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CCAAT/ENHANCER BINDING PROTEIN HOMOLOGOUS PROTEIN (CHOP) DECREASES BONE FORMATION AND CAUSES OSTEOPENIA

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Abstract

CCAAT enhancer binding protein (C/EBP) homologous protein (CHOP), is a member of the C/EBP family of nuclear proteins and plays a role in osteoblastic and adipocytic cell differentiation. CHOP is necessary for normal bone formation, but the consequences of its overexpression *in vivo* are not known. To investigate the direct actions of CHOP on bone remodeling *in vivo*, we generated transgenic mice overexpressing CHOP under the control of the human osteocalcin promoter. CHOP transgenics exhibited normal weight and reduced bone mineral density. Static and dynamic femoral bone histomorphometry revealed that CHOP overexpression caused reduced trabecular bone volume, secondary to decreased bone formation rates. One of 2 lines displayed a decrease in the number of osteoblasts, but *in vivo* bromodeoxyuridine labeling demonstrated that CHOP overexpression did not have an effect on osteoblastic cell replication. The decreased osteoblast cell number was accounted by an increase in apoptosis, as determined by DNA fragmentation measured by transferase-mediated digoxigenin-deoxyuridine triphosphate (dUTP) in situ nick-end labeling (TUNEL) reaction. In conclusion, transgenic mice overexpressing CHOP in the bone microenvironment have impaired osteoblastic function leading to osteopenia.

Keywords

osteoblasts; bone formation; CCAAT enhancer binding proteins; nuclear proteins; transgenics

INTRODUCTION

CCAAT/enhancer binding proteins (C/EBP) are a family of nuclear proteins, which include C/EBP homologous protein (CHOP), also termed growth arrest and DNA damage-inducible gene (GADD) 153, or DNA damage inducible transcript 3 (DDIT3) [1–4]. C/EBP proteins contain highly conserved DNA-binding and leucine dimerization domains, and can form homo- and hetero- dimers that bind to similar sequence motifs. C/EBPs also dimerize with the activator of transcription (ATF)/cyclic AMP response element binding protein (CREB) family of

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proteins [1,2,5]. C/EBPs play a central role in cell fate and function, and C/EBP β can interact with runt related transcription factor (Runx)-2 and regulate the commitment of the cells of the osteoblastic and of the adipocytic lineage [1,2,6,7].

CHOP is a member of the basic Zip family of transcription factors. In contrast to other C/EBPs, the presence of two proline residues in the DNA-binding region of CHOP disrupts its helical structure and alters direct binding to DNA although CHOP/C/EBP dimer binding can occur and regulate transcription [4,8–10]. In most instances, CHOP regulates transcription indirectly by interacting with other nuclear proteins. The primary partner of CHOP is C/EBP β , but CHOP can dimerize with other members of the ATF/CREB family of proteins, and interacts with the Fos/Jun family of transcription factors by a tethering mechanism [8,11–13]. CHOP is implicated in programmed cell death in response to endoplasmic reticulum (ER) stress [14–16]. However, other functions of CHOP have been postulated, including an effect on osteoblastic cell differentiation [17].

In accordance with its role in endoplasmic reticulum (ER) stress responses, CHOP expression is induced by amino acid and glucose deprivation [18,19]. CHOP expression also is induced by the pancreatic endoplasmic reticulum kinase (PERK), an effect mediated by an increase in *atf-4* translation [15,20]. *Perk* null mice and humans with *perk* gene mutations develop osteopenia due to increased osteoblast sensitivity to cellular stress, and *atf4* null mice exhibit decreased bone formation due to impaired osteoblastic function [21,22]. Similarly, *chop* null mice exhibit decreased bone formation, indicating that CHOP plays a role in osteoblastic function *in vivo* [23]. Recently, we demonstrated that CHOP transcripts accumulate as stromal cells differentiate toward the osteoblastic phenotype, and that the constitutive overexpression of CHOP *in vitro* enhances osteoblastogenesis and opposes adipogenesis [17,24]. However, the consequences of CHOP overexpression in adult skeletal tissue *in vivo* are not known.

The purpose of this study was to assess the effects of CHOP overexpression on bone remodeling *in vivo*. For this purpose, we created transgenic mice expressing CHOP under the control of the osteoblastic specific osteocalcin promoter, and determined their skeletal phenotype.

MATERIALS AND METHODS

Generation of transgenic mice

For the generation of CHOP transgenic mice, a bicistronic vector with an internal ribosomal entry site (IRES) to direct the expression of CHOP and green fluorescent protein (GFP) was used (Figure 1). A 600 nucleotide fragment coding for CHOP was cloned downstream of a 3.8 kilobase (kb) fragment of the human osteocalcin promoter (hOC) (E. Gardiner; Sydney, Australia) followed by polyadenylation sequences [25,26]. Microinjection of linearized DNA into pronuclei of fertilized oocytes from CD-1 outbred albino mice (Charles River Laboratories, Cambridge, MA), and transfer of microinjected embryos into pseudopregnant mice were carried out by the transgenic facility at the University of Connecticut Health Center (Farmington, CT). Positive founders were identified by Southern blot analysis of tail DNA. Genomic DNA was digested with the restriction endonuclease, Sca I resolved by electrophoresis on a 0.8% Seakem LE agarose gel (Cambrex Bio Science, Rockland, ME), blotted onto GeneScreen Plus charged nylon (Perkin-Elmer, Norwalk, CT), hybridized with a 600 base pair (bp) CHOP cDNA (D. Ron; New York City, NY), and washed as previously described [24]. The bound radioactive material was visualized by autoradiography on Kodak X-AR5 film (Eastman Kodak, Rochester, NY). Founder transgenic mice were bred to wild type CD-1 mice to generate individual transgenic lines. First generation (F1) heterozygous and wild type littermates were genotyped by Southern blot analysis. Heterozygous mice were intercrossed to generate a homozygous offspring, which was identified by Southern blot analysis. The results described were obtained from the analysis of two transgenic lines, derived from

independent founders, and compared to wild type littermate control mice. 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 performed on mice anesthetized with tribromoethanol (Sigma-Aldrich Corp., St. Louis, MO) on a Faxitron X-ray system (model MX 20, Faxitron X-ray Corp., Wheeling, IL). The X-rays were performed at an intensity of 30 kilovolts for 20 seconds. Total and femoral bone mineral content (gm), skeletal area (cm²), and bone mineral density (BMD; gm/cm²) and total % fat content were measured on anesthetized mice using the PIXImus small animal DEXA system (GE Medical Systems/LUNAR, Madison, WI) [27]. Calibrations were performed with a phantom of a defined value, and quality assurance measurements were performed prior to each use. The coefficient of variation for total BMD is <1% (n = 9 mice).

Bone histomorphometric analysis

Static and dynamic histomorphometry was carried out on transgenic mice and wild type controls at 4 and 12 weeks of age. Mice were injected with calcein, 20 mg/kg, and demeclocycline, 30 mg/kg, at an interval of 2 or 7 days for 4 and 12 week old mice, respectively, and sacrificed by CO₂ inhalation 2 days after the demeclocycline injection. Femurs were dissected, fixed in 70% ethanol, dehydrated and embedded undecalcified in methyl methacrylate. Longitudinal sections, 5 µm thick, were cut on a Microm microtome (Microm, Richards-Allan Scientific, Kalamazoo, MI) and stained with toluidine blue, pH 6.4 or Von Kossa. Static parameters of bone formation and resorption were measured in a defined area between 181 µm and 725 µm from the growth plate, using an OsteoMeasure morphometry system (Osteometrics, Atlanta, GA) [28,29]. For dynamic histomorphometry, mineralizing surface per bone surface and mineral apposition rate were measured in unstained sections under ultraviolet light, using a B-2A set long pass filter consisting of an excitation filter ranging from 450 to 490 nanometers (nm), a barrier filter at 515 nm, and a dichroic mirror at 500 nm. 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 [30].

Measurement of bromodeoxyuridine incorporation in decalcified bone sections

Transgenic mice and wild type controls were injected intraperitoneally with 50 µg bromodeoxyurine (BrdU) per gram of body weight at 4 weeks of age, 24 h before sacrifice. After sacrifice, femurs were dissected, fixed in 10% formalin in phosphate buffered saline (PBS) for 3 h at room temperature, decalcified with Decal-Stat (Decal Corp., Tallman NY) overnight at 4°C and embedded in paraffin. Longitudinal sections across the femurs were obtained. Prior to processing, tissue slides were exposed to 0.05% pepsin in 0.1 N HCl for 30 min at 37°C. Slides were then placed in citrate buffer, heated at 50°C for 20 min for heat induced target antigen retrieval, and incubated with 1:100 primary antibody to BrdU (Dako Corp., Carpinteria, CA) [31,32]. To identify actively proliferating cells, nuclei that had incorporated BrdU were detected using the Dako EnVision+ System, Peroxidase system (Dako Corp., Carpinteria, CA.) per manufacturer's instructions. Sections were counterstained with hematoxylin. For each section, BrdU-positive nuclei of cells lining the trabecular perimeter were counted in three consecutive fields of the primary spongiosa. Sections incubated in the absence of the primary antibody were used as negative controls for the assay. Preliminary experiments confirmed absence of BrdU positive cells in mice injected with vehicle alone.

Measurement of apoptosis in decalcified bone sections

Sections were mounted on poly-lysine-coated glass slides plus (Allegiance Healthcare Corp., McGraw Park, IL). Slides were treated with 100 μ l of 20 μ g/ μ l proteinase K in 10 mM Tris, pH 8.0 for 20 min at room temperature, rinsed with 50 and 10 mM Tris buffered sodium/potassium chloride (TBS), reincubated in 3% H₂O₂ in methanol and rinsed with 50 mM TBS, as described [29,33]. DNA fragmentation was detected by the transferase-mediated digoxigenin-deoxyuridine triphosphate (dUTP) in situ nick-end labeling (TUNEL) reaction using Klenow terminal deoxynucleotidyl transferase per manufacturer's instructions (Oncogene Research Products, Cambridge, MA) [29,33]. Sections were incubated in 0.15% CuSO₄ in 0.9% NaCl for 2 min and counterstained with 2% methyl green aqueous solution. Sections of femurs from wild type mice treated with 1 μ g/ μ l DNase I in 50 mM TBS/ 1mM MgSO₄ were used as a positive control of the assay. Sections not incubated with the transferase, but with vehicle alone, were used as negative controls. Cells in which the nuclei were clearly dark brown rather than blue-green were considered apoptotic and osteoblasts were identified as cells lining the trabecular surface.

Northern blot analysis

Total RNA was isolated from calvariae using the Trizol reagent solution as per manufacturer's instructions (Invitrogen, Carlsbad, CA). The RNA was resolved by electrophoresis on a formaldehyde agarose gel following denaturation, blotted onto GeneScreen Plus charged nylon and hybridized with a 0.6 kb CHOP cDNA and a 0.7 kb murine 18S ribosomal RNA cDNA (American Type Culture Collection ATCC, Manassas, VA), as described [24]. The bound radioactive material was visualized by autoradiography on Kodak X-AR5 film, employing Cronex Lightning Plus or Biomax MS intensifying screens. Northern analysis are representative of 3–7 samples.

Cell Culture and real time – reverse transcription – polymerase chain reaction (RT-PCR)

Parietal bones were obtained from 3 to 5 day old mice and osteoblast-enriched cells (Ob cells) were obtained by sequential collagenase digestion as described [34]. Ob cells were cultured in a 5% CO₂ incubator at 37°C, in Dulbecco's modified Eagle's medium, 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA), in the presence of 5 mM β glycerophosphate and 100 μ g/ml ascorbic acid for 10 days following confluence. Total RNA was extracted and CHOP, osteocalcin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels determined by real-time RT-PCR [35]. For this purpose, 1–10- μ g of RNA were reverse-transcribed using SuperScript III Platinum Two-Step qRT-PCR kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions and amplified in the presence of 5'-GACGCTTCACTACTCTTGACCCTGCG[FAM]C-3' and 5'-GGATGTGCGTG TGACCTCTGT-3' primers for CHOP; 5'-CACTTACGGCGCTACCTTGGGTAAGT [FAM] G-3' and 5'-CCCAGCACAACTCCTCCCTA-3' primers for osteocalcin; and 5'-CACGCTCTGGA AAGCTGTGGCG[FAM]G-3' and 5'-AGCTTCCCCTTCAGCTCTGG-3' primers for GAPDH and Platinum Quantitative PCR SuperMix-UDG (Invitrogen) at 54°–60° C for 45 cycles. Gene copy number was estimated by comparison with a standard curve constructed using *chop* or *osteocalcin* (J. Lian, University of Massachusetts, Worcester, MA) DNAs and corrected for *gapdh* (R. Wu, Cornell University, Ithaca, NY) copy number [24, 36,37]. 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

Results are expressed as means $\pm$ SEM. Statistical significance was determined by Student's *t* test.

RESULTS

Generation, identification and overall phenotype of CHOP transgenic mice

Two transgenic mouse lines overexpressing CHOP under the control of the human osteocalcin promoter were generated. Founder 1 carried 4 copies and founder 2 carried 6 copies of the transgene. Founder 1 was used to generate a homozygous offspring, whereas Founder 2 was used to generate a heterozygous offspring. CHOP transgene (2.6 kb) and endogenous transcripts (0.6 kb) were expressed in RNA extracted from calvariae of transgenic mice, whereas only endogenous transcripts were expressed in wild type controls (Figure 1). The level of expression of endogenous CHOP mRNA was less intense than the transgene-derived mRNA. Transgenic mice were compared with wild type age and sex matched controls. Both transgenic lines displayed similar skeletal phenotypes. Homozygous transgenics from Founder 1 exhibited a skeletal phenotype at 4 weeks, but not at 12 weeks of age, whereas heterozygous transgenic mice from Founder 2 displayed a phenotype at 4 weeks and 4 months of age. The time-dependent loss of the phenotypic expression of line 1 is possibly related to a decline in the activity of the osteocalcin promoter and modest levels of protein expression [38,39]. At birth and throughout the study period, CHOP overexpressing mice were visually indistinguishable from wild type controls. Their weight at 4 weeks (Figure 2), 12 weeks and 16 weeks (not shown) was not different from wild type controls.

X-rays, bone mineral density and body composition

Contact radiography of CHOP transgenics did not reveal osteopenia or obvious skeletal abnormalities (not shown). At 4 weeks of age, male homozygous from Line 1 displayed a 6 to 7% decrease in total and femoral BMD ($n = 7$; $p < 0.05$ for both) and male heterozygous from Line 2 a 6% decrease in BMD (Figure 2). Percent total body fat was not different between transgenics and controls (Figure 2).

Static and dynamic histomorphometry

Histomorphometric analysis of femurs from 4 week old male homozygous transgenics from Line 1 (Table 1) and heterozygous transgenics from Line 2 (Figure 3) revealed a ~45% decrease in trabecular bone volume. CHOP transgenics exhibited a 30% (Line 1) or 60% (Line 2) reduction in the number of osteoblasts per trabecular area (not shown), but only transgenics from Line 2 displayed a 30 to 35% reduction in the number of osteoblasts per perimeter and in osteoblast surface ($n = 6$; $p < 0.01$). This was the only difference noted between the two lines studied in one month old mice, and it may be related to different levels of CHOP expression. The number of osteoclasts and eroded surface were not different in CHOP transgenic mice from either line and wild type controls, indicating normal bone resorption in CHOP overexpressing mice. Fluorescence microscopy of CHOP transgenic male mice revealed a significant 40 to 65% decrease in mineral apposition and bone formation rates, indicating impaired osteoblastic function (Table 1, Figure 4). The number of adipocytes/marrow area were determined in Line 2, and were increased in CHOP transgenic mice proportionally to the decrease in trabecular bone volume from (means $\pm$ SEM; $n = 6 - 7$) $21.7 \pm 1.9 \text{ cm}^2$ in controls to $38.9 \pm 2.4 \text{ cm}^2$ in transgenics ($p < 0.05$). One month old female homozygous transgenic mice from Line 2 were studied and displayed a 35% ($p < 0.05$) decrease in trabecular bone volume (not shown). As indicated, homozygous mice from line 1 did not display a skeletal phenotype at 3 months of age, whereas heterozygous male mice from line 2 were osteopenic at 4 months of age. Total BMD, trabecular bone volume and bone formation rates were decreased by 10%, 48% and 75%, respectively, in 4 month old transgenics of line 2, as compared to wild type controls ($n = 3 - 4$; all, $p < 0.05$).

To investigate whether the reduction in the number of osteoblasts observed in Line 2 of CHOP transgenics was secondary to a decrease in cell proliferation, 4 week old male CHOP transgenic

mice and wild type controls were sacrificed 24 h after an intraperitoneal injection of BrdU. The BrdU label was detected in the primary spongiosa region of the femur, where most of the cells actively dividing are preosteoblastic. A small number of labeled cells were observed in the secondary spongiosa area. The number of BrdU labeled preosteoblasts/osteoblasts per area was (means $\pm$ SEM; n = 4 – 5) 106.5 ± 9.1 cells/mm² in transgenics, and 124.5 ± 11.1 cells/mm² in wild type controls, indicating that cell proliferation is not impaired in CHOP transgenic mice (Figure 5). To determine whether the decreased number of cells in CHOP transgenics from Line 2 was secondary to cell death, a TUNEL assay was performed. CHOP transgenic mice had an increased number of apoptotic osteoblasts and osteocytes when compared to wild type controls. The number of apoptotic/total osteoblasts was 26% in femurs from wild type controls and 45% apoptotic/total osteoblasts in CHOP transgenics (Figure 5).

Ob-cell culture

To examine the effect of CHOP on osteoblastic function *in vitro*, calvarial Ob cells were isolated from CHOP homozygous transgenics from Line 2 and wild type control mice, and studied at confluence and 10 days post confluence. Ob cells from CHOP transgenics expressed 8 times higher levels of CHOP mRNA than wild type controls 10 days after confluence. Osteocalcin mRNA levels increased in CHOP overexpressing and control cells as the culture progressed, and were 16 fold higher in CHOP overexpressing cells than in wild type controls 10 days after confluence (Table 2). These results suggest that *in vitro* CHOP overexpression favors osteoblast differentiation as previously documented in ST-2 stromal cells [17].

DISCUSSION

Our findings demonstrate that transgenic mice overexpressing CHOP under the control of the osteocalcin promoter develop osteopenia. The skeletal phenotype was evident at 4 weeks of age, a time of marked expression of the transgene. Histomorphometric analysis of femurs from CHOP transgenic mice revealed reduced trabecular bone volume due to decreased bone formation. Osteoblast-targeted CHOP overexpression caused an inhibition of bone formation possibly because of a decrease in osteoblastic function, since bone formation, and MAR were reduced. In one of the two lines studied there was also a decrease in the number of osteoblasts, possibly accounting for some of the inhibitory effects on bone formation. The reason why osteoblast cell number decreased in one, but not in both transgenic lines is not clear, but it is possibly related to different levels of expression of CHOP in the bone environment.

The decreased number of osteoblasts observed in one of the two lines of CHOP transgenics was secondary to an increase in cellular death and not secondary to changes in cell replication. BrdU labeling was conducted in paraffin and not methyl methacrylate embedded bones. Consequently, technical reasons did not allow us to correct labeled cells/trabecular bone area. The results are in agreement with the known effects of CHOP on programmed cell death in response to ER stress [14,40,41]. ER stress induces CHOP expression and phosphorylation, and the constitutive overexpression of CHOP in cultured cells sensitizes them to ER stress favoring cellular death and impairing cellular functions [40,42]. Consequently, it is not surprising that overexpression of CHOP *in vivo* can lead to osteoblastic apoptosis and decreased osteoblastic function. The results observed mimic those reported in chondrocytes following the induction of ER stress, which causes chondrocytic apoptosis, and decreased extracellular matrix synthesis and type II collagen production [42]. Although impaired cell growth also occurs during ER stress responses, and presumably is mediated by CHOP, this did not occur in CHOP overexpressing mice, as BrdU labeling was not different from control wild types [14,42,43]. This could be because of differences in the cells and models studied or because the impaired cell growth observed during ER stress response is mediated by CHOP, but requires the presence of additional cellular signals. It is also possible that CHOP overexpression resulted

in the dimerization of CHOP with C/EBP α , which cooperates with p21 to inhibit cyclin-dependent kinase-2 activity inducing cell growth arrest [44]. In the absence of available C/EBP α , enhanced growth arrest might not occur.

Previously, we have documented that in osteoblasts CHOP interacts with other members of the C/EBP family of transcription factors, including C/EBP α and β , and the phenotype observed in CHOP transgenics could be secondary to the binding of CHOP and C/EBP β [17]. This is reaffirmed by recent work demonstrating that mice overexpressing a dominant negative isoform of C/EBP β , p20C/EBP β , LIP, under the control of the type I collagen promoter exhibit osteopenia and a phenotype similar to the one we report in CHOP overexpressing mice [45]. These *in vivo* findings are in agreement with observations demonstrating that, by interacting with Runx-2, C/EBP β can induce osteocalcin transcription and can promote osteoblast differentiation [6,7]. Consequently, the overexpression of a dominant negative C/EBP β or of CHOP would prevent C/EBP β -DNA interactions or would result in sequestration of C/EBP β and as a consequence impaired osteoblastic function.

Overexpression of CHOP in the bone microenvironment *in vivo* also may result in important interactions with Fos and Jun or with ATF-4, a member of the ATF/CREB family, and explain the osteopenia observed in CHOP transgenics [11,12]. ATF-4 has important effects on osteoblast function and *atf4* null mice exhibit decreased osteoblastic activity, whereas forced ATF-4 expression induces osteoblastic gene markers [22,46]. CHOP interactions with Fos and Jun or ATF-4 might occur *in vivo*. However, overexpression of CHOP in ST-2 stromal cells did not alter the binding of nuclear extracts to an AP-1 consensus sequence, did not modify the transactivation of AP-1 reporter constructs, and electrophoretic mobility supershift assays failed to reveal interactions with ATF-4 (R.C. Pereira and E. Canalis, unpublished observations). Recent work has demonstrated that CHOP can inhibit Wnt/T-Cell factor signaling in response to Wnt-8, but CHOP overexpression in cells of the osteoblastic lineage results in enhanced and not in suppressed Wnt/ β catenin signaling [17,47]. Consequently, impaired Wnt/ β catenin signaling does not seem a plausible explanation for the phenotype observed in CHOP overexpressing mice.

There are differences between the results we reported *in vivo* with those obtained *in vitro* either in stromal cells constitutively overexpressing CHOP or Ob cells from CHOP transgenics. Previously, we reported that CHOP accelerates osteoblastic differentiation and opposes adipogenesis in ST-2 stromal cells [17]. Accordingly, Ob cells obtained from transgenic mice express higher levels of osteocalcin mRNA than control cells. It is important to note that the acceleration of osteoblastogenesis *in vitro* occurs after relatively brief periods of time, and that the more prolonged expression of CHOP *in vivo* could have different temporal effects. These would depend on the proteins interacting with CHOP at specific times and the long term consequences of these interactions. Although the percent total body fat was not altered in CHOP transgenics, this may be a reflection of the osteoblastic specific osteocalcin promoter used to direct CHOP expression. The increase in the number of adipocytes detected in the marrow space would not appear congruent with the inhibitory effects of CHOP on adipogenesis. However, it represents an increase in adipocytes that is proportional and related to a decrease in trabecular bone volume.

Previously, we demonstrated that CHOP is necessary for normal osteoblastic function *in vivo* and the present studies showing that CHOP overexpression *in vivo* inhibits bone formation would appear not congruent with our earlier observations in *chop* null mice [23]. It is not unusual for intracellular and extracellular proteins to be necessary for a biological effect, but when expressed in excess to be inhibitory. For example, Hes-1 is necessary for adipogenesis, but its overexpression inhibits adipogenesis, twisted gastrulation and connective tissue growth factor are necessary for BMP effects on osteoblastogenesis, but when in excess they can be

inhibitory [48–51]. Similarly, CHOP is necessary for normal osteoblastic function, but when in excess, and under specific conditions *in vivo*, it may interact with intracellular proteins necessary for osteoblastic function and become inhibitory.

In conclusion, *in vivo* overexpression of CHOP inhibits bone formation, and causes osteopenia.

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Figure 1.

Schematic representation of the construct used for the creation of transgenic mice, and relative levels of CHOP transgene and endogenous mRNA expression in CHOP homozygous (Line 1) and heterozygous (Line 2) transgenic mice and wild type (WT) controls. Total RNA was extracted from calvariae of 4 week old CHOP transgenics and controls, resolved by Northern blot analysis and hybridized with $[\alpha\text{-}^{32}\text{P}]$ -labeled CHOP and mouse 18S cDNAs.

Figure 2. Bone mineral density (BMD $\text{gm} \times \text{cm}^2 \times 10^{-3}$), weight (gm) and total body fat (%) of 4 week-old CHOP transgenic heterozygous (Line 2; black bars) and wild type control (WT, white bars) male mice. Values represent means $\pm$ SEM; $n = 6-7$. *Significantly different from wild type controls, $p < 0.05$.

Figure 3.

Static bone histomorphometry performed on femurs from 4 week old male CHOP transgenic heterozygous (Line 2; black bars) and wild type controls (WT, white bars). Stained sections were examined, and trabecular bone volume (BV/TV, %), osteoblast and osteoclast number per perimeter (Ob/B.Pm./mm; Oc/B.Pm./mm) trabecular number (Tb.N./mm) and thickness (Tb.Th.; μm) and eroded surface/bone surface (ES/BS) determined. Values are means + SEM; n = 6–7. *Significantly different from WT controls, $p < 0.05$. The lower panel shows representative femoral sections from CHOP transgenics and WT controls stained with Von Kossa and examined at a final magnification of 40x.

Figure 4.

Dynamic bone histomorphometry performed on femurs from 4 week old male CHOP heterozygous transgenic (Line 2; black bars) and wild type controls (WT, white bars). Unstained sections were analyzed by fluorescence microscopy and mineralizing surface per bone surface (MS/BS; %), mineral apposition rate (MAR; $\mu\text{m}/\text{day}$) and bone formation rate/ bone surface (BFR/BS; $\mu\text{m}^3/\mu\text{m}^2/\text{day}$) determined. Values are means $\pm$ SEM; $n = 6-7$.

*Significantly different from WT controls, $p < 0.05$. The lower panels show representative femoral sections from CHOP transgenics and WT controls and visualization of calcein and demeclocycline labels by fluorescence microscopy at a final magnification of 100x and 400x.

Figure 5.

Effect of CHOP overexpression on osteoblast cell proliferation and apoptosis in femoral sections from 4 week-old male CHOP heterozygous transgenics (Line 2) and wild type (WT) controls. A. For visualization of BrdU incorporation, mice were injected intraperitoneally with BrdU, 50 μ g/gram of body weight, 24 h before sacrifice and processed as described in Materials and Methods. Arrows point to the BrdU labeled cells in the primary spongiosa at a magnification of 400x. B. DNA fragmentation was detected by the TUNEL reaction, and osteoblasts, identified as cells lining the trabecular surface, in which the nuclei were dark brown rather than blue-green were considered apoptotic. Final magnification 200x. Data are means

$\pm$ SEM; $n = 3-4$, and are expressed as the percent apoptotic/total cells from CHOP transgenics (black bars) and a WT control (white bars). *Significantly different from WT controls, $p < 0.05$.

Table 1

Static and dynamic bone histomorphometry performed on femurs from 4 week old male CHOP homozygous transgenic (Line 1) and wild type controls.

	Wild Type	CHOP
Bone Volume/Total Volume (%)	14.0 ± 1.2	7.9 ± 0.1 [*]
Trabecular Number/mm	10.8 ± 1.1	6.6 ± 0.3 [*]
Trabecular Thickness/μm	13.1 ± 0.4	12.1 ± 0.7
Osteoblasts/Perimeter/mm	26 ± 3.6	27.4 ± 5.3
Osteoclasts/Perimeter/mm	6.7 ± 0.5	7.7 ± 0.8
Eroded Surface/Bone Surface (%)	22.7 ± 1.5	26.6 ± 2.6
Mineral Apposition Rate; μm/day	0.42 ± 0.06	0.16 ± 0.02 [*]
Bone Formation Rate/Bone Surface; μm ³ /μm ² /day	0.006 ± 0.001	0.002 ± 0.001 [*]

Values represent means ± SEM (n = 6 – 7) obtained from femoral sections stained and analyzed for static parameters of bone histomorphometry or unstained and analyzed by fluorescence microscopy.

* Significantly different from control wild type, $p < 0.05$.

Table 2

Effect of CHOP overexpression on osteocalcin mRNA expression in cultured osteoblasts.

	Wild Type	CHOP
	gene copy number/ <i>gapdh</i>	
<i>Chop/gapdh</i>		
Confluence	0.4 ± 0.1	1.2 ± 0.3
10 days post confluence	6.0 ± 1.3	28.0 ± 2.6*
<i>Osteocalcin/gapdh</i>		
Confluence	1.2 ± 0.4	3.0 ± 0.9
10 days post confluence	8.1 ± 1.5	126.1 ± 35.7*

Calvarial Ob cells were obtained from CHOP homozygous (Line 1) transgenics and wild type controls of both sexes and total RNA extracted and reverse-transcribed, and 0.1 to 0.6 µg amplified by real time RT-PCR in the presence of specific primers to detect *chop*, *osteocalcin* and *gapdh* genes. Data represent gene copy number corrected for *gapdh*. Values represent means ± SEM; n = 3.

* Significantly different from control wild type, $p < 0.05$.