenics	21·6±1·2	479±7	3·8±0·1
Male			
Wild type	26·8±0·4	470±4	5·0±0·1
C/EBPβ transgenics	26·3±0·7	463±3	4·6±0·3

Weight (g), total BMD (g/cm^2×10^4), and total body fat (g) were obtained from 1- or 3-month old C/EBPβ transgenics and wild type littermate controls using a PIXImus small animal DEXA system. BMD was calculated by dividing the bone mineral content (g)/bone area (cm^2), and total fat (g) was measured directly by the instrument. Values are means±S.E.M. (n=5–12).

Real time RT-PCR

Total RNA was extracted from cell cultures and mRNA levels determined by real-time RT-PCR (Nazarenko *et al.* 2002*a*, *b*). For this purpose, 5 μg RNA were reverse-transcribed using SuperScript III Platinum Two-Step qRT-PCR kit (Invitrogen), according to the manufacturer's instructions, and amplified in the presence of CGGTTAGGGCGT-CTCCACAGTAAC[FAM]G-3′ and 5′-CTTGGAGAGG-GCCACAAAGG-3′ primers for alkaline phosphatase; 5'-CGGATTGAACCGAGACAACTACATA[FAM]G-3' and 5'-CGCCACCTACAATCAGGCTCT-3' primers for C/EBPβ; 5'-CGAAGTTACATGACACTGGGCTT[FAM]-G-3' and 5′-CCCAGCACAACTCCTCCCTA-3′ primers for osteocalcin; 5′-CGAACCGGATAATGTGAAGTTCAA-GGTT[FAM]G-3′ and 5′-CTGCTTCAGCTTCTCTGC-CTTT-3′ primers for ribosomal protein L38 (RPL38), and Platinum Quantitative PCR SuperMix-UDG (Invitrogen) at 60 °C for 45 cycles. Transcript copy number was estimated by comparison with a standard curve constructed using C/EBPβ (S.L. McKnight), osteocalcin (J.B. Lian, University of Massachusetts, Worcester, MA USA; Lian *et al.* 1989), and alkaline phosphatase (American Type Culture Collection; ATCC, Manassas, VA, USA) cDNAs, corrected for RPL38 copy number (ATCC; Kouadjo *et al.* 2007). Reactions were conducted in a 96-well spectrofluorometric thermal iCycler (Bio-Rad), and fluorescence was monitored during every PCR cycle at the annealing step.

Statistical analysis

Data are expressed as means±S.E.M. Statistical differences were determined by Student's *t*-test or ANOVA.

Results

C/EBPβ transgenic mice

Two lines of FVB mice overexpressing C/EBPβ under the control of the 3·8 kb human osteocalcin promoter were established. The transgenic lines expressed 8 and 16 copies of the transgene. Both lines had a similar phenotype and the line

Table 2 Femoral static histomorphometry of 1-month old female C/EBPβ transgenic mice and wild type littermate controls

	Wild type	C/EBPβ transgenics
Heterozygous		
Bone volume/tissue volume (%)	11·0±1·3	6·4±0·8*
Trabecular separation (μm)	90·4±6·0	120·5±21·4
Trabecular number (mm^{-1})	9·9±0·5	6·2±0·5*
Trabecular thickness (μm)	11·1±0·8	10·3±0·6
Osteoblast surface/bone surface (%)	33·9±3·2	31·0±0·9
Number of osteoblasts/bone perimeter (mm^{-1})	61·6±5·8	60·1±2·2
Number of osteoblasts/tissue area (mm^{-2})	950·8±44·9	579·2±24·3*
Osteoclast surface/bone surface (%)	14·1±0·4	13·7±0·1
Number of osteoclasts/bone perimeter (mm^{-1})	12·7±0·3	12·6±0·4
Number of osteoclasts/tissue area (mm^{-2})	198·6±11·3	121·3±6·0*
Eroded surface/bone surface (%)	28·0±1·4	27·4±0·2
Number of adipocytes/tissue area (mm^{-2})	65·9±9·0	83·7±19·7
Homozygous		
Bone volume/tissue volume (%)	12·1±0·6	7·5±0·9*
Trabecular separation (μm)	87·6±4·7	135·7±14·8*
Trabecular number (mm^{-1})	10·1±0·5	7·0±0·6*
Trabecular thickness (μm)	11·9±0·2	10·7±0·5
Osteoblast surface/bone surface (%)	28·0±2·4	29·2±3·3
Number of osteoblasts/bone perimeter (mm^{-1})	61·0±7·0	65·4±6·9
Number of osteoblasts/tissue area (mm^{-2})	970·9±124·8	718·7±96·5
Osteoclast surface/bone surface (%)	13·3±0·8	14·5±0·7
Number of osteoclasts/bone perimeter (mm^{-1})	13·0±0·4	13·9±0·4
Number of osteoclasts/tissue area (mm^{-2})	207·5±16·0	152·3±12·8*
Eroded surface/bone surface (%)	24·4±0·7	25·3±0·6
Number of adipocytes/tissue area (mm^{-2})	145·4±16·7	150·0±11·8

Bone histomorphometry was performed on femurs from 1 month old female C/EBPβ transgenics and wild type littermate controls. Values are means±S.E.M.; n=3−9; *Significantly different from wild type controls, P<0·05.

expressing 16 copies of the transgene was characterized in detail. C/EBPβ transgenic mice were compared with wild type littermate controls of identical sex, at the age of 1 month, a time of high activity of the osteocalcin promoter, or at the age of 3 months (Frenkel *et al.* 1997). In the offspring of C/EBPβ heterozygous mice intermatings, the ratio of wild type, C/EBPβ heterozygous, and C/EBPβ homozygous was 29:60:11 for females and 32:63:5 for males. As a consequence, the number of C/EBPβ homozygous mice was limited, and this only allowed a detailed histomorphometric analysis of homozygous female mice. We suspect that the non-Mendelian distribution of the transgenic allele is due to intrauterine lethality, likely because of transgene expression in the central nervous system, where the osteocalcin promoter is also active (Frenkel *et al.* 1997). There was no significant difference in weight and total body fat in either 1- or 3-month old C/EBPβ transgenic mice, when compared with wild type littermate controls (Table 1). Contact radiography did not reveal any obvious skeletal abnormality, and there was no difference in BMD between transgenics and controls (Table 1).

Bone histomorphometric analysis of distal femurs from 1-month old female C/EBPβ transgenics revealed a decrease in bone volume/tissue volume, secondary to a reduction in the number of trabeculae (Table 2, Fig. 2A). A similar decrease in bone volume was observed in male heterozygous transgenics (Table 3, Fig. 2B). The number of osteoblasts per tissue area was decreased in C/EBPβ transgenics, possibly due to the decreased bone volume, since the number of osteoblasts per bone perimeter, and osteoblast surface/bone surface were not different from controls (Tables 2 and 3). Changes in bone volume were not associated with an increase in bone resorption since the number of osteoclasts per bone perimeter and eroded surface were not increased in transgenics when compared with controls (Tables 2 and 3). A modest decrease in the number of osteoclasts per tissue area was detected in female transgenics (Table 2). Serum concentrations of CTX (means ± S.E.M.; $n=3$–6) were 26·5 ± 3·1 ng/ml in female heterozygous C/EBPβ transgenic mice and 33·3 ± 1.9 ng/ml in wild type littermate controls; 30·7 ± 5·6 ng/ml in female homozygous transgenics and 32·3 ± 4·0 ng/ml in wild type littermate controls; and 49·4 ± 15·5 ng/ml in male heterozygous transgenics and 50·4 ± 7·4 ng/ml in wild-type littermate controls. The results indicate that C/EBPβ overexpression does not affect bone remodeling. There were no substantial differences between female and male phenotypes, and no change in mineral apposition rate (not shown) or BFR (Fig. 2) was detected in C/EBPβ transgenics. No significant change in the number of adipocytes per tissue area was observed in either female or male transgenics when compared with wild type littermate controls (Tables 2 and 3). This could be expected since C/EBPβ transcription was directed by the osteocalcin promoter, which is not active in adipocytic cells.

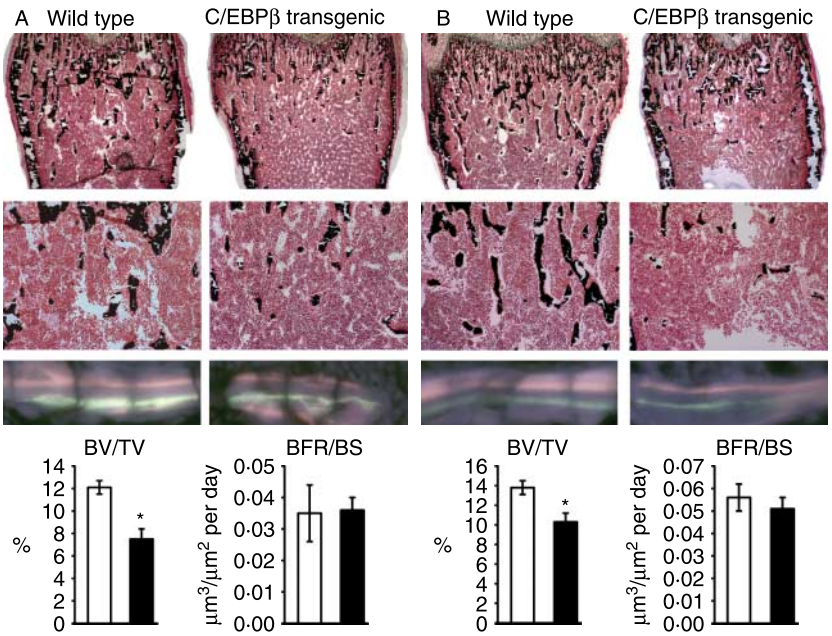

Figure 2 Representative histological sections and calcein/demeclocycline labels (upper panels) of femoral sections from 4 week old female homozygous (A) and male heterozygous (B) 1 month old C/EBPβ transgenic mice and wild type littermate controls stained with von Kossa without counter staining (final magnification 40× and 100×) or unstained and examined under fluorescence microscopy (final magnification 100×). In the lower panels, bars represent bone volume/tissue volume (BV/TV, %), and BFR/bone surface (BFR/BS, $\mu m^3/\mu m^2$/day) of C/EBPβ transgenics (black bars) and wild- type controls (white bars). Values are means ± S.E.M., $n=3$–9. * Significantly different from control mice, $P<0{\cdot}05$.

Table 3 Femoral static histomorphometry of 1-month old male C/EBPβ heterozygous transgenic mice and wild type littermate controls

	Wild type	C/EBPβ transgenics
Bone volume/tissue volume (%)	13·8±0·7	10·3±0·9*
Trabecular separation (μm)	79·9±3·5	110·3±9·3*
Trabecular number (mm^{-1})	10·9±0·4	8·4±0·6*
Trabecular thickness (μm)	12·6±0·3	12·1±0·4
Osteoblast surface/bone surface (%)	31·0±1·5	29·3±1·3
Number of osteoblasts/bone perimeter (mm^{-1})	37·4±1·3	33·8±1·5
Number of osteoblasts/tissue area (mm^{-2})	639·8±24·9	449·4±39·3*
Osteoclast surface/bone surface (%)	6·9±0·4	7·9±0·8
Number of osteoclasts/bone perimeter (mm^{-1})	5·6±0·3	6·6±0·6
Number of osteoclasts/tissue area (mm^{-2})	95·8±5·2	84·7±5·2
Eroded surface/bone surface (%)	16·8±0·8	17·6±1·1
Number of adipocytes/tissue area (mm^{-2})	269·4±24·1	306·9±40·7

Bone histomorphometry was performed on femurs from 1-month old male C/EBPβ heterozygous transgenic mice and wild type littermate controls. Values are means±S.E.M.; n=6–8; *Significantly different from wild type controls, $P<0·05$.

Bone histomorphometric analysis of distal femurs from 3-month old female C/EBPβ heterozygous transgenics revealed a phenotype analogous to that observed at 1 month, and characterized by a decrease in bone volume/tissue volume, due to a reduction in the number of bone trabeculae (Table 4). BFR and mineral apposition rate were not affected (data not shown). Serum concentrations of CTX (means ±S.E.M.; n=4) were 17·8±1·9 ng/ml in female heterozygous C/EBPβ transgenic mice and 16·3±1·1 ng/ml in wild-type littermate controls, confirming that C/EBPβ overexpression does not affect bone remodeling.

Both transgenic lines studied displayed analogous skeletal phenotypes. In accordance with the line described, 1-month old male C/EBPβ heterozygous transgenics, from the line expressing eight copies of the transgene, exhibited a decrease in bone volume. Bone volume/tissue volume was (means ±S.E.M.; n=6–8) 8·0±0·4% in controls and 5·3±0·5% in transgenics, $P<0·05$; trabecular number was 7·9±0·3 mm^{-1} in controls and 5·9±0·5 mm^{-1} in transgenics, $P<0·05$. In summary, C/EBPβ transgenics exhibited osteopenia secondary to a decrease in trabecular number without apparent changes in bone formation or bone resorption.

Bone marrow stromal cell and osteoblast cultures

To investigate the impact of C/EBPβ on osteoblastic cell function, bone marrow stromal cells from heterozygous transgenic mice and wild type littermate controls were cultured for up to 2 weeks after confluence, under conditions favoring osteoblastogenesis. C/EBPβ overexpression was confirmed by real-time RT-PCR, and C/EBPβ transcripts were two fold greater in transgenic than in control cells (Fig. 3A). Alkaline phosphatase mRNA levels were suppressed by about 40% in cells from transgenics, when compared with control cells (Fig. 3A). Accordingly, mineralization of the culture, tested after 14 days of incubation by alizarin red staining, was reduced in C/EBPβ overexpressing cells, confirming an inhibitory effect of C/EBPβ on osteoblastic differentiation (Fig. 3B). Conversely, osteocalcin mRNA levels were increased in C/EBPβ overexpressing cells (Fig. 3A). This may be explained by observations demonstrating that interactions between Runx-2 and C/EBPβ can increase osteocalcin expression (Gutierrez *et al.* 2002, Hata *et al.* 2005).

To confirm that C/EBPβ inhibits osteoblast differentiation, calvarial osteoblasts were harvested from heterozygous transgenic mice and wild-type littermate controls, and cultured for up to 10 days in differentiating conditions. C/EBPβ overexpression was confirmed by real-time RT-PCR, and C/EBPβ transcripts at confluence were 1.5-fold greater in transgenic than in control cells (Fig. 3C). Alkaline phosphatase mRNA levels were suppressed by about 50% in cells from transgenics when compared with control cells (Fig. 3C). Accordingly, calvarial osteoblasts overexpressing C/EBPβ exhibited decreased levels of APA when compared with control cells cultured with vehicle or BMP-2 (Fig. 3D). Osteocalcin transcript levels did not change in

Table 4 Femoral static histomorphometry of 3-month old female C/EBPβ heterozygous transgenic mice and wild type littermate controls

	Wild type	C/EBPβ transgenics
Bone volume/tissue volume (%)	6·9±0·5	4·7±0·4*
Trabecular separation (μm)	143·4±6·1	213·4±19·1*
Trabecular number (mm^{-1})	6·6±0·3	4·6±0·3*
Trabecular thickness (μm)	10·4±0·5	10·0±0·3
Osteoblast surface/bone surface (%)	13·0±1·3	12·1±1·3
Number of osteoblasts/bone perimeter (mm^{-1})	22·4±2·0	20·5±1·7
Number of osteoblasts/tissue area (mm^{-2})	231·1±22·2	148·4±15·2*
Osteoclast surface/bone surface (%)	13·5±1·1	12·7±1·5
Number of osteoclasts/bone perimeter (mm^{-1})	17·8±1·0	17·8±1·6
Number of osteoclasts/tissue area (mm^{-2})	184·4±12·3	127·9±11·7*
Eroded surface/bone surface (%)	33·7±1·6	34·1±2·1

Bone histomorphometry was performed on femurs from 3-month old female C/EBPβ heterozygous transgenic mice and wild type littermate controls. Values are means±S.E.M.; n=7–9; *Significantly different from wild type controls, $P<0.05$.

osteoblasts from transgenics when compared with control cells (not shown), possibly because the inhibition of differentiated osteoblast function precluded the stimulatory effect of C/EBPβ and Runx-2 on osteocalcin transcription in mature osteoblasts.

Cebpb null mice (Cebpb$^{-/-}$)

Cebpb$^{+/-}$ mice were intermated to obtain *Cebpb*$^{-/-}$ mice, and their phenotype was examined. The homozygous *Cebpb* deletion causes female infertility, not allowing the establishment of a *Cebpb*$^{-/-}$ colony, and limiting the availability of mice for subsequent studies (Sterneck *et al.* 1997). Male *Cebpb*$^{-/-}$ mice showed a tendency towards a reduced body weight and total body fat as compared with wild type age matched controls of identical genetic composition at 1·5 and 3 months. The difference in weight and body fat was statistically significant at 6 months of age (Fig. 4A and B). Contact radiography of male *Cebpb*$^{-/-}$ mice did not reveal obvious skeletal abnormalities (data not shown). However, male *Cebpb*$^{-/-}$ mice at 1·5, 3, and 6 months displayed a tendency toward a decrease in BMD, which was statistically significant at 1·5 months of age (Fig. 4C). Male *Cebpb*$^{-/-}$ mice at 1·5, 3, and 6 months exhibited decreased trabecular bone volume that was statistically significant at 3 months of age (Fig. 4D).

A complete histomorphometric analysis, performed in 3-month old *Cebpb*$^{-/-}$ male mice, revealed a 30% decrease in bone volume/tissue volume, when compared with wild type age matched controls of the same genetic composition (Table 5, Fig. 5). The decrease was secondary to a reduced number of trabeculae. *Cebpb*$^{-/-}$ male mice did not exhibit differences in the number of osteoblasts per bone perimeter and osteoblast surface/bone surface. Similarly, there were no significant changes in the number of osteoclasts/perimeter or in eroded surface in *Cebpb* null mice, when compared with wild type controls. There was no change in the number of adipocytes per tissue area possibly because C/EBPδ alone is sufficient to support adipogenesis (Table 5; Lane *et al.* 1999). No changes in mineral apposition rate (not shown) and BFR (Fig. 5) were detected. In summary, *Cebpb* null mice are osteopenic due to a decrease in trabecular number, but do not exhibit changes in bone formation or bone resorption.

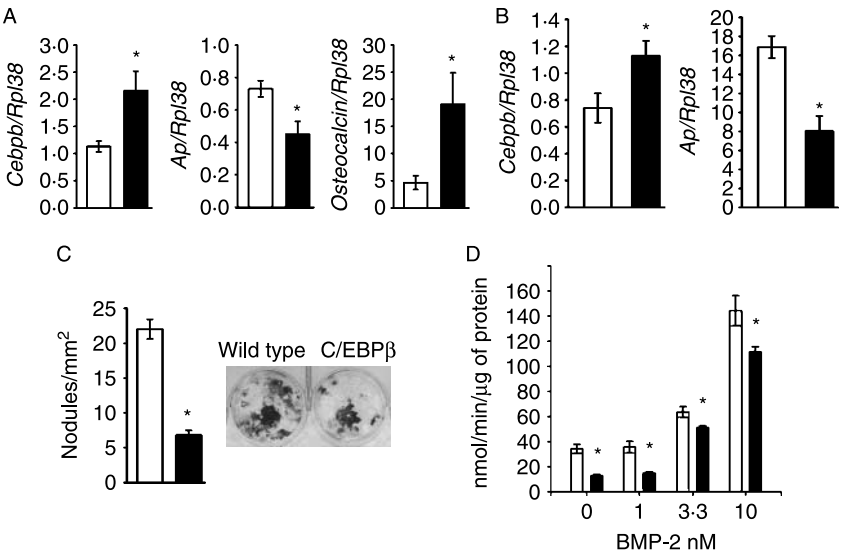

Figure 3 Effect of C/EBPβ overexpression on osteoblast differentiation in bone marrow stromal cells and calvarial osteoblasts harvested from C/EBPβ transgenic mice (black bars) and wild-type controls (white bars). In (A) stromal cells were cultured for 10 days after confluence; total RNA was reverse-transcribed, and amplified by real-time RT-PCR. Data are expressed as *Cebpb, Ap,* and osteocalcin copy number, corrected for *Rpl38* expression. Values are means±S.E.M., $n=4$. * Significantly different from wild-type control cells, $P<0{\cdot}05$. In (B) stromal cells were cultured for 2 weeks after confluence, and mineralized nodules determined by alizarin red staining. A representative culture is shown. Values are means±S.E.M., $n=6$. * Significantly different from wild-type control cells, $P<0{\cdot}05$. In (C) calvarial osteoblasts were cultured for 72 h after confluence; total RNA was reverse-transcribed, and amplified by real-time RT-PCR. Data are expressed as *Cebpb* and *Ap* copy number corrected for *Rpl38* expression. Values are means±S.E.M., $n=4$. * Significantly different from control cells, $P<0{\cdot}05$. In (D) calvarial osteoblasts were cultured to confluence, exposed to control medium or to BMP-2 at the indicated doses for 72 h, and *Ap* activity was quantified in cell extracts and expressed as nanomoles of *p*-nitrophenol/min per μg of total protein. * Significantly different from control cells, $P<0{\cdot}05$. Bars represent means±S.E.M. for six observations.

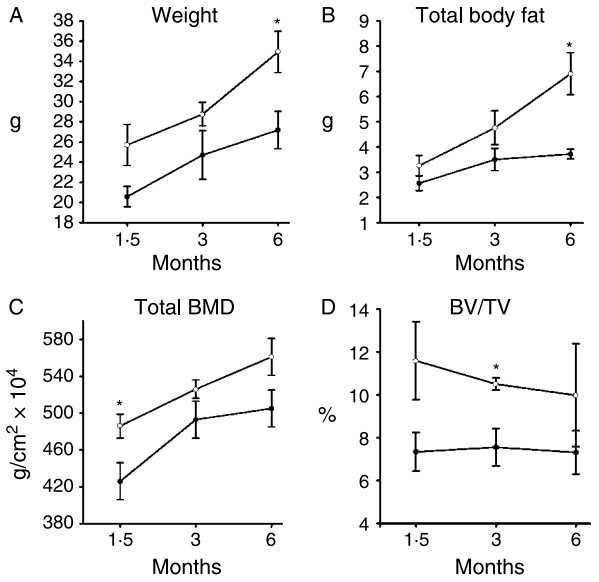

Figure 4 Weight, body composition, and bone volume/tissue volume (BV/TV) in male *Cebpb* null mice (filled circles) and wild-type age matched controls (open circles). The weight (A), total body fat (B), total BMD (BMD, C), and BV/TV (D) of male *Cebpb* null mice and wild type age matched controls of the same genetic composition at 1·5, 3, and 6 months of age is shown. *Significantly different from control mice, $P<0·05$. Bars represent means ± S.E.M., $n=5$.

Downregulation of C/EBPβ

Skeletal cells from *Cebpb*$^{-/-}$ mice were not readily available because of the low fertility of *Cebpb*$^{+/-}$ mice and infertility of *Cebpb*$^{-/-}$ mice. To investigate the function of C/EBPβ, its mRNA levels were downregulated by RNA interference (RNAi) in primary calvarial osteoblasts. C/EBPβ down-regulation was confirmed by real-time RT-PCR, and C/EBPβ transcripts were reduced by 70% in cells transfected with the C/EBPβ siRNA, when compared with control cells transfected with scrambled siRNA (Table 6). There were no changes in alkaline phosphatase or osteocalcin mRNA levels, measured by real-time RT-PCR, in C/EBPβ downregulated cells (Table 6). Accordingly, there was no effect on APA in cells transfected with C/EBPβ siRNA when compared with control cells (Table 6). These results indicate that C/EBPβ may be dispensable for osteoblast differentiation *in vitro*, and suggest that the osteopenic phenotype of the null mutation could be secondary to indirect mechanisms.

Discussion

This report demonstrates that transgenic mice overexpressing C/EBPβ under the control of the 3·8 kb human osteocalcin promoter exhibit osteopenia. The osteopenia was secondary to a reduced number of trabeculae, but C/EBPβ transgenics exhibited no change in osteoblast number or BFR. The osteopenic phenotype could not be attributed to changes in bone resorption, since, osteoclast number, eroded surface, and biochemical markers of bone remodeling were not different between transgenics and controls. The reduced number of trabeculae could be the result of impaired trabecular formation during skeletal development. This is possible since the osteocalcin promoter is expressed by mature osteoblasts as early as day 18 of embryonic life (Frendo *et al.* 1998, Kalajzic *et al.* 2002), and C/EBPβ overexpression can suppress the terminal differentiation of osteoblasts (Iyer *et al.* 2004). A possible explanation for a developmental impact of C/EBPβ could be a suppression of Runx-2 transcription, and as a consequence, impaired trabecular bone formation (Otto *et al.* 1997, Wiper-Bergeron *et al.* 2007). It is possible that osteoblastic differentiation and function are more sensitive to transcriptional changes during development, or that compensatory mechanisms are established postnatally, since osteoblast number and bone formation were not affected in 1-month old C/EBPβ transgenics.

Cebpb null mice showed reduced weight, total body fat, and total BMD. Although they were osteopenic, no changes in osteoblast or osteoclast number or BFR were observed. The osteopenic phenotype of *Cebpb* null mice is in accordance with recent reports from other investigators (Staiger *et al.* 2008, Tominaga *et al.* 2008), and with a function of C/EBPβ in the early differentiation stages of mesenchymal cells, resulting in impaired chondrocyte and osteoblast maturation. This leads to impaired cartilage and bone development, and would explain the reduced number of trabeculae and

Table 5 Femoral static histomorphometry of 3-month old male *Cebpb* null mice and male wild-type age matched controls

	Wild type	*Cebpb*$^{-/-}$
Bone volume/tissue volume (%)	10·5 ± 0·3	7·5 ± 0·9*
Trabecular separation (μm)	114·1 ± 4·0	149·3 ± 11·9*
Trabecular number (mm^{-1})	7·9 ± 0·3	6·3 ± 0·5*
Trabecular thickness (μm)	13·3 ± 0·4	12·1 ± 0·7
Osteoblast surface/bone surface (%)	11·6 ± 1·8	10·6 ± 1·2
Number of osteoblasts/bone perimeter (mm^{-1})	18·0 ± 2·5	17·7 ± 2·1
Number of osteoblasts/tissue area (mm^{-2})	226·4 ± 36·3	175·1 ± 23·0
Osteoclast surface/bone surface (%)	4·1 ± 0·8	5·7 ± 1·0
Number of osteoclasts/bone perimeter (mm^{-1})	3·5 ± 0·7	5·2 ± 0·9
Number of osteoclasts/tissue area (mm^{-2})	43·3 ± 9·0	51·0 ± 9·2
Eroded surface/bone surface (%)	14·6 ± 1·7	17·2 ± 1·2
Number of adipocytes/tissue area (mm^{-2})	35·9 ± 11·7	15·3 ± 5·2

Bone histomorphometry was performed on femurs from 3-month old male *Cebpb*$^{-/-}$ mice and male wild type age matched controls of the same genetic composition. Values are means ± S.E.M.; $n=5$; * Significantly different from wild type controls, $P<0·05$.

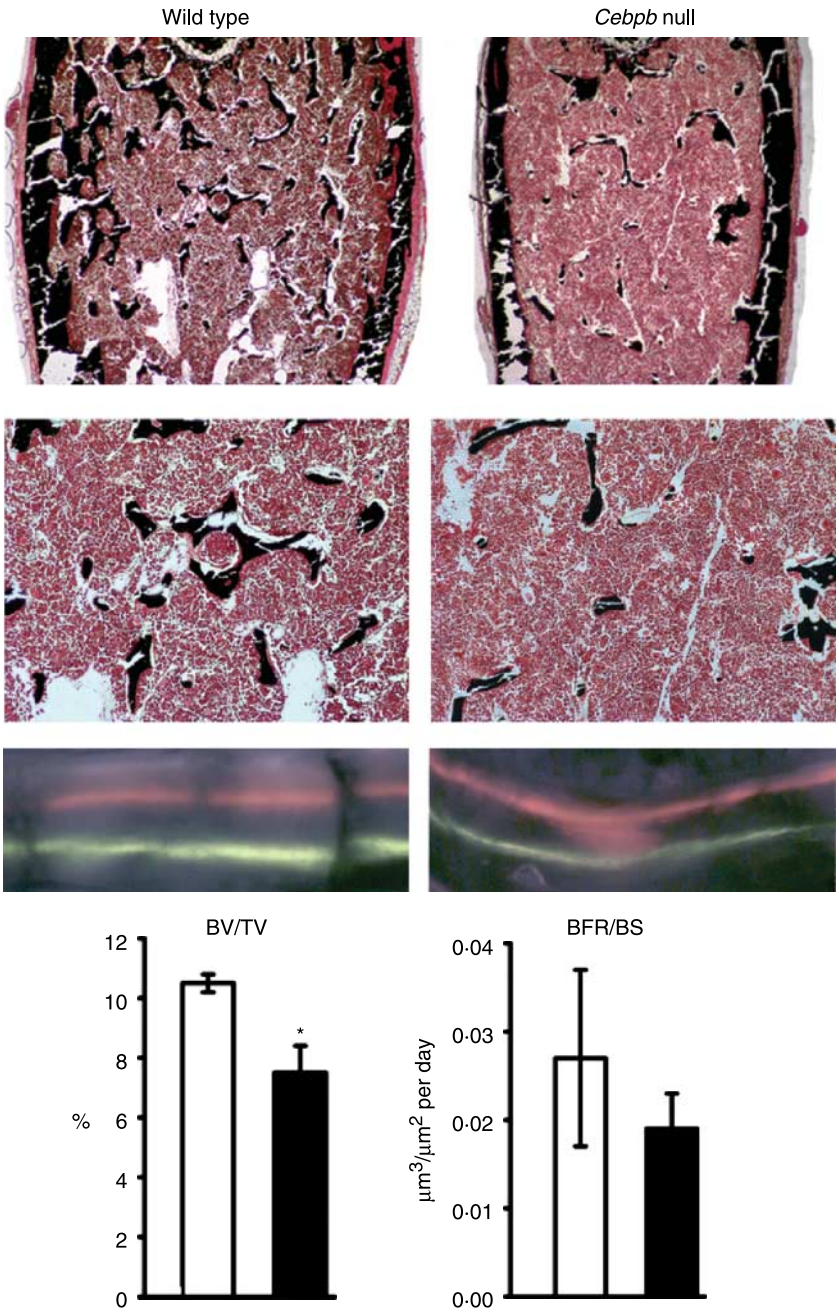

Figure 5 Representative histological sections and calcein/demeclocycline labels (upper panels) of bone femoral sections from 3-month old *Cebpb* null mice and wild-type littermate controls stained with von Kossa without counter stain (final magnification 40× and 100×) or unstained and examined under fluorescence microscopy (final magnification 100×). In the lower panels, bars represent bone volume/tissue volume (BV/TV, %), and BFR/bone surface (BFR/BS, $\mu m^3/\mu m^2$ per day) of *Cebpb* null (black bars) and wild-type controls (white bars). Values are means ± S.E.M., $n=5$. * Significantly different from control mice, $P<0{\cdot}05$.

osteopenic phenotype (Tominaga *et al*. 2008). However, other factors probably contribute to the bone loss caused by the *Cebpb* deletion, since C/EBPβ regulates gene expression in a variety of organs (Ramji & Foka 2002). *Cebpb* null mice have decreased serum levels of insulin like growth factor I (IGF-I), secondary to decreased IGF-I expression by the liver (Staiger *et al*. 2008), and systemic IGF-I plays an important role in skeletal homeostasis (Gazzerro & Canalis 2006, Yakar *et al*.

Table 6 Effect of C/EBP downregulation by RNAi on osteoblastic markers in calvarial osteoblasts

	siScrambled	siC/EBPβ
Cebpb/Rpl38	9·15±1·37	2·41±1·12*
Alkaline phosphatase/Rpl38	1·78±0·22	2·09±0·71
Osteocalcin/Rpl38	3·05±1·25	2·28±0·70
APA	6·87±0·84	5·87±0·82

Cebpb, alkaline phosphatase and osteocalcin copy number, corrected for *Rpl38* expression, were determined by real-time RT-PCR in calvarial osteoblasts transfected with siC/EBPβ or siScrambled control. APA (nmol/min per μg of protein) was determined in parallel cultures. Values are means±S.E.M., $n=4$–6. * Significantly different from control cells, $P<0·05$.

2006, Giustina *et al.* 2008). Other contributing factors to the osteopenic phenotype could be the consequences of lymphoproliferative and myeloproliferative alterations, and increased Interleukin-6 serum levels exhibited in the *Cebpb* null state (Screpanti *et al.* 1995). Uncontrolled proliferation of lymphocytes or myeloid precursors can lead to osteopenia (Roodman 1997), and overexpression of Interleukin-6 *in vivo* causes impaired growth plate development, increased bone resorption, and decreased osteoblastic differentiation (De *et al.* 2006). A systemic nature of the osteopenic phenotype, as opposed to an osteoblast specific effect, is supported by the absence of a cellular phenotype following the downregulation of C/EBPβ by RNAi in osteoblasts. However, the absence of a cellular phenotype is in contrast with recently reported findings in osteoblasts from *Cebpb* null mutants, where inactivation of *Cebpb* resulted in impaired osteoblast maturation (Tominaga *et al.* 2008). The discrepancy between our results and those of Tominaga *et al.*, could be attributed to the transient and possibly incomplete downregulation of C/EBPβ by RNAi, causing sufficient C/EBPβ expression to support osteoblast differentiation/function for the period of study. It is also possible that under the experimental conditions used in the present work, the levels of C/EBPβ were sufficient to maintain osteocalcin expression (Gutierrez *et al.* 2002). C/EBPβ can favor the early stages of osteoblast differentiation through its hetero-dimerization with activating transcription factor (ATF) 4, and by enhancing the association of ATF 4 with Runx-2 to regulate osteocalcin transcription (Hata *et al.* 2005, Tominaga *et al.* 2008). Interactions of C/EBPβ with ATF 4 may affect other steps of osteoblastic differentiation, since ATF 4 plays an essential role in this process (Yang *et al.* 2004), and could also explain the findings reported in *Cebpb* null osteoblasts and in the *Cebpb* null mice.

Evidence for direct activity of C/EBPβ in osteoblasts also can be derived from observations *in vivo*. Transgenics overexpressing either LIP, a physiological dominant negative form of C/EBPβ (Harrison *et al.* 2005), or CHOP, a member of the C/EBP family, are osteopenic (Pereira *et al.* 2007). The primary partner of CHOP is C/EBPβ and the osteopenia observed in CHOP transgenics is likely secondary to its dimerization with C/EBPβ and a decrease in C/EBPβ transactivating activity in the skeletal environment (Ron & Habener 1992, Vinson *et al.* 2002). It is of interest that inactivation of *Chop* causes decreased bone formation, but not an obvious osteopenic phenotype. The function of CHOP, like that of C/EBPβ, appears to be dependent on cell type, stage of mesenchymal/osteoblastic cell differentiation, and experimental conditions (Pereira & Delany 2004, Pereira *et al.* 2006, Shirakawa *et al.* 2006).

To understand the mechanism leading to the osteopenic phenotype resulting from C/EBPβ overexpression, we examined primary osteoblasts and bone marrow stromal cells harvested from C/EBPβ transgenic mice. The cellular phenotype is in accordance with the osteopenic phenotype observed, and with previous work demonstrating that the stable transduction of C/EBPβ in MC3T3 and C3H10T1/2 cells, by repressing Runx-2 transcription, abrogates osteoblast differentiation induced by retinoic acid (Wiper-Bergeron *et al.* 2007). Accordingly, osteoblastic cells from C/EBPβ transgenics exhibited reduced mineralization, APA, and alkaline phosphatase mRNA expression. By contrast, transduction of cells of the osteoblastic lineage with adenoviral vectors expressing C/EBPβ resulted in enhanced osteoblastic differentiation (Hata *et al.* 2005), also suggesting that experimental conditions can have a significant impact on the effect of C/EBPs in osteoblasts. C/EBPβ/ATF 4/Runx-2 interactions enhance osteocalcin transcription, and osteocalcin transcripts are increased in conditions of C/EBPβ overexpression (Gutierrez *et al.* 2002, Tominaga *et al.* 2008). Accordingly, osteocalcin mRNA levels were increased in bone marrow stromal cell cultures from C/EBPβ transgenics. However, they were not changed in calvarial osteoblasts, possibly due to the inhibitory effect of C/EBPβ on Runx-2 expression and on terminal osteoblast differentiation (Iyer *et al.* 2004, Wiper-Bergeron *et al.* 2007).

C/EBPβ is involved in cell-fate decisions and regulates the balance between osteoblasts and adipocytes *in vitro*, and the *Cebpb* deletion *in vivo* impairs the formation of white and brown adipose tissue (Tanaka *et al.* 1997, Hata *et al.* 2005). Accordingly, male *Cebpb* null mice exhibited reduced total body fat. The *Cebpb* deletion did not affect the number of bone marrow adipocytes, possibly because C/EBPδ is sufficient to sustain adipogenesis in the absence of C/EBPβ (Lane *et al.* 1999). However, the effect of the *Cebpb* deletion on the differentiation of bone marrow adipocytes *in vivo* is not known, and it is conceivable that genes regulating the development of brown and white adipose tissue could be different from genes regulating bone marrow adipogenesis. We did not detect an effect on total body fat or in the number of adipocytes in C/EBPβ transgenics. This should be expected, since C/EBPβ was expressed under the control of the osteocalcin promoter, which is expressed by mature osteoblasts and not by undifferentiated mesenchymal cells capable of adipocytic differentiation.

In conclusion, C/EBPβ plays an important regulatory role in osteoblast differentiation and skeletal function *in vivo*.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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