Glucocorticoids Inhibit Cell Cycle Progression in Differentiating Osteoblasts via Glycogen Synthase Kinase 3β

Elisheva Smith¹, Gerhard A. Coetzee² and Baruch Frenkel¹,³

Departments of ³Orthopaedic Surgery, ¹Biochemistry & Molecular Biology, ²Urology Institute for Genetic Medicine and Norris Cancer Center, University of Southern California Keck School of Medicine, Los Angeles, CA 90033

Running Title: Glucocorticoids modulate GSK3β

Address correspondence to:
Baruch Frenkel
Institute for Genetic Medicine
University of Southern California Keck School of Medicine
2250 Alcazar Street, CSC/IGM240
Los Angeles, California 90033

TEL: (323) 442-1322       FAX: (323) 442-2764       E-mail: frenkel@hsc.usc.edu
Summary

Differentiating osteoblasts in culture undergo a commitment stage, during which cobblestone-like cells grow to high density past confluence. In contrast to earlier proliferative stages, the cell cycle during this commitment stage is inhibited by glucocorticoids (GC). Chronic GC treatment also impedes mineral deposition, if steroid administration commences early enough during commitment. The present study defines a role for the glycogen synthase kinase 3β (GSK3β) and its target, c-MYC, in the GC-sensitive, osteoblast persistent cell cycle. c-MYC levels decrease as cells reach confluence, but then increase during growth to high density. GC administration at this stage results in down-regulation of c-MYC. This is accompanied by GC-mediated attenuation of GSK3β Ser⁹ inhibitory phosphorylation and increased GSK3β kinase activity. The down-regulation of c-MYC is attributable to enhanced Thr⁵⁸ phosphorylation, leading to accelerated degradation. In contrast, GC did not inhibit c-MYC synthesis rate or the level of β-catenin, a transcriptional co-activator of c-myc. The attenuated cell cycle, as well as the reduced c-MYC level, was rescued back to control levels by specific inhibition of GSK3β using lithium chloride. These results suggest that tonal GSK3β repression at the cobblestone stage of osteoblast differentiation permits osteoblast growth to high density. GC interfere with this growth-permissive axis by GSK3β activation, resulting in c-MYC down-regulation and impediment of the G1/S cell cycle transition.
**Introduction**

Osteoporosis is a major adverse effect of glucocorticoid (GC) treatment for the amelioration of symptoms of autoimmune and inflammatory diseases (1). The most important single mechanism contributing to bone loss in GC-treated patients is inhibition of osteoblastic bone formation (for review see (2,3)). We recently identified a commitment stage during osteoblast differentiation in culture (the “cobblestone” stage) in which GC exert their inhibitory effect (4). During this stage, proliferation persists, but is regulated uniquely compared to control of the cell cycle prior to confluence. At this commitment stage, but not earlier, GC inhibit cell cycle progression (4).

Growth to high cell density often reflects loss of cell cycle control. However, controlled growth to high density occurs during cell differentiation not only in cultured osteoblasts (4) but also in cultured adipocytes (5,6) as well as during the early definition of skeletal elements *in vivo* (50). Differentiating non-transformed osteoblasts that grow to high density do exhibit growth inhibitory signals, e.g., increased levels of the cyclin-dependent kinase inhibitor (cdi) p27kip1, decreased cyclin E and reduced free E2F DNA-binding activity (4). To explain the persistent growth, we postulated that the Wnt signaling pathway, which links cell-cell and cell-matrix interactions to the cell cycle (7-10), may account for the post-confluent osteoblast persistent cell cycle, and may be involved in the inhibition of this cell cycle by GC.

Wingless/Wnt-mediated cell-cell signaling molecules play a major role in organogenesis, and are conserved among vertebrates, flies, as well as primitive multicellular organisms (11-13). Components of the Wingless/Wnt signaling pathway participate in cell-cell adhesion, body patterning, cell growth and tumorigenesis (11,12). Forced activation of
the Wnt signaling pathway by over-expression of either Wnt1 (18) or its target, β-catenin (19) results in cell growth to high density.

The β-catenin pool relevant to Wnt signaling resides in the cytoplasm, and is distinct from a membrane-associated pool that plays a role in cell-cell adherence. In response to Wnt signaling, cytoplasmic β-catenin increases and enters the nucleus, where it cooperates with LEF/Tcf in the transcriptional activation of cell cycle stimulatory genes, such as c-myc (9) and cyclin D1 (10). A major regulator of β-catenin is Glycogen Synthase Kinase 3β (GSK3β). β-catenin and GSK3β are found in a cytoplasmic complex called β-catenin destruction complex, which also contains axin and APC (25). The association of β-catenin with GSK3β results in rapid degradation of β-catenin, probably mediated by phosphorylation. Activation of the Wnt pathway results in decreased GSK3β intrinsic activity, and, more importantly, dissociation from GSK3β, leading to increased stability and accumulation of β-catenin.

GSK3β also plays a role in regulating growth signals elicited by molecules such as Insulin, IGF-I (14), EGF (15), FGF-1 (16) and the fibronectin-activated Integrin-linked kinase (ILK) (17). These signals inhibit the intrinsic activity of GSK3β, but do not usually lead to disruption of the β-catenin destruction complex.

In the current study, we examined the involvement of the GSK3β—β-catenin—c-myc axis in GC-inhibition of the persistent cell cycle in post-confluent differentiating osteoblasts. We found that GC-treated differentiating osteoblasts display increased GSK3β activity and decreased levels of c-MYC; however, c-MYC down-regulation by GSK3β was not mediated via β-catenin, but rather by direct phosphorylation and enhanced degradation of c-MYC itself.
Experimental Procedures

Cell Culture – MC3T3-E1 osteoblastic cells used in this study are derived from a subclone recently isolated based on its robust mineralization potential (4). This subclone was expanded to approximately $10^9$ cells, at which time frozen stocks were prepared and defined as passage 1. Cells were maintained up to passage 12 in $\alpha$-minimum essential medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. All experiments were performed under differentiation conditions, i.e., in the presence of 50 $\mu$M ascorbic acid and 10 mM $\beta$-glycerophosphate. SAOS-2 human osteosarcoma cells, stably transfected with either wild type or a dimerization-defective (dim) glucocorticoid receptor, were a kind gift from Dr. M.J. Garabedian (20). These cells were maintained in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum and 400 $\mu$g/ml Geneticin.

Cell fractionation – Nuclear and cytoplasmic fractions were prepared essentially according to Verona et al. (21). Cell pellets were resuspended in two packed cell volumes of hypotonic buffer containing 10 mM HEPES (pH=7.5), 10 mM KCl, 3 mM MgCl$_2$, 1 mM EDTA (pH=8), 1 mM PMSF, 1 mM DTT, 10 $\mu$g/ml aprotinin, 10 $\mu$g/ml leupeptin, 10 mM NaF and 0.1 mM Na$_3$VO$_4$. Cells were left to swell on ice for 10 min., then vortexed for 10 sec, and spun at 500 X g for 5 min. The supernatant, containing the cytoplasmic lysate, was supplemented with one-third the volume of 80% glycerol and clarified by centrifugation at 20,000 X g for 30 minutes. The nuclear pellet was washed twice in hypotonic buffer, and lysed in two nuclear pellet volumes of lysis buffer containing 100 mM HEPES (pH=7.4), 0.5 M KCl, 5 mM MgCl$_2$, 28% glycerol, and
protease and phosphatase inhibitors as above. The nuclear extracts were centrifuged at 20,000 X g for 1 hour to remove cell debris.

**Western analysis** – Between 60-100 µg protein of nuclear extract, cytoplasmic lysate, or whole cell pellets were subjected to SDS-PAGE. Separated proteins were transferred to a 0.2 µm nitrocellulose membrane using Mini Trans-Blot Transfer Cell (Biorad), and immuno-detection was performed using ECL (Amersham) according to the manufacturer’s recommendations, followed by exposure of the membrane to X-ray film. Results were quantitated by photodensitometry using the AlphaImager 2000 system (Alpha Innotech Corporation), and are expressed as Mean±SD. Differences are considered significant when p≤0.05 as determined by paired t-test.

**GSK3β Immunoprecipitation and kinase assay** were performed essentially as described by Diehl et al. (22). Cells were lysed in 1% Triton-X-100 buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.27 M sucrose, 1 mM DTT, 1 mM PMSF, 0.4 mM NaF and 0.4 mM NaVO₄. GSK3β was immunoprecipitated from 200 µg of total protein extract using 1 µg mouse monoclonal anti GSK3β antibodies (Transduction Laboratories, Lexington, KY) and A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoprecipitate was resuspended in 24 µl kinase buffer containing 50 mM HEPES buffer (pH 7.5), 10 mM MgCl₂, 1 mM EGTA, 14 µM ATP, 83 µM p-GS-II peptide, 10 µCi [γ⁻³²P] ATP (6000 Ci/mmole) and protease and phosphatase inhibitors as above. Following 30 min at 30°C, 8 µl of 4 X loading buffer was added, containing (final concentrations) 4% SDS, 12% glycerol, 50 mM Tris-HCl (pH 6.8), 2% β-mercaptoethanol and 0.01% Serva blue G. The mixture was boiled...
for 5 min and loaded onto a 16.5% acrylamide-tricine gel (23) followed by autoradiography and quantitation as above.

**Flow cytometry.** Cell cycle analysis was performed according to Darzynkiewicz et al. (24). Briefly, cells were lightly trypsinized, resuspended in Hanks buffer, fixed in cold 70% ethanol, washed with Hanks buffer and suspended in 1 ml of Hanks buffer containing 20 µg/ml propidium iodide and 5 Kunitz U of DNase-free RNase A. The percentage of cells in G1, S, and G2/M was determined using an EPICS® Profile Analyzer.

**Reagents** – Tissue culture reagents were purchased from Gibco BRL. Antibodies against GSK3β (#G22320) and β-catenin (#C19220) were purchased from Transduction Laboratories (Lexington, KY). Anti [phospho-Ser^9^]GSK3β (#9336) and anti [phospho-Thr^58^]c-MYC (#9401) antibodies were from Cell Signaling Technology (Beverly, MA). Anti c-MYC (SC-764) antibodies and protein A/G Agarose beads were from Santa Cruz Biotechnologies (Santa Cruz, CA). Phospho-glycogen synthase peptide II (p-GS-II) was from Upstate Biotechnology (Lake Placid, NY). MG132 was from Calbiochem (San Diego, CA). Protein concentration was determined using Micro BCA protein assay kit (Pierce).
Results

GC activate GSK3β by attenuating its inhibitory Ser⁹ phosphorylation. We have recently shown that GC inhibit persistent cell cycle progression at a commitment stage during osteoblast differentiation, characterized by cobblestone appearance. We postulated that components of the Wnt signaling pathway might play a role during this commitment stage in mediating cell cycle persistence and its inhibition by GC. One of these components is GSK3β, a cell cycle guardian, which is inactivated by a variety of growth stimuli. We evaluated GSK3β in non-treated and GC-treated MC3T3-E1 cells at and immediately after the “cobblestone” stage. As determined by Western analysis, DEX-treatment of cells that had just become confluent resulted in a small but consistent increase in the total cellular levels of GSK3β (Figure 1A). Because nuclear localization of GSK3β has recently been proposed to play a cell cycle-related role (22), we also performed Western analysis on nuclear extracts, and found that the increase in GSK3β level was even more pronounced in the nuclear fraction of DEX-treated cells (Figure 1A). Notably, this effect was attenuated in cultures that had already grown to high density (Figure 1A, day 9). Accordingly, we thereafter confined our work specifically to cells that just initiate growth to high density.

GSK3β activity is subject to functionally critical post-translational modifications. We therefore measured GSK3β activity by immunoprecipitation and kinase assays using a glycogen synthase-derived peptide as substrate. As exemplified in Figure 1B, left top panel, DEX significantly increased GSK3β activity, and this increase amounted to 1.77±0.1-fold in three independent experiments.
An important post-translational modification of GSK3β is the phosphorylation of serine–9, which results in GSK3β inhibition, such as seen following growth factor stimulation (14-16). We therefore evaluated the effect of GC on GSK3β Ser⁹ phosphorylation. Indeed, as shown in Figure 1B, DEX decreased the level of [phospho-Ser⁹]GSK3β (left middle panel), while increasing total GSK3β (left bottom panel). In three independent experiments, DEX induced an average 2.4-fold decrease in the level of [phospho-Ser⁹]GSK3β (Figure 1B, right panel, pGSK). When corrected for the total levels of GSK3β, DEX reduced by 3-fold the relative level of GSK3β Ser⁹ phosphorylation (Figure 1B, right panel, pGSK/GSK). These results suggest that GC attenuate inhibitory phosphorylation of GSK3β mediated by growth stimulatory signals. Higher activity of GSK3β, a cell cycle guardian, would result in cell cycle impediment, as observed in GC-treated post-confluent MC3T3-E1 osteoblasts (4).

**GC down-regulate c-MYC in MC3T3-E1 cells in a β-catenin-independent fashion.**

An important downstream target of the Wnt signaling pathway is β-catenin, which participates in the transcriptional activation of cell cycle stimulatory genes, such as c-myc (9) and cyclin D1 (10). GSK3β-mediated phosphorylation promotes the degradation of β-catenin. We therefore examined if β-catenin was down-regulated in GC-treated MC3T3-E1 cells. However, as shown in Figure 1C, DEX did not decrease either the nuclear or the cytoplasmic β-catenin level, suggesting that β-catenin is resistant to GC-mediated activation of GSK3β in MC3T3-E1 osteoblasts. This is consistent with the notion that some GSK3β exists in pools that are physically and functionally distinct from those found complexed with APC, axin and β-catenin (see ref. (25) for review).
GSK3β can down-regulate cell cycle regulatory factors, such as c-MYC and cyclin D1, in both β-catenin-dependent and –independent manners. The β-catenin-independent pathway entails direct phosphorylation of c-MYC on Thr$^{58}$ followed by ubiquitination and proteasomal degradation (26,27). Indeed, despite the persistence of β-catenin in GC-treated MC3T3-E1 cells, Western blot analysis of c-MYC revealed reduced levels in DEX-treated compared to non-treated cultures (Figure 1D). In three independent experiments, c-MYC levels were significantly decreased by 2.3±0.2 fold.

**Osteoblast growth to high density is associated with c-MYC upregulation.** c-MYC has been previously found to express in osteoblast and chondrocyte *in vivo* (28,29). We therefore analyzed c-MYC by Western blot analysis during MC3T3-E1 osteoblast differentiation *in vitro*. Expectedly, as cells reached confluence (Figure 2A, day 5), c-MYC levels decreased (Figure 3B, day 5 vs. day 3). The same was observed in NIH3T3 cells under similar culture conditions (data not shown). However, two days after the “cobblestone stage” (Figure 3A, day 7) c-MYC was up-regulated back to levels observed in pre-confluent cultures (Figure 3B, day 7). This was not seen with the NIH3T3 cells (data not shown). In contrast to c-MYC, cyclin A, which is down-regulated by GC in post-confluent persistently cycling osteoblasts (4), is expressed at steady levels during pre-confluence, confluence, and until at least two days after confluence (Figure 3C). Taken together, this expression pattern and the effect of DEX (Figure 1D) suggest a role for c-MYC in driving the osteoblast post-confluent persistent cell cycle.

**GC promote Thr$^{58}$ phosphorylation and enhanced degradation of c-MYC.** Based on the activation of GSK3β (Figure 1B), without an accompanying decrease in β-catenin (Figure 1C), the decreased c-MYC level in GC-treated MC3T3-E1 cells (Figure 1D) is
attributable to protein instability. We therefore studied c-MYC synthesis and degradation in GC-treated cultures. Initially, we evaluated c-MYC synthesis rates by blocking degradation using the proteasomal inhibitor MG132; this was immediately followed by concomitant MG132 withdrawal and administration of cyclohexemide to block protein synthesis during the measurement of c-MYC degradation rate. As shown in Figure 3, representing one of three experiments with similar results, DEX did not inhibit c-MYC synthesis. However, c-MYC degradation was significantly accelerated in DEX-treated cells (Figure 3). Half-life values can only be approximated in these experiments because proteasomal recovery following MG132 withdrawal is not instantaneous. Assuming complete recovery at 1.5 hours post MG132 withdrawal, c-MYC was degraded in the DEX-treated cells over the following hour with an apparent half-life value 3.2±0.7 times greater than that measured in the non-treated cells (p=0.02, n=3).

GSK3β has been shown to target c-MYC for proteasomal degradation by phosphorylation on Thr\textsuperscript{58} (26,27). We therefore tested the c-MYC-Thr\textsuperscript{58} phosphorylation status in DEX-treated versus non-treated cells. Samples from 4-hour MG132-treated cultures (as in Figure 3A) were subjected to Western analysis using a [phospho-Thr\textsuperscript{58}]c-MYC-specific antibody. As shown in Figure 3C, DEX increased c-MYC Thr\textsuperscript{58} phosphorylation level. Corrected for total c-MYC, the relative DEX-mediated increase in c-MYC Thr\textsuperscript{58} phosphorylation was 1.7- and 2.6-fold in two independent experiments. These results are consistent with the insensitivity of β-catenin to GC (Figure 1C), and strongly suggest that increased c-MYC phosphorylation by GSK3β in GC-treated osteoblasts leads to accelerated degradation and reduced c-MYC steady state levels.
GC effects on GSK3β and c-MYC precede alterations in the cell cycle profile. The increased GSK3β activity in GC-treated cells (Figure 1B) may be a result, not a cause of cell cycle attenuation (30). We therefore tested GSK3β activity following short periods of GC treatment, before an effect on the cell cycle is mounted. Triton extracts of 2-hour and 5 hour-treated cultures were subjected to GSK3β immunoprecipitation and kinase assay. These short treatment periods did not alter the distribution of cells among the phases of the cell cycle (data not shown). As shown in Figure 4, top panel, DEX activated GSK3β within as little as two hours of treatment, with somewhat lesser activation observed at 5 hours.

Next, Triton extracts as above were subjected to Western blot analysis using antibodies to either GSK3β or [phospho-Ser⁹]GSK3β. As shown in Figure 4, two-hour DEX treatment significantly reduced [phospho-Ser⁹]GSK3β, and this reduction amounted to 47±5% in three independent experiments. Corrected for total GSK3β, the change in the relative Ser⁹ phosphorylation level [calculated, as in Figure 1B, as the ratio between pGSK3β(DEX)/pGSK3β(control) and GSK3β(DEX)/GSK3β(control)] was significantly reduced within the 2-hr treatment period by 46±20% (n=3). The glucocorticoid effect on the relative Ser⁹ phosphorylation of GSK3β was similar at the 5-hr time point, amounting to a significant 50±18% reduction (n=3). Because Ser⁹ phosphorylation is a major (albeit not the only (31)), determinant influencing GSK3β activity, our data suggest that the fast decrease in GSK3β Ser⁹ phosphorylation following 2 hours of treatment may be the initial event leading to GSK3β activation, c-MYC down-regulation and cell cycle attenuation. Consistent with this, a reduction in c-MYC levels was apparent at the 5-hr, although not at the 2-hr treatment time point (Figure 4, bottom panel).
GC attenuate GSK3β Ser⁹ phosphorylation that follows serum stimulation. c-MYC plays a critical role in the response of the cell cycle machinery to growth signals. To examine c-MYC immediate response to serum stimulation, MC3T3-E1 cells were incubated in serum-free medium for 48 hours and then re-fed with medium containing 10% serum. DEX was administered 2 hours prior to, and again along with serum stimulation. Following serum stimulation, c-MYC immediately began to accumulate in the non-treated cells (Figure 5). In three independent experiments, c-MYC levels significantly increased by 2.5±1.1-fold within 20 minutes of serum stimulation. In contrast, c-MYC accumulation was blunted in the DEX-treated cells, where an insignificant 27±25% increase was measured following serum stimulation. The antimitogenic effect of DEX in serum-stimulated cells was confirmed by flow cytometry analysis at 12 hours following serum stimulation with or without DEX (Figure 5B).

Following serum stimulation, [phospho-Ser⁹]GSK3β consistently accumulated in the non-treated cultures (Figure 5); the accumulation rate in the DEX-treated cultures was reduced by an average of 27% as compared to the control cultures, but this difference did not reach statistical significance (p=0.15, n=3). However, when corrected for the respective total GSK3β values, the increase in relative GSK3β Ser⁹ phosphorylation was significantly attenuated in the DEX-treated cultures, to levels 63% lower than the respective control values (p=0.03, n=3). Thus, GC attenuate the inhibitory Ser⁹ phosphorylation of GSK3β in serum-stimulated MC3T3-E1 cells, potentially blunting the c-MYC accumulation.

Lithium protects the MC3T3-E1 cell cycle from GC. If GSK3β plays a role in the antimitogenic effect of GC, then inhibition of GSK3β may ameliorate the inhibitory
effect of GC on cell cycle progression. We therefore tested the antimitogenic effect of DEX on the osteoblast post-confluent cell cycle in the presence of lithium, a specific GSK3β inhibitor (32). As shown in Figure 6A, exposure to lithium accelerated cell cycle progression in both DEX-treated and non-treated post-confluent osteoblast cultures. However, the lithium effect on the DEX-treated cells was more pronounced, bringing, within 9 hours, the cells in S+G2+M to virtually the same percentage as measured in the DEX-free cultures. Figure 6B shows representative cell cycle profiles from this 9-hour time point. As can be seen, 9-hour treatment was clearly sufficient for DEX to induce a G1/S block, and, more importantly, lithium completely equalized the cell cycle profile in non-treated and DEX-treated cells. This could be explained by lithium counteracting the DEX-mediated c-MYC down-regulation within as early as 4 hours (Figure 6B, insert). Interestingly, the GC-inhibition of cyclin A (4) was not rescued by lithium at this time point (Figure 6B, insert). By 18 hours of treatment, DEX further inhibited cell cycle progression (Figure 6A). Again, lithium almost entirely counteracted the decrease in the percentage of cells in S+G2+M (Figure 6A). It should be noted that the distribution of cells between S and G2/M in the DEX-treated cultures was different from that observed in the absence of DEX (data not shown), possibly reflecting effects of DEX and lithium on other cell cycle transitions (33). As opposed to lithium, potassium did not reverse the inhibitory effect of DEX on the G1/S transition (Figure 6A). In conclusion, our results indicate that GSK3β is a critical regulator of the post-confluent cell cycle, and that GSK3β activation contributes to the antimitogenic effect of GC in differentiating osteoblasts.
GC-inhibition of GSK3β Ser⁹ phosphorylation requires receptor dimerization. As a first step towards elucidating mechanisms by which GC activate GSK3β, we asked whether GC receptor (GR) dimerization was required for this activation. Receptor dimerization is a pre-requisite for DNA binding and classical transcriptional activation, but is not required for DNA-binding-independent action, such as AP-1 trans-repression (34). We employed SAOS-2 human osteosarcoma cell lines (originally GR-negative), which had been stably transfected with either the wild type or a dimerization defective (R479D/D481R) rat GR (35). Each of these cell lines was treated with DEX, and GSK3β Ser⁹ phosphorylation status evaluated by Western blot analysis. Similar to MC3T3-E1 osteoblasts (Figure 1B), DEX significantly inhibited GSK3β Ser⁹ phosphorylation in SAOS-2 cells expressing wild type GR (Figure 7). In contrast, DEX did not inhibit GSK3β Ser⁹ phosphorylation in SAOS-2 cells expressing the dimerization-defective GR (Figure 7). These results indicate that DEX-mediated GSK3β activation requires receptor dimerization, suggesting that it occurs via classical transcriptional activation of genes yet to be identified.
Discussion

Cell proliferation and differentiation are traditionally perceived as reciprocal processes, cell cycle withdrawal being a prerequisite for terminal differentiation. However, stages that precede expression of an ultimate cellular phenotype may occur as cells are cycling, such as during the differentiation of lymphocytes (36), adipocytes (5,6) and osteoblasts (4). Our earlier work with osteoblasts suggest that the differentiation-related cell cycle, occurring in cultures that have reached confluence, is regulated in a unique manner, rendering it susceptible to inhibition by GC.

The current study suggests that c-MYC plays a crucial role in the persistent proliferation of post-confluent osteoblasts in culture. c-MYC steady state levels decrease as osteoblasts reach confluence, similar to what is observed in non-osteoblastic cells. However, soon thereafter during osteoblast differentiation, c-MYC rises back to levels observed prior to confluence and the cells grow to high density. GC decrease c-MYC serum response and steady-state levels, and attenuate the persistent cell cycle in the post-confluent differentiating cells.

The increase in c-MYC levels in post-confluent differentiating osteoblasts may explain the persistent cell cycle despite high levels of the cdi p27Kip1 (4,37), and even when p27Kip1 is forcibly elevated further using viral transduction (4). The role of c-MYC in osteoblast differentiation may be to delay exit from the cell cycle, thus allowing differentiation steps, which require cell division. Alternatively, c-MYC may play a role in regulating differentiation-related genes, with the post-confluent cell cycle being a non-essential accompanying event. It is difficult to identify a differentiation stage in vivo, which may be analogous to the post-confluent cell cycle observed in vitro. However, in

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favor of a physiological role for c-MYC in mineralizing tissues, this proto-oncogenic transcription factor has been shown to express in osteoblasts and in hypertrophic chondrocytes in vivo (28, 29). These observations, and the inhibitory effects of GC on c-MYC and on the osteoblast phenotype, suggest a role for c-MYC and possibly the persistent cell cycle in osteoblast differentiation.

The increase in c-MYC levels in post-confluent differentiating osteoblasts may also shed light on mechanisms of osteosarcoma development. It is intriguing to speculate that physiological up-regulation of c-MYC at a specific stage of osteoblast differentiation provides a window of opportunity for the action of osteoblast-transforming agents such as FOS (38, 39). In this context, the c-MYC up-regulation is consistent with E2F4/p130 being the predominant and functionally critical E2F/pocket complex in post-confluent differentiating osteoblasts (4), because both c-MYC (40) and E2F4 (41) cooperate with RAS in cellular transformation.

How do GC exert their inhibitory effects on c-MYC and on the osteoblast persistent cell cycle? In search for upstream effectors, it is important to remember that the osteoblast cell cycle becomes sensitive to GC only at confluence. It therefore seemed logical that the GC-inhibition may involve the Wnt signaling pathway, which bridges cell-cell and cell-matrix interactions with the cell cycle machinery. Interestingly, what we observe in GC-treated cultures is increased intrinsic activity of GSK3β, but not decreased nuclear β-catenin levels, suggesting that GC primarily affect GSK3β in the growth factor pathway. However, we feel it is premature to rule out an effect of GC on GSK3β in the Wnt pathway. In fact, GC decreased reporter activity in cells transiently transfected with a Tcf/LEF-controlled luciferase construct (data not shown). Thus, we believe that GC
affect GSK3β in both the Wnt and the growth factor pathways. Importantly, inhibition of GSK3β by lithium neutralizes the inhibitory effects of GC, indicating causal relationship between GSK3β activation and cell cycle attenuation.

The lack of GC-mediated decrease in nuclear β-catenin is consistent with the unaffected c-MYC synthesis rate, which is regulated by β-catenin (9, 42,43). However, GSK3β may work directly on c-MYC, phosphorylating and accelerating its degradation (26,27). This mechanism clearly contributes to the antimitogenic effect of GC in differentiating osteoblasts, as indicated by the increased Thr58 phosphorylation and the accelerated degradation of c-MYC in response to GC treatment. Preferential effect of GC on GSK3β in the growth factor pathway versus the Wnt pathway may be explained by selective requirements for substrate priming (44,45).

GSK3β is a cell cycle guardian, which is inactivated in response to growth factors, typically by Ser9 phosphorylation. Our results show that GC attenuate this inactivating phosphorylation. GC decreased GSK3β Ser9 phosphorylation in cultures 2 hours (Figure 4B) as well as 48 hours (Figure 1B) after feeding fresh medium with or without DEX. The GC effect on GSK3β Ser9 phosphorylation was also apparent during the immediate response to serum stimulation (Figure 5). Thus, GC activate GSK3β by prevention of the serum-induced Ser9 inhibitory phosphorylation, which is sustained for as long as 48 hours.

A limitation of our study is that it employs cell lines, either transformed (SAOS-2) or untransformed (MC3T3-E1), which may be regulated differently than osteoblasts in vivo. Preliminary results with primary murine bone marrow-derived mesenchymal stem cells indicated that, like in the MC3T3-E1 cultures, DEX inhibited both condensation and
calcium deposition (data not shown). Also, GSK3β Ser⁹ phosphorylation was decreased in the DEX-treated cultures, but this effect did not reach statistical significance. It is possible that a DEX effect on the bone marrow-derived osteoblasts was diluted by different responses of non-osteoblasts and/or osteoblasts at DEX-insensitive stages of differentiation. Thus, the role of GSK3β in osteoblast differentiation and in glucocorticoid-induced osteoporosis remains to be confirmed in primary cultures and in vivo.

Among cyclins, cyclin-dependent kinases and cdks that were analyzed, GC’s most significant effect in MC3T3-E1 differentiating osteoblasts was down-regulation of cyclin A and its dissociation from E2F4/p130 complexes (4). It is possible that GC down-regulate c-MYC and cyclin A via independent mechanisms, and that both of these effects contribute to attenuation of the cell cycle. However, the present study demonstrates rapid inhibitory effects of DEX on c-MYC, which occurred prior to any significant effect on cyclin A (data not shown). Furthermore, rescue of the GC-inhibited cell cycle by lithium was accompanied by restoration of c-MYC, but not cyclin A levels (Figure 6B). These results suggest a more crucial role for c-MYC in GC-inhibition of the osteoblast persistent cell cycle. However, less than 2 hours of treatment was insufficient for DEX to mount any effect on c-MYC or GSK3β (data not shown). This, and the requirement for GR dimerization for the Ser⁹-dephosphorylation of GSK3β (Figure 7), suggests that GC-mediated GSK3β activation occurs via transcriptional stimulation of an intermediary gene(s).

Are our results with lithium relevant to bone and mineral metabolism in lithium-treated psychiatric patients? Limited work in this controversial field indicates lithium-induced
hyperparathyroidism, but no hypercalcemia or bone loss in these patients (46); one study reported increased levels of alkaline phosphatase (47), a serum marker of bone formation. Preserved bone mass in the face of hyperparathyroidism has been attributed to enhancement of renal calcium reabsorption (46,48). Based on our in vitro studies, it is interesting to speculate that enhanced osteoblast function may also contribute to bone preservation in lithium-treated patients with hyperparathyroidism.

Maintenance of bone mass throughout life requires that bone formation equals bone resorption. GC impair this balance primarily by inhibiting osteoblastic bone formation. The present study demonstrates that GC activate GSK3β in osteoblasts. This may interfere with important differentiation cues generated by cell-cell contacts, cell-matrix interactions, or by growth factors signaling through GSK3β, in particular IGF-I, known to play a role in osteoblast growth and differentiation (49). Our current findings suggest that impediment of osteoblast function by GSK3β activation may be mediated by the down-regulation of c-MYC, potentially via inhibition of a differentiation-related cell cycle. Thus, GC-activation of osteoblast GSK3β may play an important role in the pathogenesis and potentially future therapies for GC-induced osteoporosis.
Acknowledgements: The authors are grateful to M.J. Garabedian (NYU), J.R. Woodgett and A. Ali (Toronto) for suggestions and reagents, to Abel Valdovinos (USC) for technical assistance and to M.J. Roberts (USC) for the preparation of primary mesenchymal stem cell cultures from murine bone marrow. These studies were supported by grants from the National Institutes of Health (RO1-AR47052 and T32 CA 09659), from the Arthritis Foundation and from the Zumberge Foundation.
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Legends to Figures

Figure 1. Glucocorticoids activate GSK3β in differentiating osteoblasts. A. MC3T3-E1 cells were maintained under differentiation protocol and treated with 1 µM DEX at either the “cobblestone” stage (days 5-7) or thereafter (days 7-9). Whole cell triton extracts (WCE) and nuclear extracts (Nuc) were prepared and analyzed by Western blotting using GSK3β antibody. B. Left top, The Triton extracts from the day 7 cultures above were subjected to GSK3β kinase activity assay. GSK3β was immunoprecipitated and incubated with $^{32}$P-γATP and phospho-glycogen synthase peptide II, followed by electrophoresis and autoradiography. Left middle, SDS-whole cell extracts were prepared from non-treated and DEX-treated cultures on day 7 and subjected to Western blot analysis using antibodies against [phospho-Ser$^9$] GSK3β. Left bottom, Western analysis of total GSK3β was performed on the same membrane used in the middle panel. Right, Graph representing the DEX/Control ratios (Mean±SD, n=3) for [phospho-Ser$^9$]GSK3β ($pGSK$), total GSK3β ($GSK$) and the relative GSK3β Ser$^9$ phosphorylation level ($pGSK/GSK$). The latter value was calculated for each experiment by dividing the $pGSK$ ratio by the $GSK$ ratio. C. Nuclear and cytoplasmic extracts of cells harvested on day 7 were subjected to Western blot analysis using anti β-catenin antibodies. D. Differentiating MC3T3-E1 cells were treated with 1 µM DEX on day 5 and harvested on day 7 for Western analysis of c-MYC in SDS-whole cell extracts.

Figure 2. Up-regulation of c-MYC in post-confluent differentiating osteoblasts.

A. Morphology of MC3T3-E1 osteoblasts during the GC-sensitive commitment (“cobblestone”) stage. The left frame presents cells on day 3, just prior to confluence, the
middle frame shows cells with cobblestone appearance (day 5) and the right frame
demonstrates growth to high density (day 7). Magnification X100.  B,C. Cells were
harvested at the three developmental stages represented in A and Western blot analysis
was performed using either anti c-MYC (Panel B) or anti cyclin A (Panel C) antibodies.

Figure 3. Glucocorticoids accelerate c-MYC degradation.  A. Post-confluent MC3T3-E1 cells were treated on day 6 with either 1 µM DEX or vehicle. At 16 hours of DEX-
treatment, 50 µM MG132 in 0.1% DMSO (final concentrations) was administered, and
cells collected for c-MYC Western blot analysis following 0, 1, 2, 3 or 4 hours. At the 4-
hour time point, the MG132 was washed and cells re-fed with fresh medium containing
50 µM cyclohexemide. Cultures were again harvested at 1, 1.5, 2, 2.5 and 3 hours
following MG132 withdrawal for Western analysis of c-MYC. B. Graph illustrating the
degradation of c-MYC in treated (circles) versus non-treated (squares) cells. Values were
derived by densitometry of the autoradiogram shown in A (right panels). Similar results
were obtained in three independent experiments. C. Western analysis of 4-hr MG132-
treated cells using antibodies that recognize either all forms or just the Thr58-
phosphorylated from of c-MYC.

Figure 4. DEX-mediated GSK3β activation precedes cell cycle attenuation.
MC3T3-E1 cells were treated on day 6 with either 1 µM DEX or vehicle and harvested
following either 2 or 5 hours for preparation of Triton whole cell extracts. The same
extracts were subjected to either GSK3β kinase assays (Top) as in Figure 1B, or Western
blot analysis using the indicated antibodies.
Figure 5. Glucocorticoids attenuate serum-induced GSK3β serine 9 phosphorylation. A. Post-confluent differentiating cells were deprived of serum for 48 hours, and then re-fed serum-containing medium. DEX treatment (1 µM) initiated 2 hours prior to serum stimulation. SDS-cell extracts were prepared at 0, 10 and 20 minutes of serum stimulation and Western blot analysis performed using the indicated antibodies. B. Parallel DEX-treated and non-treated cultures were harvested 12 hours following serum stimulation, fixed and stained with propidium iodide for flow cytometry analysis. The percentage of cells in the S+G2+M phases of the cell cycle is depicted (Mean±SD, n=3).

Figure 6. Inhibition of GSK3β by lithium rescues the glucocorticoid-inhibited cell cycle. Post-confluent MC3T3-E1 cells were treated with either 0.1 µM DEX or vehicle, together with 40 mM LiCl, a specific GSK3β inhibitor, or KCl as control. Cells were collected following either 9 hours (only the lithium group) or 18 hours (lithium and potassium treatments). Cells were ethanol-fixed, stained with propidium iodide and the cell cycle profile was analyzed by flow cytometry. A. Percentage cells in S+G2+M is presented for each experimental group. Bars represent mean±SD of three plates, except for the KCl-treated groups, for which the bar represents mean of duplicate measurements, which differed by <5%. B. Representative cell cycle profiles are shown for the 9-hour DEX-treated and non-treated cells in the absence and presence of lithium. The percentages of cells in G1, S and G2/M are depicted underneath each histogram. Insert shows Western analysis of c-MYC and cyclin A (cycA) in 18-hour DEX-treated cells, to
which lithium was administered 4 hours prior to collection (D+L). c-MYC and cyclin A of DEX-treated cells in the absence of lithium (D) and of non-treated cells (C) are shown for comparison.

**Figure 7. Glucocorticoid-mediated attenuation of GSK3β Ser⁹ phosphorylation is dependent on receptor dimerization.** SAOS-2 osteosarcoma cells expressing either the wild type (wt) or a dimerization mutant form (dim) of the rat glucocorticoid receptor were treated for 48 hours with 1 µM DEX and subjected to Western analysis using antibodies against either [phospho-Ser⁹]GSK3β or total GSK3β, as indicated. A. Autoradiograms from a representative experiment. B. Graph representing the DEX/Control ratios (Mean±SD, n=3) for [phospho-Ser⁹]GSK3β (pGSK), total GSK3β (GSK) and the relative GSK3β Ser⁹ phosphorylation level (pGSK/GSK). The latter value was calculated for each experiment by dividing the pGSK ratio by the GSK ratio.
Figure 1

A.

B.

C.

D.
Days of culture

A

B

C

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Figure 2
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Figure 3
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Figure 4
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Figure 5
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Figure 6A
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Figure 6B
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Figure 7
Glucocorticoids inhibit cell cycle progression in differentiating osteoblasts via glycogen synthase kinase β
Elisheva Smith, Gerhard A. Coetzee and Baruch Frenkel

*J. Biol. Chem.* published online February 27, 2002

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