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Analysis of Osteoblast Proliferation, Differentiation and Apoptosis in Co13,6-p20C/EBPbeta transgenic Mice

Piero Palacios

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Analysis of Osteoblast Proliferation, Differentiation and Apoptosis in Col3.6-p20C/EBPβ Transgenic Mice

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Analysis of Osteoblast Proliferation, Differentiation and Apoptosis in Col3.6-p20C/EBPβ Transgenic Mice

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ABSTRACT

C/EBP transcription factors have been recognized as key regulators of cellular differentiation in a variety of cells, including adipocytes, hepatocytes, granulocytes and macrophages. A growing literature also suggests an important role for C/EBP family members in the growth and differentiation of osteoblasts. To better comprehend the role of C/EBP in osteogenesis, a transgenic mouse model has been generated in which the function of C/EBP in cells of the stromal and osteoblast lineage has been blocked by overexpression of a dominant negative isoform of C/EBPβ, known as p20C/EBPβ.

Preliminary studies of these p20C/EBPβ transgenic mice demonstrated varying degrees of osteopenia. Although only three out of the four lines of transgenic mouse models created exhibited an osteopenic bone phenotype, all four possessed alterations in osteoblastic marker gene expression. The analysis of such markers in femurs and calvaria of transgenic mice indicated two possible sites of action at which C/EBPβ affected the osteoblast lineage. The first site of action occurred late in osteoblast differentiation, manifested by a decrease in terminal markers of osteoblastic differentiation such as osteocalcin and pOBCol 2.3 GFP. The latter is a transgenic reporter of osteoblast differentiation in which the visible GFP protein is expressed under control of a 2.3 kb Col1 promoter construct. The second site appears to be in early osteoblast differentiation characterized an increase in early differentiation markers such as bone sialoprotein (BSP) and alkaline phosphatase. These preliminary studies have also shown an increase in cell number in transgenic mouse stromal cells cultures. The same phenomenon was observed in primary cell cultures, with an increase in cell number in the TG cultures after day 7,
reaching a plateau at day 15. It was unknown if the increase in cell number in TG cultures was due to an increase in cell proliferation or a decrease in cell apoptosis.

Therefore, in the present study, primary osteoblast and BMSC cultures from TG mice were be used to measure proliferation and apoptosis in the early stages of culture. Studies were performed on a pOBCo13.6-GFP genetic background to provide a real-time marker of the stromal-osteoprogenitor pool and to allow changes in this cell population to be specifically tracked within a heterogeneous population of cells. Proliferation and apoptosis were assessed by flow cytometry analysis. In order to measure cell proliferation, cells were pulsed with BrdU, because it can be incorporated into DNA in place of thymidine. Cells were pulsed in a range from 2 to 12 hours, and detected using a monoclonal antibody against BrdU. Apoptosis was assessed using annexin V, a marker of early apoptosis, and 7AAD, a marker of late apoptosis, as measured by flow cytometry analysis.

By analyzing the results of these experiments we will be determine if the role of eC/EBP transcription factors in early osteoblast differentiation and whether they act to increase proliferation or as a protective factor decreasing the number of cells undergoing programmed cell death.
Introduction

Objective of Research

In order to better understand the process of bone formation, both in health and disease, it is necessary to understand the molecular and genetic mechanisms underlying this process. The precise molecular events occurring during osteoblast differentiation are not yet understood. A more thorough understanding of osteoblastic differentiation and proliferation will be required for the elimination of certain bone diseases and will improve our understanding of orthodontic tooth movement.

With the objective of over-expressing a dominant negative C/EBP to assess the role of C/EBP in bone, four lines of mice carrying the pOBCol3.6 FLp20C/EBP𝛽 transgene (a dominant negative form) have been established by pronuclear microinjection and designated as lines 00-59, 50-10, 63 and 65. The only difference between these four lines is the position of the transgene within the mouse genome. These lines were subsequently crossed with homozygous POBCol2.3-GFP mice. These mice, expressing a GFP fluorescent reporter, were developed at the University of Connecticut and express the osteoblast-specific GFP marker which allows for in vivo imaging of the terminal stages of osteoblast differentiation. Initial work in our lab on these four lines of p20C/EBP𝛽 transgenic mice has shown varying degrees of osteopenia secondary to reduced bone formation. It was demonstrated that there were alterations in osteoblastic marker gene expression with an overall increase in BSP expression and an overall decrease in osteocalcin and POBCol2.3-GFP expression. It is important to note that these latter two markers are in fact terminal markers of osteoblastic differentiation. An overall decrease in POBCol2.3-GFP expression in
vivo was also noted in these transgenic lines. These preliminary studies help illustrate how C/EBP transgenic factors are required either for osteoblast differentiation or for the maintenance of the osteoblast phenotype. In order to better understand the role of C/EBP's during osteoblast proliferation, the previously mentioned lines of mice were crossed with pOBCol3.6-GFP mice, an early marker of osteoblast differentiation. Using this model the expression of GFP in our TG cultures should be directly correlated with the expression of our transgene, since they are driven by the same promoter. To be able to identify and quantify the number of cells undergoing proliferation a flow cytometry analysis using BrdU incorporation into the cellular DNA was undertaken. The amount of BrdU labeling was analyzed by flow cytometry analysis. At the same time, using a two color analysis, GFP was determined. With this design we were be able to determine the cells that not only are undergoing proliferation, but those that were expressing the p20C/EBPβ transgene. We also attempted to determine the apoptosis rate occurring in primary calvarial cells over-expressing C/EBPβ. To identify the number of cells undergoing programmed cell death a 7AAD-Annexin V incorporation assay was used followed by flow cytometry analysis in order to measure incorporation of these apoptotic markers. By identifying the cell responses accounting for the increases in cell number in early TG cultures, we would improve our understanding of the role of C/EBP in the regulation of pluripotent progenitor pools and in early osteoblast differentiation.


**Background**

Osteoblasts are the primary bone-forming cells in the body. Their presence and activity are essential for acquiring and maintaining normal bone mass. It is being recognized that impaired generation of bone cells, either due to an underperforming lineage or to a diversion of the lineage to other cell types, is a primary cause for diminished bone mass.[1] Identifying factors that regulate osteoblast differentiation will be necessary to understand diseases of bone and for designing effective therapeutic strategies. [1]

Osteoblast precursors are recruited from a multipotential mesenchymal progenitor that can give rise to several differentiated cell phenotypes including fibroblasts, chondrocytes, adipocytes and osteoblasts. [1] Undifferentiated osteoprogenitors are present in the bone marrow and periosteum. These undifferentiated osteoprogenitor cells eventually differentiate into preosteoblasts, osteoblasts and finally osteocytes (figure 1). Unfortunately, the differentiation and ultimate fate of these osteoprogenitor cells is determined and influenced by a complex and still poorly understood interaction of hormones and local factors.
C/EBP and Cellular Differentiation

The C/EBP family is known to be involved in the regulation of cell growth and differentiation of several cell types. Common structural features of these proteins are highly conserved DNA binding and leucine zipper dimerization domains at the carboxyl terminus and divergent amino termini containing regulatory and transactivation domains[2, 3] C/EBPβ has been linked to hepatocyte specific gene regulation because it shows high expression in liver cells [4]and binds to regulatory elements in the promoter of liver specific genes. In addition it has been suggested that C/EBPβ contributes in the regulation of the acute phase response of the liver. Lately, it has become clear that C/EBPβ also play a role in other tissues. Results derived from experiments in cell culture and with knockout mice have demonstrated that this protein is essential for lymphocyte and adipocyte differentiation [5, 6] The role of different C/EBP proteins during adipocyte differentiation is thought to follow a sequential model in which C/EBPδ and C/EBPβ precede expression of C/EBPα which functions to inhibit proliferation and promote terminal differentiation. In fact expression of C/EBPα is primarily restricted to highly differentiated cells such as hepatocytes, adipocytes and type II cells of the lung.[7]. This sequential expression of C/EBP isoforms, together with the fact the C/EBPα promoter contains a C/EBP response element, has led to the idea that C/EBPβ can activate the expression of the C/EBPα promoter during differentiation processes such as adipose conversion.

A large body of literature suggests important roles for C/EBP family members in growth and differentiation of osteoblasts. In primary rat osteoblast cultures, C/EBPβ and C/EBPδ are abundantly expressed in proliferating cells, decline during the mid-stage of
differentiation, and then subsequently increase at late stages of differentiation. C/EBPβ and C/EBPδ can also act synergistically with Runx2/Cbfa1, a transcription factor and nuclear matrix binding protein, to enhance osteocalcin transcription in cell culture systems. [8] Correlating C/EBPβ and δ function with Runx2 presents a critical link between C/EBP factors and osteoblast differentiation given that Runx2/Cbfa1 is essential for osteoblastic response to extracellular matrix signals and is needed for the expression of genes associated with osteoblast differentiation. [9] Several key studies have established that Runx2 is required for both in vivo bone formations as well as for osteoblast differentiation. [10] It has also been demonstrated that forced expression of Runx2 in non-osteoblastic cells leads to osteoblast-specific gene expression. [11] Furthermore, Runx2-deficient mice completely lack bone formation, due to an absence of osteoblasts, demonstrating that Runx2 is an essential factor for osteoblast differentiation. [12] Also, humans with cleidocranial dysplasia, an autosomal dominant condition characterized by phenotypic changes in skeletal patterning and growth, have been shown to have deletions within the human Runx2/Cbfa1 gene [13]. The C/EBP family has also been shown to play vital roles in regulating adipocyte commitment, differentiation and gene expression. C/EBPβ and C/EBPδ have been shown to be expressed very early in the pathway from multi-potential progenitor cells to adipocytes. [5, 6] C/EBPβ and C/EBPδ then induce the expression of C/EBPα and PPARγ which direct the expression of terminal adipocyte marker genes. [14] Disruption of the C/EBPα [15] C/EBPβ and C/EBPδ genes [16] prevented normal development of adipose tissue. The role of C/EBP transcription factors in adipogenesis is important when appreciating that osteoblasts and adipocytes share common pluripotent progenitor cells. It has been
shown that an inverse relationship exists between the differentiation of adipocytes and osteoblasts in rats. [17] Hopefully, the role of C/EBP’s in adipogenesis can provide some insight into their potential role in regulating osteogenesis.

**CCAAT/Enhancer-Binding Proteins (C/EBP)**

The C/EBP’s are a family of transcription factors that function in tissue differentiation, healing, immune response and tissue metabolic functions. [18] There are currently six members of the C/EBP family that have been isolated and characterized: C/EBP -α, -β, -δ, -γ, -ε, and -ζ. [19] The majority are expressed in liver, spleen and adipocytic tissues. [8] The C/EBP are classified as members of the basic leucine zipper (bZIP) transcription factor family [Fig 2]. [7] They are structurally related to each other, each consisting of an amino terminal transactivation region, a central basic DNA-binding domain, and a Carboxy-terminal dimerization interface termed the leucine zipper. [18]

The terminal leucine zipper mediates dimerization between C/EBP polypeptides. This dimerization is required for DNA binding and for the activation of transcription in target genes. [20] (Fig 3) C/EBP proteins can form either homodimers or heterodimers with one another, as well as with other members of the bZIP family. They can also form protein-protein interactions with non-bZIP transcription factors. [21] The central DNA binding domain is highly conserved and rich in basic amino acids. The amino-terminal region has amino acid sequences that are unique to each C/EBP member and are critical for transcriptional activity. [20]

C/EBPβ mRNA has three in-frame AUG start codons which initiate the translation of three protein isoforms of 38 kDa, 35kDa and 20 kDa. [9] The larger
products, p38 and p35C/EBPβ (originally called LAP) contain the N-terminal transactivation domain and function as transcriptional activators. [22] The 20 kDa isoform (originally LIP) lacks this N-terminal transactivation domain but contains the C-terminal bZIP domain; p20C/EBPβ therefore acts as a dominant negative regulator of C/EBP function. [22]. This isoform can also arise by a proteolytic cleavage of full length C/EBPβ that is C/EBPα independent. [23]

A number of studies have suggested important roles for members of the bZip transcription factor superfamily in regulating skeletal development. Wang et al. reported that c-fos null mice exhibit osteopetrosis and a failure of tooth eruption due to a lack of osteoclasts[15]. Two models of bZip overexpression have been reported that result in osteosclerosis due to increased bone formation; overexpression of ΔFosB, a naturally occurring splice variant of FosB[24], and overexpression of Fra-1[25], the product of the c-fos-related fsll gene. The overexpression of ΔFosB with the NSE promoter also resulted in a decrease in adipogenesis, leading to speculation that diversion of pluripotent progenitors from adipocyte to osteoblast commitment could be the cellular mechanism responsible for the osteosclerosis. However, a follow-up study in which ΔFosB was targeted with the osteoblast-specific OC promoter resulted in an osteopetrotic phenotype with normal adipogenesis, indicating a cell autonomous action of ΔFosB on osteoblast function[26]. Interestingly, in this study ΔFosB and the further truncated isoform Δ2ΔFosB were found to interact with C/EBPβ and alter its ability to bind to a C/EBP consensus sequence. This resulted in an inhibition of adipogenesis in multipotent ST2 cells but not in the committed pre-adipocytic line 3T3-L1. This observation was
interpreted as evidence that the interaction of these transcription factors at a very early stage could prevent commitment to the adipocyte lineage.

Recently, a role in osteoblast differentiation has been demonstrated for ATF4, a member of the CREB-ATF transcription factor family. Loss of ATF4 function by gene ablation in mice resulted in osteopenia associated with a failure of terminal differentiation and reduced type I collagen protein synthesis[27]. This study identified ATF4 as a substrate for RSK2, a growth factor regulated kinase. Loss of RSK2 function also resulted in a low bone mass phenotype[27]. In subsequent work, it was shown that over-expression of ATF4 in non-osteoblastic cells can induce osteoblast-specific gene expression[28]. Further, a cooperative interaction between ATF4 and Runx2 results in high level expression of the terminal osteoblast marker gene OC[29].

C/EBP transcription Factors: Role in Osteoblast Differentiation and Function

A number of reports have supported a positive role for C/EBP in osteoblast differentiation. C/EBP’s as well as bone morphogenetic proteins (BMP) play essentials roles in mammalian cell differentiation by shaping adipogenic and osteoblastic lineages. Recent evidence suggested that adipocytes and osteoblast share a common mesenchymal precursor cell phenotype, Using the C2C12 engineered pluripotent cell line (myocyte, adipocyte, osteoblast), Fux et al. [30] found that C/EBPα may differentiate cells into either osteoblasts or adipocytes, depending on the level of expression. With low levels of expression osteoblasts were generated, but when maximum levels of C/EBPα were expressed adipocytic differentiation occurred. In these same cells Gu [31] overexpressed BMP7 and observed a shift in the differentiation pathway from myoblast to osteoblast. It
was observed that the level of Runx2 mRNA, a bone specific transcription factor was also stimulated. Using real time PCR the level of C/EBPβ were increased but it was not noticed any changes on the level of C/EBPβ were observed. In a recent report McCarthy [32], observed that the 3' proximal region of the C/EBPδ gene promoter contains a binding sequence for Runx2, indicating a possible regulation of C/EBPδ expression by Runx2. The effects of C/EBPβ and p20C/EBPβ on adipocyte and osteoblast differentiation were studied by Hata et al. [33] in C3H10T1/2 cells. In this model, the expression of p20C/EBPβ blocked adipocyte differentiation and, in cooperation with Runx2, led to an increase in osteoblast differentiation.

Preliminary studies done in our laboratory have seen two possible sites of action for C/EBPβ during osteoblast differentiation; one occurring late in maturation as seen by a decrease in osteocalcin and POBCol2.3-GFP and the second occurring early in osteoblast differentiation manifested by an increase in alkaline phosphatase and bone sialoprotein (Figure 4).

C/EBP Transcription Factors and Proliferation

Recent evidence suggests that cell proliferation can be regulated by members of the C/EBP family. C/EBPα, the first member of the family to be identified, has been associated with an antimitotic activity.[34]. Some reports have been published showing that C/EBPα can interact with p21, a potent inhibitor of cyclin depended kinases (CDKs), the enzymes that are crucial for the orderly progression of the cell cycle. It has been found that C/EBPα mediated growth arrest occurs through protein interaction and is independent of its transcriptional activity.[35, 36].
In a recent study published by Wang it was showed that C/EBPα interacts directly with cdk2 and cdk4, arresting cell proliferation by inhibiting these cells kinases. As expected, the activities of cdk2 and cdk4 are increased in C/EBPα knockout mice, resulting in enhanced proliferation.[37]

C/EBPβ and Cell Proliferation

A number of studies have reported an effect of C/EBPβ on cell proliferation, in a study done by Buck using HepG2 hepatocarcinoma cells, it was seen that expression of full-length C/EBPβ caused arrest of cells at the G1/S boundary. This effect required the leucine zipper and N-terminal transactivation domains. p20C/EBPβ having a dominant negative transcriptional activity had opposite effects to the ones observed with the full length protein. A similar anti-proliferative effect of C/EBPβ was observed in a study done by Zhu in keratinocytes [38]. In primary mouse fibroblasts, over-expression of some oncogenes, including Ras^{V12}, causes cells to exit the cell cycle and enter G1 phase o [39].

Contrary to these observations it has been observed that, C/EBPβ appears to be a positive regulator of the cell cycle in mammary epithelial cells. In an experiment done by Seagroves, on C/EBPβ null mice it was observed that mammary epithelium fail to proliferate, and resulted in poor ductal morphogenesis with inability to lactate [40, 41]. The p20C/EBP isoform has been also correlated with hyperproliferation and transformation in mammary epithelial cells. Transgenic mice with overexpression of p20C/EBPβ in mammary tissue show focal and diffuse alveolar hyperplasia and occasional neoplasias, and invasive and noninvasive carcinomas [42].
Substantial evidence supports a role for C/EBPβ and other family members in the control of apoptotic cell death. The stimuli that initiate the apoptotic process include extracellular signals such as the Fas ligand. Stimulation of the Fas pathway in response to Fas ligand or agonistic anti-Fas receptor antibodies results in recruitment of proteins that form the death inducing signaling complex (DISC) including procaspase 8 and Fas-associated Death Domain (FADD). Formation of the DISC is followed by activation of procaspase 8, which cleaves Bid, a proapoptotic member of the Bcl 2 family, resulting in release of cytochrome c from the mitochondria, activation of caspase 3, and cleavage of cellular substrates, cellular breakdown and ultimately cell death. Several inhibitors of mitochondrial cytochrome c release including Bcl-2 and Bcl-x mediate resistance to Fas-induced cell death. C/EBPβ has been linked to apoptotic targets in the liver; in a recent publication[43] apoptosis induced by an activating anti-Fas antibody was greatly reduced in hepatocytes of C/EBPβ null mice, suggesting a anti-apoptotic role in these cells. This decrease was associated with a 10-fold increase in expression of Bcl-x protein, an anti-apoptotic member of the Bcl family [43]. In Neuro2A cells, C/EBPβ was shown to enhance apoptosis via activation of p53 and the cdk inhibitor p21 [44].

Contrasting these studies, C/EBPβ can also act as an inhibitor of apoptosis. In a publication by Sterneck and Zhu [45] C/EBPβ knockout mice exhibited increased apoptosis in epidermal keratinocytes in response to carcinogen treatment and are completely resistant to carcinogen induced skin tumorigenesis. Their findings showed that C/EBPβ exerts an essential, keratinocyte-intrinsic role in cell survival in response to
carcinogen treatment. The elimination of C/EBPβ in keratinocytes is sufficient to confer complete resistance of the skin chemical carcinogenesis. C/EBPβ appears to inhibit apoptosis in different ways. A study by Buck suggested that phosphorylation of C/EBPβ by RSK (ribosomal protein S6), enzyme responsible for propagation of the EGFR (epidermal growth factor receptor), prevents activation of procaspases 1 and 8 and therefore prevents apoptosis [46]. Another mechanism by which C/EBPβ might prevent programmed cell death is by promoting cell survival through enhanced expression of survival factors. For example, Myc/Raf transformed macrophages required C/EBPβ expression to prevent cell death. This effect was mediated by C/EBPβ-dependent expression of IGF-I, an autocrine survival factor for these cells [47].
The immunofluorescent staining of incorporated bromodeoxyuridine (BrdU) and flow cytometric analysis provide a high resolution technique to determine the frequency and nature of individual cells that have synthesized DNA. In this method, BrdU (an analog of the DNA precursor thymidine) is incorporated into newly synthesized DNA by cells entering and progressing through the S (DNA synthesis) phase of the cells cycle (Fig 7).[48] The incorporated BrdU is then detected with specific anti-BrdU fluorescent antibodies using flow cytometry. Often, staining with a dye that binds to total DNA such as 7-amino-actinomycin D (7-AAD) is coupled with immunofluorescent BrdU staining. With this combination, two-color flow cytometric analysis permits the enumeration and characterization of cells that are actively synthesizing DNA (BrdU incorporation) in terms of their position in the cell cycle (i.e., G0/G1, S, or G2/M phases defined by 7-AAD staining intensity as an indicator of ploidy)[49]

Prolonged exposure of cells to BrdU allows for the identification and analysis of actively cycling, as opposed to non-cycling, cell fractions. Pulse labeling of cells with BrdU various time points, permits the determination of cell-cycle kinetics.[50]

Annexin V

Apoptosis is characterized by a variety of morphological features such as loss of membrane asymmetry and attachment, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA. One of the earliest indications of apoptosis is the translocation of the membrane phospholipids phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. Once exposed to the extracellular environment,
binding sites on PS become available for Annexin V, a 35-36 KDa, Ca\(^{2+}\) dependent, phospholipid binding protein with a high affinity for PS (Fig. 7).\[51\]

The translocation of PS precedes other apoptotic processes such as loss of plasma membrane integrity, DNA fragmentation, and chromatin condensation. As such, Annexin V can be conjugated to biotin or to fluorochrome such as FITC, PE, APC, Cy5, or Cy5.5, and used for the easy flow cytometric identification of cells in the early stage of apoptosis.

Because PS translocation also occurs during necrosis, Annexin V is not an absolute marker of apoptosis. Therefore, it is often used in conjunction with vital dyes such as 7-amino-actinomysin (7AAD) or propidium iodide (PI), which bind to nucleic acids, but can only penetrate the plasma membrane when membrane integrity is breached, as occurs in the later stages of apoptosis or in necrosis (Fig. 8).\[52\]

Cells that are negative for Annexin V and the vital dye have no indications of apoptosis: PS translocation has not occurred and the plasma membrane is still intact.

On the other hand cells that are Annexin V-positive and vital dye-negative, however are in early apoptosis as PS translocation has occurred, yet the plasma membrane is still intact.

Cells that are positive for both Annexin and the vital dye are either in the late stages of apoptosis or are already dead, as both PS translocation and the loss of plasma membrane integrity have occurred. When measured over time, Annexin V and vital dye can be used to monitor the progression of apoptosis: from cell viability, to early-stage apoptosis, and finally to late-stage apoptosis and cell death.\[53\]
**Rationale**

Substantial evidence has been generated to support that pOBCol3.6-targeted p20C/EBPβ expression increases the availability of multipotent progenitor cells to the osteoblast and adipocyte lineage pathways. From our preliminary studies we know that pOBCol3.6-targeted p20C/EBPβ affect the osteoblastic lineage in two different locations, the first one occurring late in differentiation, as shown by the decrease in terminal markers as osteocalcin and pOBCol2.3-GFP, suggesting a late differentiation arrest. The second site of action occurs early in differentiation, as seen by an increase in alkaline phosphatase (Alk-phos) and bone sialoprotein (BSP), as well as an increase in cell number in the TG cultures.

We have taken advantage of our ex vivo model systems to perform mechanistic studies on the role of cellular proliferation and apoptosis in regulating the pool of progenitor cells. The advantage of this approach is that it should be more physiologically relevant than the use of osteoblastic or multipotent immortalized cell lines, which may not faithfully reflect lineage progression pathways that occur in vivo. Moreover, primary calvarial cell cultures represent well-studied and widely accepted models of lineage progression in vitro, exhibiting the full range of differentiation from pluripotent-progenitor cell to terminally differentiated cell. A major limitation of this approach is the heterogeneity of the culture system, making it difficult or impossible to ascribe an observed effect to a specific cell population. To circumvent this problem, these experiments will be performed in a genetic background harboring pOBCol3.6-GFP to provide a real-time fluorescent marker of the pluripotent osteoblast lineage.
We have focused primarily on flow cytometry-based methods for measuring cellular proliferation and apoptosis, allowing us to compare changes not only between WT and TG cultures, but also between the GFP-positive and GFP-negative cell populations. Our data suggests that GFP-positive cells at early stages of culture represent pluripotent progenitor cells capable of giving rise to adipocytes or osteoblasts. Following commitment to the osteoblast lineage, GFP-positive cells express early osteoblast markers such as AP and BSP. GFP expression then persists alongside late osteoblast differentiation markers such as OC, DMP-1 and pOBCol2.3-GFP [1, 54]. Importantly, since GFP and the p20C/EBPβ transgene are under control of the same pOBCol3.6 promoter, GFP will serve as a visual surrogate marker to indicate which cells are expressing the p20C/EBPβ transgene. This allowed us to track cell-autonomous effects on cell proliferation and/or apoptosis by differential analysis of transgene effects (WT vs. TG) in GFP-positive and GFP-negative populations.
Hypothesis

1. Primary cells from TG mice show enhanced or prolonged proliferation relative to WT cells.

2. Primary osteoblast from transgenic mice show enhanced apoptosis compared to WT cells.

Specific Aims

1. Flow cytometry analysis of proliferation in primary osteoblasts will be assessed by:

   - BrdU labeling
   - Analysis of proliferation specifically in TG expressing cells using a surrogate GFP marker
   - pOBCol3.6-GFP (early osteoblast marker, p20 TG promoter)

2. Analysis of cells undergoing apoptosis will be assessed by:

   - Incorporate Annexin V and 7-AAD and flow cytometry analysis
Experimental design, procedures, and methods

Creation of FLAG-tagged p20C/EBPβ transgenic mice

In order to identify the cells that express the C/EBP transgene, an amino-terminal FLAG epitope was incorporated into the p20C/EBPβ construct. This allows the C/EBP transgene to be detected by Western blotting against the FLAG epitope and immunocytochemistry using the M2 FLAG antibody. We can therefore discriminate between p20C/EBPβ and endogenously occurring C/EBP isoforms. The dominant negative function of p20C/EBPβ is not altered by the incorporation of the FLAG epitope due to the modular nature of transcription factors. The FLAG p20C/EBPβ construct was created by PCR using the CMV-LIP plasmid as a template and confirmed by sequencing. Experiments also confirmed the FLAG-tagged p20C/EBPβ exhibits the expected DNA binding, nuclear localization, and dominant negative function.

Targeting of FLp20C/EBPβ to osteoblastic cells:

A pOBCo13.6-FLp20/C/EBPβ construct was used to target expression of FLp20C/EBPβ throughout the osteoblastic lineage. PCR3.1-FLp20/C/EBPβ was digested with HindIII and XbaI and the resulting FLp20/C/EBPβ fragment was subcloned into an intermediate Cla vector which contains a polylinker and the bovine growth hormone (bGH) polyadenylation sequence flanked by two ClaI sites. Following digestion of ClaI, the FLp20/C/EBPβ-bGH cassette (792 bp) was isolated and subcloned into pBCSK+ColInt5.2Xba, which contains a ClaI site downstream of the 3.6 kb Col1a1 promoter and 1.6 kb Col1a1 first intron. Clones containing FLp20/C/EBPβ in the correct orientation were identified using asymmetric restriction sites.
Because there is differential utilization of Collα1 promoter elements as the cell progresses through differentiation, FLp20C/EBPβ expression can be targeted toward cells at multiple stages of the osteoblast lineage. pOBCol3.6GFP has been reported as an early marker of the osteoblast lineage, being expressed in early pluripotent progenitor cells, while pOBcol2.3GFP expression is restricted more to differentiated osteoblasts [55]. Therefore, FLp20C/EBPβ driven by pOBCol3.6 was used to target expression broadly throughout the osteoblast lineage. Heterozygous transgenic and wild type mice were crossed with homozygous pOBCol3.6-GFP reporter mice, providing us a osteoblast lineage that is also a surrogate reporter of the early p20C/EBPβ transgene expression, since the p20C/EBP transgene and GFP are driven by the same promoter in the resulting transgenic offspring.

*Preparation of Transgenic FLp20C/EBPβ Overexpressing Mice*

A pronuclear microinjection technique of pOBCol3.6-FLp20C/EBPβ was used to prepare transgenic CD-1 mice that overexpress FLp20C/EBPβ. A dot blot analysis of tail snip DNA from potential founders was screened by using a probe directed against the human growth hormone polyadenylation site, which is cloned downstream of p20C/EBPβ. Mice that screened positive for the human growth hormone 3'-UTR were confirmed by PCR using a 5'-primer directed against the Collα1 first intron (5'-ACCCTCCTCCATTTTAGCC-3') and a 3'-primer directed against the FLAG epitope (5'-CATCGTCGTCGTCTTCTAGTC-3'). Four transgenic founders (2 male, 2 female) were obtained and bred with wild type CD-1 mice to establish transgenic lines. Progeny
are screened for the presence of the transgene at the time of weaning by tail snip, DNA extraction and PCR. The four transgenic lines are: 00-50-9, 00-50-10, 00-63 and 00-65.

Primary cell culture model

Transgenic pOBCol3.6-FLp20 C/EBPβ mice were crossed with homozygous pOBCol2.3GFP mice. The GFP, or green fluorescent protein, provides an osteoblast-specific marker which can be visualized and photographed in living cells under a microscope.

Calvaria, comprising the frontal and parietal bones, were dissected from six to eight week old wild type and pOBCol3.6-FLp20 C/EBPβ transgenic mice. Through a series of five digestions, primary cells were isolated and plated in 8cm² wells. Cells were counted and plated to a density of 17,500cells/cm² for a total of 140,000 cells per well. Cultures were fed on the second and fifth day with DMEM (Dulbecco’s modified Eagle Medium) media supplemented with 10% heat-inactivated fetal calf serum (HI-FCS), 100μ/ml penicillin and 100μ/ml streptomycin (P/S). From day 7 through day 28, the cultures will be fed every 48 hours with alpha-MEM, 10% HI-FCS, penicillin (100μ/ml), streptomycin (50μg/ml), and supplemented with 50μg/ml phosphoascorbate and 4mM \( \beta\)-glycerol phosphate.

The osteogenic capacity of these cells was assessed via visualization of both mineralized bone nodules and GFP fluorescent of colonies under a microscope. The cell colonies were imaged via phase/contrast and fluorescence microscopy on days 14, 17 and 21 at both 10x and 40x magnification. Additionally, the distribution and quantification of GFP expression in the cell cultures was observed at the macroscopic level via a fluorimager.
**Cell Proliferation: BrdU-APC**

At days number 8, 10, an 12 the cultures were labeled with 1mM BrdU (20 μl into 2ml well) and then incubated 120 minutes at 37°C.

After washing the cultures with PBS two times, 0.5 ml of the following digestion solution (30 ml of 2.5% trypsin, 300 mg of Collagenase A, 300 mg of Hyaluronidase) was added to each well. Cultures were placed in 37°C incubator for 20 minutes. Floating cells were then collected into 15 ml collecting tube (one tube per three wells). Wells were rinsed with 2 ml of DMEM and then transferred to the same collecting tube. Cells were collected by centrifugation. Supernatants were removed and cells were suspended in 2 ml of PBS. Then cells were filtered through a 35 μm strainer using a 10 ml syringe and collected into 15ml tube.

Once cells were harvested, using the BD Biosciences Pharmingen BrdU labeling Kit, they were fixed with buffer 1(BD Cytofix/Cytoperm Buffer), then permeabilized with buffer 2 and buffer 3 (BD Perm/Wash Buffer, BD Cytoperm Plus Buffer), and later treated with DNase for 1 hour at 37°C to expose BrdU epitopes. Cells were then washed and stained with an APC labeled anti-BrdU antibody. Negative controls had the BrdU pulse omitted but were otherwise handled in an identical manner.
BrdU Flow Cytometry Analysis

A calibur II machine was used for the flow cytometry analysis, a forward/side scatter profile was obtained for each sample. BrdU was gated using the APC gate (FL-4) and GFP was gated on FL-1. BrdU and GFP were plotted individually by the use of histograms, and also BrdU was also plotted as a function of GFP. A quadrant plot was used to measure the percentage of cells incorporating BrdU and GFP, these quadrant were set up with the use of the control samples.

Cell Apoptosis: Annexin V

Primary calvarial cells will be cultured for 6 to 12 days and harvested as described above, but without fixation or permeabilization. Cells were labeled with APC-conjugated annexin V and 7-AAD, which in this context is used as a vital stain. No 7-AAD labeling of DNA should be observed in intact cells. Flow cytometry in this case involved measurement of fluorescent signals in FL3 (7-AAD) and FL4 (annexin V), with appropriate compensation for overlap. Using the combination of annexin V and 7-AAD labeling allowed us to track the progress in cell death over time in culture, as cells proceed from early apoptosis (annexin V labeling only) to later stages of apoptosis (annexin V and 7-AAD labeling). Negative controls were provided by omission of annexin V and/or 7-AAD from the labeling reaction.
Flow Cytometry Analysis of Annexin-7AAD Labeling

The same machine used for BrdU incorporation was used to measure the amount of annexin-7AAD labeling. Annexin V was measured using the APC gate (FL-4) and 7AAD using the FL3 gate. A forward a side scatter was obtained for each sample. Annexin V was plotted as function of 7AAD, and in very similar manner quadrants plots were used to measure the percentages of cells labeled with annexin V and 7AAD. Control samples were used to set up these quadrants. Since we observed some limitations in the measuring of the annexin V positive labeling when the quadrants plots were used, we decided to gate the area that was miss out on the quadrant plot, to measure the annexin V positive cells, this area was name G1 gated annexin. To determine the type of cells that were undergoing apoptosis, annexin V was plotted as a function of the forward scatter, and subsequently quadrants plot were drawn as settled by control samples.
Significance

There is mounting evidence that C/EBP transcription factors are important regulators of proliferation, cellular differentiation, and apoptosis. Previous studies using a naturally-occurring dominant negative C/EBP transcription factor to broadly target C/EBP function throughout the stromal-osteoblast lineage have unmasked both early and late effects of this transcription factor family. Late inhibitory effects on and bone formation and the expression of osteoblast-specific markers suggest that C/EBP function is required for terminal osteoblast differentiation. Determining the molecular mechanisms by which C/EBP regulates maturation of these cells will therefore enhance our understanding of the transcriptional control of gene expression, cell morphology and matrix elaboration in bone and in other mineralized tissues.

Osteoblasts play a significant role in bone growth, bone development and orthodontic tooth movement. Osteoblastic differentiation from mesenchymal progenitor cells is an extremely complex process. The precise molecular mechanisms behind it are still largely unknown. Understanding the factors involved in the process of osteoblastic differentiation could help eradicate skeletal diseases such as cleidocranial dysplasia, osteoporosis, periodontitis and other diseases of bone. Additionally, comprehension of the growth and activity of the osteoblast could lead to a better understanding of the mechanism and rate of tooth movement in the field of orthodontics.
Results

Transgenic pOBCol3.6-FLp20 C/EBPβ mice were crossed with homozygous pOBCol3.6-GFP mice. The pOBCol3.6-GFP, provided an osteoblast-specific marker which can be visualized and photographed in living cells under a microscope.

The cellular mechanisms regulating expansion of progenitors were assessed by flow cytometry. The cellular autonomy of transgenic effects was assessed by the behavior of GFP-positive and negative populations with respect to proliferation and apoptosis. The experiments were done on the time period between 6 and 14 days of culture.

BrdU Incorporation

Cells were cultured from 8 to 14 days, and BrdU was pulsed from 2 to 12 hours. The initial BrdU incorporation of study (experiment 253) was performed on day 8 (Figure 9 and 10), using a BrdU pulsed of 2 hours. Harvested cells were analyzed for expression of GFP (FL1) and incorporation of BrdU (FL4). The increased in cell number at day 8 was associated with an increase in the percentage of GFP-positive cells. GFP positive cells in TG cultures represent 33% of the total compared to 28% in WT cultures consistent with expansion of the transgene-expressing population.(table I and II).

However, in this experiment we did not find differences in BrdU incorporation in the GFP negative population (BrdU+/GFP-) between WT (WT mean, 9.31%) and TG cells (TG mean 7.36%). Nor was any difference found in BrdU incorporation in the double positive cells (BrdU+/GFP+), as shown by comparison of their mean values (WT,4.62 ; TG,3.61). (Tables I and II; Figure 11)
The results for the BrdU incorporation using flow cytometry analysis on done on day 10; (Figure 12) are consistent with those on day 8. Increased cell number at day 10 was also associated with an increase in the percentage of GFP-positive cells. Transgenic GFP-positive cells represented a mean of 33% of the total cell population compared with 22% in WT cultures (Table III, IV and Figure 14). In this experiment BrdU incorporation in GFP negative cells (BrdU+/GFP-) was actually higher in WT cells (Mean 4.53%) compared with their TG counterpart (mean 1.18%). Although BrdU incorporation was higher in WT cultures this difference needs to be interpreted cautiously due to the preliminary nature of this study and small sample size (n=2). Wild type cultures also showed a modest increase in BrdU incorporation in the GFP positive cells.

The results of the BrdU incorporation on day 12 from the same experiment (Figure 13), showed no increases in cell number or on the amount of GFP-positive cells. TG cultures contained 24% GFP positive cells compared with 21% in the WT group (table V, VI; Figure 15). By day 12 BrdU incorporation was very low and did not differ between WT (WT mean, 0.48%) and TG (TG mean, 0.17%) cells in the GFP negative population (BrdU+/GFP-). In the double positive cells, (BrdU+/GFP+) BrdU was extremely low (0.15% labeling in WT) with no difference seen between WT and TG cells. (Tables V, VI; Figure 15).

Since no positive results were obtained from the 2 hours BrdU pulse experiments, it was decided to increase the time of pulsing, aiming to increase the chances of getting cells going through the S phase of the cell cycle. The results for this experiment done on day 9 (tables VII, VIII; Figures 16-18) were similar to those seem on the previous
experiments, the BrdU incorporation on the GFP negative cells (BrdU+/GFP-) showed no
difference between the WT and TG cell cultures as seem by comparison of their mean
values, (Mean WT, 5.56; TG, 5.07). In the double positive cells (BrdU+/GFP+), the WT
cells showed more BrdU incorporation than the TG cells, (Mean WT, 2.55; TG, 1.14),
but since the incorporation was very little and as mentioned previously a small sample
sized was used (n=3), these results should be interpreted cautiously. The number of GFP
positive cells on the WT group observed in this study was very similar to that observed
on the TG. (Mean WT, 24.9%; TG, 23.5%).

Interestingly even when small sample sizes were used and regardless of the BrdU
pulsing time, there was no evidence that showed increase BrdU incorporation in TG
cultures, that explained the increment in cell number. However examination of the
forward scatter/side scatter (FSC/SSC) plot showed a difference between WT and TG
cells. WT cells showed an increase in a population with high FSC and low SSC often
referred to as “scatter apoptotic” cells (Fig.9, 11 and 13 shaded circles), due to the fact
that cells undergoing programmed cell death often exhibit such a profile, indicative of
small cells exhibiting a high degree of granularity.

To determine whether there might be differences in apoptosis between WT and
TG cultures, cells were harvested and labeled with annexin V-APC and 7-AAD.
Annexin V binds to exposed phosphatidyl-serine on the plasma membrane of early
apoptotic cells, whereas 7-AAD binds to DNA of cells that have lost plasma membrane
integrity.
Annexin V Labeling

The results for annexin V incorporation between WT and TG cell cultures done on day 6 showed reduced Annexin V labeling on the TG group. (Figures 16, 17 and Tables IX, X). The mean annexin incorporation in the WT group ( Annexin+/7AAD-) was 15.29 and in the TG group this was only 5.49, representing a 3 fold difference. The double positive cells showed higher incorporation in the WT group, while the 7AAD positive group showed very little difference between the WT and TG groups.

The amount of annexin incorporation as seen by the G1 gated annexin, was higher on the WT group with a mean G1 annexin V labeling of 41% compared to the TG group with 22% annexin labeling (figure 22).

The results for the plot between annexin V labeling and forward scatter on day 6 (Table XI and figure 23), showed a higher annexin V incorporation in the WT group for the cells that have a low forward scatter (scatter apoptotic). Mean WT annexin labeling was 15.95% compared to TG 5.28% in TG cells with low forward scatter, this was also true for the cells with high forward scatter which likely represent early apoptotic cells mean WT labeling in the high forward scatter population was 10.85% compared to 5.37% in the TG group. The TG cultures showed a higher number of cells with a high forward scatter and no annexin incorporation (scatter viable cells), with a mean of 62.0% compared to only 32.0% in the same population of WT cells.

The results for day 10 showed were very similar to those done on day 6, with the TG group showing less annexin V labeling (figure 24-26;Table XII-XIII) The mean WT annexin+/7AAD- was 4.70% whereas and in the TG group this was only 1.33%, this time the difference was not as high as on day 6. The double positive cells showed very
similar incorporation on the WT and TG group and the 7AAD positive group showed higher incorporation on the TG group than the WT, but since the normal are so low, they were not considered to be important.

The G1 gated annexin results showed higher annexin incorporation in the WT group than the TG, with a mean WT incorporation of 20% and a mean TG incorporation of 9.5% (figure 27).

The analysis of annexin V labeling as a function of forward scatter on day 10 (Table XIV and figure 28), showed a higher annexin V labeling on the WT group for the cells that have low forward scatter. (scatter apoptotic). Mean WT labeling was 2.0% of the cells compared to 1% of the TG cells. This was also true for the cells with high forward scatter (early apoptotic cells), with a mean WT incorporation of 4.5% compared to 1.2% on the TG group. The TG cultures showed a higher number of cells with a high forward scatter and no annexin labeling (scatter viable cells), with a mean of 70.5% compared to 44.9% in the WT cultures.

The results of different experiments done on day 7 showed consistency with those done on day 6 and 10 the TG group showed smaller annexin V labeling (figure 29-31 and tables XV-VII) The Mean WT Annexin+/7AAD- was 13.67% and on the TG group this was only 7.43 %, this was considered a significant difference between the two groups. The double positive cells showed similar incorporation on both groups and the 7AAD positive group showed very little difference between the groups.
The G1 gated annexin results showed higher annexin labeling in the WT group than the TG, with a mean WT annexin labeling of 23.2% compared to 21.5% in their TG counterpart (figure 32).

The results of annexin V labeling as a function of forward scatter on day 7 (Table XVII and Figure 33), showed a higher annexin V labeling in the WT group for the cells that have a low forward scatter (scatter apoptotic), mean WT incorporation 9.55% compared to TG 7.45% and this was also true for the cells with high forward scatter (early apoptotic cells) mean WT incorporation 15.45% compared to 7.4% on the TG group. The TG cultures showed a higher number of cells with a high forward scatter and no annexin V labeling (scatter viable cells), with a mean of 37.85% compared to 30.46% on the WT.

On the experiment done on day 7 (Figure 34) it was attempted to do a three color analysis between annexin incorporation and GFP expression, but the lack of GFP control and the possible spilled between channels made the analysis to difficult.
Discussion

Preliminary studies done in our laboratory using femurs and calvaria of transgenic mice indicated two possible sites of action at which p20C/EBPβ affected the osteoblast lineage. The first site of action occurred late in osteoblast differentiation, manifested as observed by a decrease in terminal markers of osteoblastic differentiation such as osteocalcin and POBCol2.3-GFP because the latter is a transgenic reporter of osteoblast differentiation in which the visible GFP protein is expressed under control of a 2.3 kb Cola1 promoter construct. The second site appears to be in early osteoblast differentiation characterized an increase in early differentiation markers such as BMP and alkaline phosphatase. These preliminary studies have also shown an increase in cell number in transgenic mouse stromal cells cultures.

The same phenomenon was observed on primary cell cultures, with an increase in cell number in the TG cultures after day 7, reaching a plateau at day 15. It was unknown if the increase in cell number in the TG cultures was due to an increase in cell proliferation or a decrease in cell apoptosis. We hypothesized that primary osteoblast from transgenic mice over expressing the p20C/EBPβ would have an enhanced proliferation when compared to WT cells.

There are numerous studies that suggest an antiproliferative effect of C/EBPβ as in hepatocytes and keratinocytes. [56, 57]. In contrast to these actions, C/EBPβ seems to be a positive regulator of the cell cycle in mammary epithelial cells [40, 41]. The p20C/EBP dominant negative isoform has also been associated with hyperproliferation and transformation in mammary epithelial cells[42].
In the present study we used transgenic pOBCol3.6-FLp20 C/EBPβ mice on a pOBCol3.6-GFP genetic background. GFP therefore served as a surrogate marker, for the transgene, with the purpose of tracking the TG cells that were undergoing cell replication. This approach allowed us to assess whether the p20C/EBPβ transgene, exerted cell autonomous effect on proliferation, BrdU was pulsed into the cultures to label the cells that were going through S phase of the cell cycle, and the results were analyzed using flow cytometry.

The results observed on day 8, showed no increase in BrdU incorporation in either the GFP negative or positive TG cells populations. We expected to see differences at this time point based on growth curves indicating the divergence of WT and TG cell number during this phase of the cultures. Indeed BrdU incorporation was maximal at day 8, then tended to decrease on day 10 and day 12, which was expected due to a decrease in cell proliferation at later stages of the culture. At all the time points, BrdU incorporation was lower in GFP positive when compared with the GFP negative population of cells). This may be due to the more advanced differentiation stage of GFP positive cells when compared with the GFP negative cell population.

The results for longer pulses (12 hours) of BrdU were analyzed and very similar results were observed; no difference in the mean BrdU incorporation between WT and TG cultures in either the GFP negative or positive populations. The prolonged pulse of BrdU, given at the time of cells feeding, increased the likelihood labeling cells going through the S phase of the cell cycle that might have been missed using a shorter pulse.
It was observed that in both population groups (GFP negative and positive cells) there was no evidence of increased BrdU incorporation in the TG cultures that would explain the difference in cell number between the groups.

The increased cell number at day 8 was associated with an increase in the percentage of GFP-positive cells, consistent with expansion of the transgene-expressing population. This was also seen on at days 10 and 12, albeit with a smaller difference between groups.

Although the cellular proliferation data were negative, examination of the forward scatter/side scatter (FSC/SSC) profile showed a difference in the cell distribution between WT and TG cells. WT cells showed an increase in a population with high FSC and low SSC often referred to as “scatter apoptotic” cells (Fig. 9, 11 and 13 shaded circles), because cells undergoing programmed cell death often exhibit a small size and high degree of granularity. That led us to hypothesize that primary osteoblasts from transgenic mice showed decreased apoptosis compared to wild type cells.

C/EBPβ has been linked to enhance apoptosis in Neuro2A cell [44], but contrasting these results, C/EBPβ can also act as an inhibitor of apoptosis as has been observed in keratinocytes [56], and in hepatic stellate cells [46].

In our study to identify the number of cells undergoing programmed cell death a 7AAD-Annexin V incorporation assay was used followed by flow cytometry analysis in order to measure labeling with these apoptotic markers. The results for annexin V labeling for the experiments done on day 6, showed increased annexin labeling on the annexin positive WT cultures compared to their counterpart in the TG group; very similar results were observed when the experiments were done on day 7 and 10, with the TG
cultures showing a two-three fold lower annexin labeling than the WT cultures. This is a novel finding since no studies have been published using osteoblasts with over expression p20C/EBPβ to evaluate programmed cell death. The two-three fold difference found in these experiments could explain the difference in cell number between the WT and the TG group observed from day 7 to 15. For example, if we use a pure mathematical model and spanning 5 cell doublings, a 10% increase in the apoptotic rate WT cells could account for a two-fold increase in the total number of TG cells, compared to the WT group.

An interesting observation was seen when the annexin V labeling was examined as a function of forward scatter. Cells with smaller forward scatter had a higher labeling of annexin V in the WT group. This result was seen on days 6, 7 and 10, and was expected, since cells undergoing programmed cell death tend to decrease in size. However, annexin labeling was also higher in the WT group in the cells with high forward scatter, likely representing that are just starting to present apoptotic features. Consistent with this interpretation annexin V binding to the phosphatidyl serine residues, is known to be an early marker of apoptosis. The number of viable cells (no annexin/high forward scatter) was higher in the TG group in all the experiments, which led us to conclude that the p20C/EBPβ could potentially increase the number of pluripotent viable cells. These progenitors may then undergo either osteoblast or adipogenic differentiation depending on the differentiation stimulus provided. Recent studies in our laboratories have demonstrated enhanced adipocyte differentiation in TG bone marrow stromal cells.
In all of our experiments the amount of 7AAD labeling was extremely low in both WT and TG cultures. We believe one of the possible reasons for these discrepancies might be detachment of apoptotic cells from the tissue culture dishes when medium was changed. The other possibility is that 7AAD is not a sufficiently sensitive marker to measure apoptosis. In future experiments, propidium iodide will be used to determine whether it provides better sensitivity for identification of late apoptotic cells.
Conclusions

- Overexpression of the p20C/EBPβ transgene had no stimulatory effect on cellular proliferation in early primary osteoblast cultures, as was seeing in our experiments that did not show differences in the BrdU incorporation between the WT and TG groups.

- The p20C/EBPβ appeared to inhibit apoptosis in early primary osteoblast cultures, as observed by the 10% increase in apoptosis rate observed in the WT group compared with their TG counterpart.

- Transgenic overexpression of p20C/EBPβ may regulate the availability of the pluripotent osteoblast progenitor pool, explaining the increase in early osteoblast markers (eg. AP and BSP) seen in TG cells

- C/EBP transcription factors may regulate survival of multipotent mesenchymal progenitor cells
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Legend of Tables and Figures

**Table I** Shows the raw data obtained on an experiment done on day 8. BrdU incorporation and GFP expression were evaluated on WT and TG cultures.

**Table II** Shows the results of experiments done on day 8. As can be observed no difference was found in the mean BrdU incorporation between WT and TG cultures.

**Table III** Shows the raw data obtained on an experiment done on day 10. BrdU incorporation and GFP expression were evaluated on WT and TG cultures.

**Table IV** Shows the results of experiments done on day 10. As can be observed no difference was found in the mean BrdU incorporation between WT and TG cultures.

**Table V** Shows the raw data obtained on an experiment done on day 12. BrdU incorporation and GFP expression were evaluated on WT and TG cultures.

**Table VI** Shows the results of experiments done on day 12. As can be observed no significant difference was found in the mean BrdU incorporation between WT and TG cultures.

**Table VII** Shows the raw data obtained on an experiment done on day 9. BrdU incorporation and GFP expression were evaluated on WT and TG cultures.

**Table VIII** Shows the results of experiments done on day 9. As can be observed no significant difference was found in the mean BrdU incorporation between WT and TG cultures.

**Table IX** Shows the raw data obtained on an experiment done on day 6. Annexin V and 7AAD were evaluated on WT and TG cultures.

**Table X** Shows the results of experiments done on day 6. As can be observed difference was found in the mean Annexin V incorporation between WT and TG cultures.

**Table XI** Shows the results obtained from the annexin vs forward scatter in experiment done on day 6.

**Table XII** Shows the data obtained on an experiment done on day 10. Annexin V and 7AAD labeling were evaluated on WT and TG cultures.

**Table XIII** Shows the results of experiments done on day 10. As can be observed significant difference was found in the mean Annexin V incorporation between WT and TG cultures.
Table XIV Shows the results obtained from the annexin vs forward scatter in experiment done on day 10.

Table XV Shows the raw data obtained on an experiment done on day 7. Annexin V and 7AAD labeling were evaluated on WT and TG cultures.

Table XVI Shows the results of experiments done on day 7. As can be observed significant difference was found in the mean Annexin V incorporation between WT and TG cultures.

Table XVII Shows the results obtained from the annexin vs forward scatter in experiment done on day 7.

Table XVIII Shows the data obtained on an experiment done on day 7. Annexin V labeling and GFP expression were evaluated on WT and TG cultures.

Table XIX Shows the results of experiments done on day 7. As can be observed significant difference was found in the mean Annexin V incorporation between WT and TG cultures.

Fig. 1 Lifecycle of an osteoblast: Osteoblast precursors are recruited from a multipotential mesenchymal progenitor that can give rise to several differentiated cell phenotypes including fibroblasts, chondrocytes, adipocytes and osteoblasts. Undifferentiated osteoprogenitors are present in the bone marrow and periosteum. These undifferentiated osteoprogenitor cells eventually differentiate into preosteoblasts, osteoblasts and finally osteocytes.

Fig. 2 Schematic representation of the Leucine zipper.

Fig. 3 C/EBPβ mRNA encodes multiple isoforms by alternative translational initiation from multiple AUG start sites. Major isoforms include a 35-kd transcriptional activator and a 20-kd dominant negative inhibitor.

Fig. 4 Showed the two possible action sites for C/EBPβ on the osteoblast differentiation as seen by an increase in early markers observed during the normal osteoblast, pOBCol 3.6-GFP and alkaline phosphatase and a decrease in late markers such as POBCol2.3-GFP and osteocalcin.

Fig 5. Growth of primary calvarial cells is increased in TG cultures after day 7.

Fig. 6 BrdU is incorporated into newly synthesized DNA by cells entering and progressing through the S phase (DNA synthesis) of the cells cycle. The incorporated BrdU is stained with specific anti-BrdU fluorescent antibodies.

Fig. 7 Schematic representation of Annexin V incorporation to the cytoplasmic membrane, as can be seen in Figure 4A it would not incorporate on normal cells, but it will incorporate to cells on the early stage of apoptosis on which
translocation of the membrane phospholipids phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane has occurred. (Figure 4B)

Fig. 8 Schematic representation of 7AAD incorporation to the nuclear DNA at later stages of apoptosis, once the cytoplasmic membrane has been disrupted.

Fig. 9 Analysis of proliferation by flow cytometry at day 8 in representative WT and TG samples. Forward scatter-side scatter plots (left plot in each panel) showed a reduction in “scatter apoptotic” (low FSC/high SSC) cells in TG cultures (compare yellow shaded circles). In quadrant plots (right plot in each panel), X-axis (FL1) represents GFP, Y-axis (FL4) is BrdU incorporation. Numbers represent the percentage of cells in each quadrant. TG cultures showed an increase in GFP-positive cells (mean 33% vs. 28% in WT). No significant differences in BrdU incorporation were seen in this experiment.

Fig. 10 Analysis of proliferation by flow cytometry at day 8 in representative control samples. In quadrant plots (right plot in each panel), X-axis (FL1) represents GFP, Y-axis (FL4) is BrdU incorporation. Numbers represent the percentage of cells in each quadrant.

Fig. 11 BrdU incorporation and GFP expression in day 8, WT and TG cultures. As can be seen no difference exist on the BrdU incorporation between WT and TG cultures. But it can be observed an increase in the amount of GFP positive cells on the TG (33%) cultures vs WT (28%).

Fig. 12 Analysis of proliferation by flow cytometry at day 10 in representative WT and TG samples. Forward scatter-side scatter plots (left plot in each panel) showed a reduction in “scatter apoptotic” (low FSC/high SSC) cells in TG cultures (compare yellow shaded circles). In quadrant plots (right plot in each panel), X-axis (FL1) represents GFP, Y-axis (FL4) is BrdU incorporation. Numbers represent the percentage of cells in each quadrant. TG cultures showed an increase in GFP-positive cells (mean 33% vs. 22% in WT). No differences in BrdU incorporation were seen in this experiment.

Fig. 13 BrdU incorporation and GFP expression on WT and TG cultures on day 12. BrdU incorporation was higher on the WT cultures on the GFP positive and negative cells, but not considered to be significant due to high standard error and very low incorporation. TG cultures showed a small increase in GFP positive (24%) cells vs WT cultures (21%).

Fig. 14 BrdU incorporation and GFP expression in WT and TG cultures on day 10. WT cells showed an increase in BrdU incorporation in the GFP positive and negative cells compared to TG cultures, but not considered significant due to high standard error between samples. TG cultures showed an increase in GFP positive (32%) cells compared to WT cultures (19%)
Fig. 15 Analysis of proliferation by flow cytometry at day 12 in representative WT and TG samples. Forward scatter-side scatter plots (left plot in each panel) showed a reduction in “scatter apoptotic” (low FSC/high SSC) cells in TG cultures (compare yellow shaded circles). In quadrant plots (right plot in each panel), X-axis (FL1) represents GFP, Y-axis (FL4) is BrdU incorporation. Numbers represent the percentage of cells in each quadrant. TG cultures showed a decrease in GFP-positive cells (mean 18% vs. 21% in WT). No significant differences in BrdU incorporation were seen in this experiment.

Fig. 16 Analysis of proliferation by flow cytometry at day 9 in representative WT and TG control samples. Forward scatter-side scatter plots (left plot in each panel) showed a reduction in “scatter apoptotic” (low FSC/high SSC) cells in TG cultures (compare yellow shaded circles). In quadrant plots (right plot in each panel), X-axis (FL1) represents GFP, Y-axis (FL4) is BrdU incorporation. Numbers represent the percentage of cells in each quadrant.

Fig. 17 Analysis of proliferation by flow cytometry at day 9 in representative WT and TG samples. Forward scatter-side scatter plots (left plot in each panel) showed a reduction in “scatter apoptotic” (low FSC/high SSC) cells in TG cultures (compare yellow shaded circles). In quadrant plots (right plot in each panel), X-axis (FL1) represents GFP, Y-axis (FL4) is BrdU incorporation. Numbers represent the percentage of cells in each quadrant. TG cultures showed a decrease in GFP-positive cells (mean 23.5% vs. 24.8% in WT). No significant differences in BrdU incorporation were seen in this experiment.

Fig. 18 BrdU incorporation and GFP expression on WT and TG cultures on day 9. WT cells showed an increase in BrdU incorporation in the GFP positive and negative cells compared to TG cultures, but not considered significant due to high standard error between samples. TG cultures showed similar expression of GFP positive cells (23.5%) compared to WT cultures (24.8%).

Fig. 19 A negative control without annexin-V-APC from experiment 329 day 6. The polygon on the upper diagrams represent the G1 gated annexin. On the lower left can be seen the plot from the forward vs side scatter, and the lower right shows the annexin vs forward scatter.

Fig. 20 Analysis of apoptosis by flow cytometry in replicate day 6 WT and TG cultures. Labeling was performed with annexin V-APC (FL4; Y axis) and 7-AAD (FL3; X axis) to visualize early and late apoptotic cells. Numbers represent the percentage of cells in each quadrant. These data indicate that early WT cultures contain a higher percentage of annexin V labeling than TG cultures. The polygon represents the G1 gated annexin, as settled by controls. The lower left diagrams show the forward and side scatter plot from both samples, and the lower right diagrams show the annexin vs forward scatter as can be seen WT cultures have higher annexin V incorporation than TG cultures, in cells with high and low forward scatter.
Fig. 21 Annexin V and 7-AAD labeling in WT and TG cultures on day 6. WT cultures incorporated more annexin V than TG cultures. Very low number can be seen from the double positive cells and 7AAD positive cells.

Fig. 22 Annexin V labeling on WT and TG cultures on day 6 as obtained from G1 gated annexin. As can be seen WT cultures incorporated higher amounts of annexin V than TG cultures.

Fig. 23 Annexin V incorporation expressed as a function of forward scatter in WT and TG cultures on day 6. WT cultures incorporated higher amounts of annexin V on the low forward scatter (scatter apoptotic) and high scatter (early apoptotic) than TG cultures. TG cultures show a higher amount of cells with no annexin and high forward scatter (scatter viable cells)

Fig. 24 A negative control without annexin-V-APC from experiment 329. The polygon on the upper diagrams represent the G1 gated annexin. On the lower left can be seen the plot from the forward vs side scatter, and the lower right shows the annexin V incorporation expressed as function of the forward scatter.

Fig. 25 Analysis of apoptosis by flow cytometry in replicate day 10 WT and TG cultures. Labeling was performed with annexin V-APC (FL4; Y axis) and 7-AAD (FL3; X axis) to visualize early and late apoptotic cells. Numbers represent the percentage of cells in each quadrant. These data indicate that early WT cultures contain a higher percentage of annexin V labeling than TG cultures. The polygon represents the G1 gated annexin, as settled by controls. The lower left diagrams show the forward and side scatter plot from both samples, and the lower right diagrams show the annexin expressed as function of the forward scatter as can be seen WT cultures have higher annexin V incorporation than TG cultures, in cells with high and low forward scatter.

Fig. 26 Annexin V and 7-AAD labeling in WT and TG cultures on day 10. Annexin V and 7AAD labeling were evaluated on WT and TG cultures. WT cultures incorporated more annexin V than TG cultures. Very low number can be seen from the double positive cells and 7AAD positive cells.

Fig. 27 Annexin V labeling in WT and TG cultures on day 10 as obtained by the G1 gated annexin. As can be seen WT cultures incorporated higher amounts of annexin V than TG cultures.

Fig. 28 Annexin V labeling expressed as a function of the forward scatter in WT and TG cultures on day 10. WT cultures incorporated higher amounts of annexin V on the low forward scatter (scatter apoptotic) and high scatter (early apoptotic) than TG cultures. TG cultures show a higher amount of cells with no annexin and high forward scatter (scatter viable cells).

Fig. 29 A negative control without annexin-V-APC from experiment done on day 7. The polygon on the upper diagrams represent the G1 gated annexin. On the lower left
can be seen the plot from the forward vs side scatter, and the lower right shows
the annexin vs forward scatter

Fig. 30 Analysis of apoptosis by flow cytometry in replicate day 7 WT and TG cultures.
Labeling was performed with annexin V-APC (FL4; Y axis) and 7-AAD (FL3; X axis) to visualize early and late apoptotic cells. Numbers represent the percentage of cells in each quadrant. These data indicate that early WT cultures contain a higher percentage of annexin V labeling than TG cultures. The polygon represents the G1 gated annexin, as settled by controls. The lower left diagrams show the forward and side scatter plot from both samples, and the lower right diagrams show the annexin vs forward scatter as can be seen WT cultures have higher annexin V incorporation than TG cultures, in cells with high and low forward scatter.

Fig.31 Annexin V and 7 AAD labeling in WT and TG cultures on day 7. WT cultures incorporated more annexin V than TG cultures. Very low number can be seen from the double positive cells and 7AAD positive cells.

Fig. 32 Annexin V labeling in WT and TG cultures on day 7 as obtained by G1 gated annexin. As can be seen WT cultures incorporated higher amounts of annexin V than TG cultures.

Fig. 33 Annexin V expressed as a function of forward scatter in WT and TG cultures on day 7. WT cultures incorporated higher amounts of annexin V on the low forward scatter (scatter apoptotic) and high scatter (early apoptotic) than TG cultures. TG cultures show a higher amount of cells with no annexin and high forward scatter (scatter viable cells).

Fig. 34 Analysis of apoptosis by flow cytometry in replicate day 7 WT and TG cultures. Labeling was performed with annexin V-APC (FL4; Y axis) and GFP (FL1; X axis) to visualize early and late apoptotic cells. Numbers represent the percentage of cells in each quadrant. These data indicate that early WT cultures contain a higher percentage of annexin V labeling than TG cultures.
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Table XII
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Table XIII
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Table XVI
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Table XVII
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Table XIX
Fig. 1
Fig. 3
Fig. 4
Expt 297: Growth Curve

Fig 5.
Fig. 11
Fig 12.
Fig. 13
Fig 14
Fig. 15
Fig. 17
Fig. 18
Fig. 20.
Fig. 21
Fig. 22
Fig. 23
Fig. 24
Fig. 25
Fig. 26
Fig. 27
Figure 28
Fig. 29
Fig. 31
Fig. 32
Fig. 33
Fig. 34