Gene expression profiling of glucocorticoid-inhibited osteoblasts

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Abstract

Glucocorticoid (GC) treatment for the management of autoimmune and inflammatory diseases is associated with decreased bone formation and increased risk for fracture. In MC3T3-E1 cell cultures, 0·1 – 1 µM dexamethasone (DEX) arrests development of the osteoblast phenotype when administration commences at a commitment stage around the time of confluency. To gain new insights into GC-induced osteoporosis, we performed microarray-based gene expression analysis of GC-arrested MC3T3-E1 cultures, 2·5 days after the administration of DEX. Of the 12 000 transcripts interrogated, 74 were up-regulated and 17 were down-regulated by at least 2·5-fold (P≤0·05). Some of these genes, such as Mmp13, Serum/GC-regulated kinase and Tieg, have previously been reported as GC-responsive. Others are shown here for the first time to respond to GCs. DEX strongly repressed Krox20/Egr2 at both the mRNA and the protein level. This is especially significant because mice lacking this transcription factor develop osteoporosis. The data also suggest that the bone morphogenetic protein (BMP) pathway, which is involved in regulating bone mass, and other pathways that influence BMP signaling, are abrogated by GCs: (i) DEX increased the mRNA levels of the BMP antagonists Follistatin and Dan; (ii) DEX increased the levels of p21 Rasgap3 and Ptpn16/MKP-1 mRNAs, negative regulators of the MAP kinase pathway; and (iii) DEX decreased Cox mRNA levels. DEX also increased thrombospondin mRNA levels, which negatively regulate bone mass in vivo, as well as the adipocytic marker Fkbp51. These and other observations disclose novel gene targets, whose regulation by GCs in osteoblasts may shed light on and provide new therapeutic approaches to osteoporosis.

Journal of Molecular Endocrinology (2004) 33, 175–193

Introduction

Glucocorticoids (GCs) are used extensively for the treatment of autoimmune and inflammatory diseases, including arthritis, asthma, multiple sclerosis, systemic lupus erythematosus, inflammatory bowel disease and chronic active hepatitis. In addition, GCs are used in combination with other drugs to reduce inflammation associated with hematologic and other cancers, and to suppress the immune system following organ transplantation. Perhaps the most disturbing side effect of GC treatment is rapid bone loss and increased risk of fracture (Van Staa et al. 2000). Although bone resorption has been suggested to contribute to GC-induced osteoporosis (Luckert & Kream 1996, Luckert 1997), it is now clear that the main mechanism underlying long-term GC-induced bone loss is the impairment of osteoblast function and bone formation (Canalis 1996, Manolagas & Weinstein 1999, Rubin & Bilezikian 2002).

Regarding their adverse effects on osteoblasts, GCs have been reported to attenuate cell proliferation and differentiation, promote apoptosis, abrogate collagen metabolism, interfere with the autocrine/paracrine action of insulin-like growth factor-I and bone morphogenetic protein-2 (BMP-2), and inhibit the bone-specific transcription
factor Runx2 (Chen et al. 1983, Delany et al. 1995a, Linkhart et al. 1996, Lian et al. 1997, Rogatsky et al. 1997, Chang et al. 1998, Weinstein et al. 1998, Luppen et al. 2003a, b). While some of these effects, such as the induction of apoptosis, were demonstrated in vivo, most of the studies were performed in vitro, alas under conditions that do not fully recapitulate the inhibitory action of GCs on the ultimate osteoblast phenotype – deposition of mineralized extracellular matrix. In fact, in many in vitro studies, GCs surprisingly stimulated osteoblast proliferation and function (Tenenbaum & Heersche 1985, Bellows et al. 1987, Pockwinse et al. 1995). Inhibition of mineralized extracellular matrix formation by GCs was recapitulated in vitro using the MC3T3-E1 cell line and occurred only when GC treatment commenced prior to the seventh day of culture (Lian et al. 1997).

In our quest for molecular mechanisms underlying GC-induced osteoporosis, we continued to employ the MC3T3-E1 culture model and identified a narrow developmental window during which cells are susceptible to the adverse effect of GCs. This window encompasses a commitment stage, occurring around the time of confluency, during which the cells undergo a uniquely regulated post-confluent cell cycle. Unlike cell cycle progression prior to confluency, the differentiation-related cell cycle is abrogated by GCs, concomitant with the inhibition of mineralization. At the commitment stage, GCs activate the enzyme glycogen synthase kinase 3β, a key negative regulator of the Wnt and other signaling pathways (Smith et al. 2002).

In order to gain further insights into molecular events associated with the adverse effects of GCs, we employed oligonucleotide microarrays to compare gene expression profiles between MC3T3-E1 cultures that have just undergone commitment and those arrested by DEX. Here we discuss the potential significance of the effects of DEX on subsets of these genes which encode transcription factors, secreted proteins, receptors, signaling molecules, and metabolic enzymes.

Materials and methods

Cell culture

MC3T3-E1 cells were plated in 100-mm dishes (300 000 cells/plate) for all experiments with the exception of those shown in Fig. 1, where cells were plated in 12-well plates (25 000 cells/well). Cultures were maintained in α-MEM (Invitrogen Corp., Carlsbad, CA, USA) containing 10% FBS (Invitrogen Corp.) and 1·5% penicillin-streptomycin (Invitrogen Corp.). To support differentiation, 50 µg/ml ascorbic acid (Sigma) and 10 mM β-glycerophosphate (Sigma) were added to confluent cultures and the medium was then changed every other day.

Calcium deposition

Calcium deposition was demonstrated by alizarin red staining or quantitated using the Sigma Diagnostics Kit 587 as previously described (Luppen et al. 2003a, b).

Apoptosis

Apoptosis was evaluated using either acridine orange staining as previously described (Mandavilli & Rao 1996) or the Apopercentage dye (Biocolor, http://www.bicolor.co.uk).

RNA preparation and cRNA array hybridization

Total RNA was isolated from control and DEX-treated cells using Trizol reagent (Invitrogen Corp.) according to the manufacturer’s instructions, and further cleaned by two successive phenol/chloroform extractions, followed by selective binding to a silica-gel-based membrane using RNeasy spin columns (Qiagen). Double-stranded cDNA was synthesized from 10 µg total RNA using an oligo-dT primer with a 5’-T7 RNA polymerase promoter sequence and the SuperScript II cDNA synthesis kit (LifeTechnologies, Gaithersburg, MD, USA). The cDNA was phenol/chloroform extracted and recovered by ethanol precipitation. Three micrograms double-stranded cDNA were used for the synthesis of biotin-labeled cRNA (Enzo Eukaryotic Gene Expression Labeling Kit; Affymetrix Inc., Sant Clara, CA, USA), which was hybridized to Affymetrix murine MG-U74Av2 gene chips (Affymetrix Inc., Santa Clara, CA, USA). Unbound cRNA was washed, and hybridized cRNA was labeled with a phycoerythrin-streptavidin conjugate. Fluorescent hybridization signals were detected using a Hewlett Packard GeneArray Scanner.
Microarray data analysis

Raw gene expression scores were generated for every gene in every sample using GENECHIP Software v5.0 (Affymetrix Inc.) with the default setups. To normalize the relative hybridization signal for each transcript, multiplicative scaling factors were calculated based on the median intensity of the 60th to 95th percentile of gene expression scores. All scores were set to a minimum value of 100 to minimize noise associated with rare transcripts (Karaman et al. 2003). The averages of normalized data from three control and three DEX-treated samples were calculated, and only differences of at least 2.5-fold with \( P \leq 0.05 \) (equal variance two-tail t-test) are reported herein.

Reverse transcription and polymerase chain reaction (RT-PCR)

The double-stranded cDNA from above was subjected to gene-specific PCR amplification to confirm expression results for selected genes. The cDNA was PCR-amplified (MJ Research Inc., Waltham, MA, USA) in a total volume of 25 µl containing 5 pmoles of each primer, 1 µCi \([\alpha-32P]dCTP\) (Perkin Elmer Life Sciences Inc., Boston, MA, USA), 0.25 nmoles unlabeled dCTP, 1.25 nmoles each of dATP, dTTP, and dGTP, and 0.5 units Taq polymerase (Invitrogen Corp.). For each of the analyzed genes, preliminary experiments were performed with increasing amounts of input cDNA to achieve close-to-linear conditions of signal/input ratio, and equal cDNA input for all samples was based on amplification of ribosomal protein L10A (rpL10A) cDNA, which itself varied by less than 25% between samples. The PCR primers are listed in Table 1. The amplified PCR products were resolved by electrophoresis in a 5% polyacrylamide gel. Following drying, the gels were exposed to a storage phosphor screen (Molecular Dynamics, Sunnyvale, CA, USA) and the signal associated with the products was measured using Storm 840 Phosphor Analyzer and Image Quant Software (Molecular Dynamics).

Western blot analysis

Cells were washed and collected in phosphate-buffered saline, centrifuged, and pellets were resuspended in 1:5 packed cell volume of lysis buffer (100 mM Hepes pH 7.5, 500 mM KCl, 5 mM MgCl₂, 0.5 mM EDTA, 28% glycerol) containing protease and phosphatase inhibitors (5 mM NaF, 0.1 mM Na₃VO₄, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20 µM MG132). The cells were further subjected to successive passes through 18.5-, 20.5-, and 23-gauge needles, followed by centrifugation at 16 000 r.p.m. for 30 min at 4 °C to remove cell debris. The protein concentration in the cell extract was determined using the Micro BCA Protein Assay (Pierce, Rockford, IL, USA) and 30 µg were subjected to electrophoresis in a 10% polyacrylamide denaturing gel. The proteins were then transferred onto a nitrocellulose membrane and Krox20 was detected using specific antibodies (1:150 dilution) from Covance (Richmond, CA, USA) and the enhanced chemoluminescence system (Amersham).

Results

Dexamethasone specifically inhibits mineralization when administration commences at a commitment stage associated with confluency

We utilized the MC3T3-E1 cell culture model, in which GCs severely inhibit development of the osteoblast phenotype (Smith et al. 2000). As shown by biochemical determination of calcium deposition, DEX inhibited mineralization in a manner strongly dependent on both the concentration and the timing of DEX administration (Fig. 1A). First, consistent with previous reports (reviewed by Ishida & Heersche 1998), DEX was inhibitory only at the pharmacological concentrations of 100 and 1000 nM, whereas 10 nM was stimulatory. Secondly, DEX at 100 and 1000 nM had a maximal inhibitory effect when administered commencing on day 3, as cells became confluent. Consistent with our previous work (Smith et al. 2000), little or no inhibition was observed when DEX was administered after the cells had committed to mineralization, around day 4. Most remarkably, exposure of the cultures to 100 or 1000 nM DEX commencing at early stages – prior to confluency – rendered them resistant to the later inhibitory potential of the drug at the same doses (Fig. 1A). Moreover, the 100 nM dose, which was inhibitory when treatment commenced on days 2–4, was stimulatory when treatment commenced
<table>
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<th>Annealing temp (°C)</th>
<th>No. of cycles</th>
<th>Product size (base pairs)</th>
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<td>245</td>
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Primers usually anneal with two adjacent exons, to confirm lack of genomic DNA contamination. However, the primer pairs for Enpp2, Follistatin, Haptoglobin, and Thrombospondins 1 and 2 targeted the 3' UTR sequences present on the microarray chip.
on day 0 or day 1 (Fig. 1A). Cultures treated with 1000 nM DEX commencing on day 1 were less inhibited than those treated from day 2 or day 3. Figure 1B presents an independent experiment with 1000 nM DEX, demonstrating similar results of calcium deposition by alizarin red staining.

In order to identify genes that link the GC inhibitory activity at the commitment stage to the abrogation of the osteoblast phenotype, we treated MC3T3-E1 cultures with 1000 nM DEX commencing on day 3, and performed a comprehensive gene expression analysis after 2.5 days. This experimental design provides a comparison between steady-state mRNA levels in osteoblasts undergoing normal, uninterrupted differentiation and those not allowed to undergo commitment. The morphological difference between differentiating and arrested cultures is demonstrated in Fig. 2A–B. Alizarin red staining of day-14 cultures, plated and treated in parallel to those used for the microarray analysis, is shown in Fig. 2C-F. Interestingly, the DEX-induced arrest was not associated with increased, but rather with decreased, apoptosis (Fig. 2G–J). In vitro inhibition of osteoblast apoptosis has been demonstrated by several investigators, although promotion of apoptosis in vivo and in vitro has been reported by others (reviewed by Zalavras et al. 2003).

The total RNA collected from the day-6 cultures was interrogated by MG-U74Av2 gene chips (Affymetrix Inc.). Of the over 12,000 transcripts represented on the microarray, expression of the vast majority (99%) did not change by more than 2.5-fold ($P \leq 0.05$) in response to DEX. In contrast, 74 genes were up-regulated and 17 were down-regulated by $\geq 2.5$-fold ($P \leq 0.05$). The expression levels of the 91 DEX-responsive genes, including 82 with known functions and 9 expressed sequence tags (ESTs), as well as the unedited list of raw data, are posted on our web site (http://hacialab.usc.edu/supplement/frenkel, Tables S1, S2). The potential significance of some of these findings is discussed below.

**Dexamethasone-responsive genes encoding transcription factors**

DEX treatment altered by $\geq 2.5$-fold the expression of zinc finger transcription factors (Krox20, Kruppel-like factor 13, transforming growth factor (TGF)-β inducible early growth response), basic leucine-zipper DNA-binding proteins (CAAT/enhancer binding protein δ and β, nuclear factor I/B), and a basic helix-loop-helix transcription factor (Drosophila period homolog) (Table 2).

**Krox20 (Early growth response 2, Egr2)**

The *Egr* genes (*krox* for the murine homologs) are mitogen-inducible, immediate early genes encoding transcription factors with three zinc fingers. Egr proteins control cell proliferation and differentiation in a variety of tissues including the brain and peripheral nerves, the vasculature, the kidney and the skeleton (McMahon *et al.* 1990, Sukhatme 1992, Beckmann & Wilce 1997, Khachigian & Collins 1998). Although *Krox20/Egr2* is expressed mainly in the adult thymus and in the embryonic central and peripheral nervous systems (Wilkinson *et al.* 1989, Topilko *et al.* 1994), the gene is also...
expressed in differentiating osteoblasts (Levi et al. 1996). Krox20/Egr2 null mice have a severe osteoporotic phenotype, attributable to reduced bone formation (Levi et al. 1996). In our microarray analysis, Krox20/Egr2 mRNA levels were repressed 5·8-fold by DEX (Table 2) and this result was confirmed by RT-PCR (Fig. 3A). Furthermore, as demonstrated by Western analysis, DEX strongly reduced Krox20 protein levels in MC3T3-E1 cultures (Fig. 3B). Thus, decreased Krox20/Egr2 in response to GCs may contribute to bone loss by abrogating the transcriptional control of Krox20/Egr2 target genes. Such targets may include the Biglycan, α1(I) and α2(I) Collagen genes, encoding components of the bone extracellular matrix, which have been shown to respond to another Krox family member, c-Krox (Galera et al. 1996, Heegaard et al. 1997). In addition, the decrease in Krox20/Egr2 may be involved in the DEX-induced up-regulation of Follistatin (Table 3, Fig. 4 and see below), which is negatively regulated by Krox20/Egr2 (Seitanidou et al. 1997).

**CONTROL**

**DEX**

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**Figure 2** The effects of DEX on cell morphology and apoptosis. MC3T3-E1 cells were plated (day 0) in 100-mm plates and treated with 1000 nM DEX commencing on day 3. (A, B) Brightfield micrographs of day-5 cultures (×200). (C, D) Micrographs of alizarin red-stained cultures on day 14 (×200). (E, F) Scans of the same stained plates. (G, H) Acridine orange staining of day-14 cultures. Bright nuclei denote cells undergoing apoptosis. (I, J) Apopto percentage staining of cells undergoing apoptosis on day 7; reproduced with permission from CRC Press (Zalavras et al. 2003). Several stained cells are indicated by arrows. This method is based on the flip-flopping of plasma membrane phosphatidylserine (Biocolor).
was up-regulated 3.0-fold by DEX (Table 2). Klf13 is a positive regulator of erythropoiesis (Asano et al. 2000), and is also expressed in the skeleton during mouse development (Martin et al. 2001). The role of Klf13 in osteoblasts, and its possible involvement in the effects of GCs in these cells, remains to be explored.

**Dexamethasone-responsive genes encoding cell surface and secreted proteins**

**Follistatin and Dan (Differential screening-selected gene aberrant in neuroblastoma)**

DEX stimulated the expression of two genes encoding secreted BMP antagonists. **Follistatin** mRNA level was increased by 3.1-fold (Table 3) and this increase, possibly related to the suppression of Krox20/Egr2 (see above), was confirmed by RT-PCR (Fig. 4). The mRNA for Dan (aka neuroblastoma suppressor of tumorigenicity) was increased by 3.3-fold (Table 3). Since both follistatin and Dan bind to and inactivate BMPs (reviewed by Canalis et al. 2003), and since BMPs are required for osteoblast differentiation in vitro and control bone mass in vivo (Yamaguchi et al. 1991, Sampath et al. 1992, Yamaguchi et al. 2000, Xiao et al. 2002, Styrkarsdottir et al. 2003, Yang et al. 2003), the stimulation of Follistatin and Dan mRNAs may contribute to GC-induced osteoporosis. We have recently shown that GC-inhibition of MC3T3-E1 osteoblast differentiation was also associated with a 100-fold decrease in Bmp-2 gene expression, and that addition of recombinant BMP-2 rescued the osteoblast phenotype (Luppen et al. 2003a, b). The GC-mediated repression of Bmp-2 was not disclosed by the microarray analysis because the overall level of its mRNA was close to the background noise level of the microarray assay. The relationship between the down-regulation of Bmp-2 and the up-regulation of Follistatin and Dan remains to be explored.

**Thrombospondins**

DEX increased the mRNA levels for *Thrombospondin 1* and 2 by 5.3-fold and 2.9-fold respectively (Table 3), and these results were confirmed by RT-PCR (Fig. 4). Thrombospondins are non-collagenous proteins.

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**Table 2** Transcription factor genes altered by DEX. Data are means±S.E.M. (n=3). Only genes altered by ≥2.5-fold are listed.

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Affymetrix identification</th>
<th>Control</th>
<th>DEX</th>
<th>Fold induction/repression by DEX</th>
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<tbody>
<tr>
<td>Period homolog (<em>Drosophila</em>)</td>
<td>AF022992</td>
<td>93619_at</td>
<td>236±17</td>
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<td>CCAAT/enhancer binding protein (C/EBP), δ</td>
<td>X61800</td>
<td>160894_at</td>
<td>1101±57</td>
<td>4881±239</td>
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<td>Nuclear factor I/B</td>
<td>Y07685</td>
<td>160859_s_at</td>
<td>619±29</td>
<td>2199±83</td>
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<tr>
<td>Zinc finger protein 99</td>
<td>U62906</td>
<td>104010_at</td>
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<td>Kruppel-like factor 13 (Klf13)</td>
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<td>TGF-β inducible early growth response (Tieg)</td>
<td>AF064088</td>
<td>99603_g_at</td>
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<td>120±21</td>
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<td>102661_at</td>
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**Figure 3** DEX represses the transcription factor Krox20/Egr2 at the mRNA and protein levels. MC3T3-E1 cells were plated (day 0) in 100-mm plates and treated with 1000 nM DEX commencing on day 3. (A) RT-PCR analysis of Krox20/Egr2 mRNA in representative cultures treated for 60 h with either DEX or vehicle. Ribosomal protein L10A (rpL10A) serves as control. (B) Western blot analysis of Krox20 in representative cultures treated for 72 h. The top panel shows the 56 kDa band representing Krox20 and the bottom panel shows two non-specific bands of 63 and 68 kDa demonstrating equal loading.
secreted glycoproteins that associate with the extracellular matrix, but do not appear to contribute directly to tissue integrity. Rather, the proteins influence cell functions such as adhesion, motility, proliferation, apoptosis, differentiation, cytoskeletal organization and transcriptional regulation by modulating the assembly of cell surface multi-protein complexes. The two Thrombospondin genes are expressed with different developmental and spatial patterns (Iruela-Arispe et al. 1993, Tooney et al. 1998). Thrombospondin 1 is widely expressed and is involved in cytoskeletal organization during tissue repair, possibly mediating some of the effects of TGF-β1 (reviewed by Chen et al. 2000). Thrombospondin 2 has a more restricted expression pattern and is synthesized primarily in connective tissues (reviewed by Bornstein et al. 2000). Both thrombospondin 1 and 2 are found in sites of new bone formation and both are produced by cultured osteoblasts (Robey et al. 1989, Carron et al. 1999). Although Thrombospondin 1 mRNA level was shown to increase during MC3T3-E1 culture progression (Sherbina & Bornstein 1992), thrombospondins seem to negatively regulate osteogenesis, based on the increase in osteoblast number, endosteal bone formation and cortical bone thickness observed in Thrombospondin 2 knock-out mice (Hankenson & Bornstein 2002). Therefore, the GC-induced Thrombospondin mRNA levels may contribute to bone loss in vivo. Furthermore, because both thrombospondin 1 and 2 inhibit angiogenesis (reviewed by Bornstein 2000 Table 3 Genes for cell surface and secreted proteins altered by DEX. Data are means±S.E.M. (n=3). Only genes altered by ≥2.5-fold are listed

<table>
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<tr>
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<td>92414_at</td>
<td>140±31</td>
<td>445±48</td>
<td>3·2</td>
</tr>
<tr>
<td>Follistatin</td>
<td>Z29532</td>
<td>98817_at</td>
<td>938±428</td>
<td>2929±258</td>
<td>3·1</td>
</tr>
<tr>
<td>Thrombospondin 2</td>
<td>L07803</td>
<td>94930_at</td>
<td>1203±364</td>
<td>3500±85</td>
<td>2·9</td>
</tr>
<tr>
<td>Collagen, type XI, alpha 1</td>
<td>D38162</td>
<td>100481_at</td>
<td>645±106</td>
<td>1853±331</td>
<td>2·9</td>
</tr>
<tr>
<td>Chondroitin sulfate proteoglycan 2</td>
<td>D45889</td>
<td>100019_at</td>
<td>708±106</td>
<td>1758±165</td>
<td>2·5</td>
</tr>
</tbody>
</table>

Figure 4 RT-PCR analysis. Expression level of the indicated genes was quantitated in day-6 MC3T3-E1 cultures by RT-PCR using primers listed in Table 1. Shown are results from a representative DEX-treated and a representative control culture. The arrow indicates the Tieg cDNA amplicon. Ribosomal protein L10A (rpL10A) amplification serves as internal control.
and by Chen et al. 2000), their stimulation by GCs may compromise the perfusion to bone and other organs, and contribute to GC-induced apoptosis in vivo.

Collagens

Ninety percent of the organic bone matrix is collagen, mostly type I. GCs are known to inhibit the expression of type I collagen genes in several cell types (reviewed by Kucharz 1988), including osteoblasts (Choe et al. 1978, Delany et al. 1995a, Mahonen et al. 1998, Luppen et al. 2003b). In the present study, the genes encoding the a1(I) and the a2(I) Collagen chains were inhibited by 1·9-fold and 1·7-fold respectively (Table S2; http://hacialab.usc.edu/supplement/frenkel), missing the 2·5-fold cutoff set for the initial analysis of the microarray data. It is noteworthy that GCs further inhibit osteoblast collagen synthesis at the protein level (Luppen et al. 2003b).

In contrast to type I collagen chains, DEX increased the mRNA levels for a1(VIII) and a1(XI) Collagen chains by 5·9-fold and 2·9-fold respectively (Table 3). a1(VIII) Collagen was further tested by RT-PCR analysis, which confirmed the microarray data (Fig. 4). Type VIII collagen, a short-chain network-forming collagen prominently expressed by endothelial cells of the vasculature and the Descemet’s membrane of the cornea, is also found in newborn mouse calvaria and skin (Muragaki et al. 1992). Type XI collagen, a fibril-forming collagen, is also expressed in the cornea but is mainly found in cartilage, where it copolymerizes with type II collagen and regulates chondrocyte differentiation and spatial organization of the growth plate (Li et al. 1995). The potential impact of increased collagens type VIII and XI on the physiological assembly of the bone extracellular matrix remains to be explored. It would also be interesting to test whether GCs induce collagens type VIII and XI in the cornea, because this could provide an insight into GC-induced glaucoma (Schacke et al. 2002).

Matrix metalloproteinase 13 (Mmp13, aka collagenase 3)

Collagens are primarily degraded by collagenases, specific proteinases of the matrix metalloproteinases (MMP) family. Among all the MMPs represented on the microarray (Table S2; http://hacialab.usc.edu/supplement/frenkel), DEX altered the expression of only one gene, Mmp13, which was increased by 5·2-fold (Table 3). This increase was confirmed by RT-PCR (Fig. 4) and is consistent with previous reports (Shalhoub et al. 1992, Delany et al. 1995b). Induction of Mmp13 mRNA may contribute to GC-induced osteoporosis by interfering with collagen type I homeostasis and should also be considered in the context of GC treatment of arthritis patients, given the high affinity of Mmp13 for type II collagen (Knauper et al. 1996).

A disintegrin and metalloprotease domain 12 (Adam 12)

Adam12 gene expression was up-regulated 3·2-fold by DEX (Table 3). Adams are membrane-anchored cell surface glycoproteins involved in cell fusion, cell adhesion, proteolysis and subsequent signaling. In MC3T3-E1 cultures, Adam12 is highly expressed prior to confluency and is down-regulated immediately thereafter and throughout differentiation (Inoue et al. 1998). It remains to be seen whether persistence of Adam12 mRNA contributes to GC-inhibition of osteoblast differentiation.

Ectonucleotide pyrophosphatase/phosphodiesterase 2 (Enpp2)

Enpps exist as both membrane and soluble proteins, and are expressed in cells from most tissues, including osteoblasts (Goding et al. 1998). They hydrolyze phosphodiester bonds in nucleoside triphosphates, leading to the generation of inorganic pyrophosphate, a physiologic calcification inhibitor (Bollen et al. 2000, Terkeltaub 2001, Johnson et al. 2003). Enpp2 mRNA level was up-regulated several-fold in response to DEX (Table 3, Fig. 4), potentially contributing to the decreased accumulation of calcium (Figs 1 and 2). Besides its role in regulating pyrophosphate levels, Enpp2 is also involved in the synthesis of lysophosphatidic acid (Tokumura et al. 2002), which can promote osteoblast proliferation and survival (Grey et al. 2001, 2002). While opposite actions of DEX apparently override the mitogenic potential of Enpp2, the putative increase in lysophosphatidic acid may help explain the inhibition of apoptosis observed in DEX-treated

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MC3T3-E1 (Fig. 2) and other osteoblastic cell cultures (Zalavras et al. 2003).

Cysteine-rich protein 61 (Cyr61)
The gene encoding Cyr61 is a growth factor-inducible immediate early gene. Its product is an extracellular matrix-associated signaling protein that promotes cell proliferation, migration and adhesion (Kireeva et al. 1996, Yang & Lau 1991). Unexpectedly, our microarray analysis indicated a 3.5-fold DEX-mediated increase in Cyr61 mRNA level (Table 3). Cyr61 is expressed during embryogenesis of the circulatory system and the cartilaginous skeleton (O’Brien & Lau 1992). Cyr61 mRNA has been detected in human osteoblasts (Schutze et al. 1998, Lechner et al. 2000), and is up-regulated by cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, which are important stimulators of bone resorption (Schutze et al. 1998). Interestingly, Cyr61 was the most up-regulated gene in a microarray analysis of mice fed with cadmium, an in vivo model for cadmium-induced osteoporosis (Regunathan et al. 2003). It is possible that osteoblast-derived Cyr61 contributes to the increased bone resorption observed in the initial phase of GC-induced osteoporosis.

Acute phase proteins
The microarray analysis revealed the stimulation by DEX of five genes encoding proteins with known functions in the immune system: haptoglobin, orosomucoid 1 and 2, the 1α-associated invariant chain of the major histocompatibility complex class II, and sterile alpha motif domain and hydrolase domain-containing protein 1 (Table S2; http://hacialab.usc.edu/supplement/frenkel). Of these, the first three are known as acute phase proteins, and they were induced by 8.0-, 17.8- and 5.3-fold respectively (Table 3 and Table S2; http://hacialab.usc.edu/supplement/frenkel). Notably, a structural role for orosomucoid 1 in bone was suggested based on its presence in bone extracts (Mbuyi et al. 1982). Haptoglobin was shown to affect bone cells in an organ culture system, probably through induction of prostanoids (Lerner & Frohlander 1992). Specifically, treatment with haptoglobin resulted in higher levels of prostaglandin (PG) E2 and increased bone resorption. The GC-stimulation of osteoblastic Haptoglobin mRNA level was confirmed by RT-PCR (Fig. 4).

Integrin binding sialoprotein (aka bone sialoprotein, Bsp)
Consistent with previous reports (Ogata et al. 1995, Chen et al. 1996), DEX stimulated Bsp gene expression by 7.4-fold (Table 3). Bsp is a late marker for osteoblast differentiation, and the gene product constitutes approximately 12% of the non-collagenous bone extracellular matrix proteins (reviewed by Ganss et al. 1999). It stimulates osteoblastic cell adhesion and proliferation (Mintz et al. 1993, Zhou et al. 1995) and has been proposed to function in the initiation of hydroxyapatite crystal formation (Hunter & Goldberg 1993). Given the general inhibitory effect of DEX on the osteoblast phenotype, including the down-regulation of other late differentiation markers such as osteocalcin (Lian et al. 1997, Luppen et al. 2003b), the stimulation of Bsp mRNA level is somewhat surprising. While it cannot explain the inhibition of the osteoblast phenotype in this study, it may explain the positive effect that GCs have on cultured osteoblasts under different conditions (Tenenbaum & Heersche 1985, Bellows et al. 1987, Leboy et al. 1991, Shallhoub et al. 1992).

Dexamethasone-responsive genes involved in signal transduction
Protein kinases and phosphatases
Our microarray analysis revealed ≥2.5-fold alteration in the mRNA levels for nine protein kinases and phosphatases in response to DEX. The data provide evidence for increases in both protein tyrosine kinases and phosphatases, and for a decrease in MAP kinase (MAPK) activity.

Protein tyrosine phosphatase (PTP) non-receptor type 16 (Ptpn16)
PTPs are either membrane receptors or non-receptor proteins. DEX increased the expression of Ptpn16 mRNA level by 4.4-fold (Table 4). Ptpn16 is an immediate early gene that encodes the PTP non-receptor MAPK phosphatase-1 (MKP-1), also called dual specificity phosphatase 1 for its ability to dephosphorylate both phospho-tyrosines and phospho-threonines (Keyse & Emslie 1992). MKP-1 inactivates MAPKs (Assenti et al. 1993), and GCs have previously been shown to inhibit MAPKs by elevating Mkp-1 mRNA level.
in fibroblasts and mast cells (Kassel et al. 2001). Moreover, a recent study linked MKP-1 up-regulation to the GC-inhibition of osteoblast proliferation (Engelbrecht et al. 2003), and administration of the tyrosine phosphatase inhibitor sodium orthovanadate prevented bone loss in GC-treated rats (Hulley et al. 2002). The MAPK pathway plays a role in osteoblast differentiation by potentiating BMP signaling (Suzawa et al. 2002), parathyroid hormone/parathyroid hormone-related peptide (PTH/PTHrP) signaling (Carpio et al. 2001, Miao et al. 2001), and by phosphorylating and activating the osteoblast master regulator Runx2 (Xiao et al. 2000). Thus, DEX stimulation of Ptpn16 mRNA probably plays an important role in GC-induced osteoporosis.

Table 4 Genes for signaling molecules altered by DEX. Data are means±S.E.M. (n=3). Only genes altered by ≥2.5-fold are listed

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>Affymetrix identification</th>
<th>Control</th>
<th>DEX</th>
<th>Fold induction/repression by DEX</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-mer proto-oncogene</td>
<td>U21301</td>
<td>92698_at</td>
<td>208±55</td>
<td>1051±83</td>
<td>5.0</td>
</tr>
<tr>
<td>Growth arrest specific 6 (Gas6)</td>
<td>X59846</td>
<td>99067_at</td>
<td>1639±173</td>
<td>7978±598</td>
<td>4.9</td>
</tr>
<tr>
<td>Cdc42 guanine nucleotide exchange factor 9-like (EST C79050)</td>
<td>Al647003</td>
<td>100958_at</td>
<td>342±102</td>
<td>1584±151</td>
<td>4.6</td>
</tr>
<tr>
<td>Protein tyrosine phosphatase, non-receptor type 16 (Ptpn16)</td>
<td>X61940</td>
<td>1004598_at</td>
<td>192±16</td>
<td>850±126</td>
<td>4.4</td>
</tr>
<tr>
<td>Leukemia inhibitory factor receptor</td>
<td>D17444</td>
<td>104658_at</td>
<td>166±16</td>
<td>727±42</td>
<td>4.4</td>
</tr>
<tr>
<td>Rho GTPase-activating protein 8 (expressed sequence C78947)</td>
<td>Al843580</td>
<td>103511_at</td>
<td>100±0</td>
<td>321±45</td>
<td>3.2</td>
</tr>
<tr>
<td>Fibroblast growth factor receptor 2</td>
<td>M63503</td>
<td>93091_s_at</td>
<td>1271±194</td>
<td>3789±393</td>
<td>3.0</td>
</tr>
<tr>
<td>p21 Ras GTPase-activating protein 3 (p21 Rasgaps3)</td>
<td>U20238</td>
<td>93319_at</td>
<td>714±94</td>
<td>1947±184</td>
<td>2.7</td>
</tr>
<tr>
<td>Aplysia ras-related homolog B (RhoB)</td>
<td>X99963</td>
<td>101030_at</td>
<td>1127±126</td>
<td>3002±142</td>
<td>2.7</td>
</tr>
<tr>
<td>Rho guanine nucleotide exchange factor 5 (RhogeF5)</td>
<td>D13903</td>
<td>93896_at</td>
<td>151±50</td>
<td>387±54</td>
<td>2.6</td>
</tr>
</tbody>
</table>

**Receptor protein tyrosine phosphatase type D (RptpD)**

DEX also increased RptpD mRNA level by 2.6-fold (Table 4). RptpD belongs to the leukocyte antigen related (LAR) family of protein tyrosine phosphatases (Mizuno et al. 1993). Although RptpD expression is largely restricted to the brain, our data demonstrate that it is also expressed in osteoblasts. Known substrates for LAR phosphatases include the insulin receptor and the epidermal growth factor receptor (Hashimoto et al. 1992, Zhang et al. 1996). Because signals mediated by these receptors contribute to osteoblast proliferation and differentiation (Canalis 1985), increased RptpD expression may adversely affect osteoblast function.

**C-mer proto-oncogene and Growth arrest specific 6 (Gas6)**

Along with the increase in PTP mRNA levels, DEX also stimulated the genes encoding C-mer, a receptor tyrosine kinase, and its ligand, Gas6 (Nagata et al. 1996, Chen et al. 1997). The 5.0-fold stimulation of both genes (Table 4) was supported by RT-PCR (Fig. 4). Gas6 is a member of the vitamin K-dependent family of growth factors, and has mitogenic, survival and adhesive properties in a variety of normal and cancer cell types (Goruppi et al. 1996, Li et al. 1996, Nakano et al. 1996, Yanagita et al. 2001, Funakoshi et al. 2002, Shankar et al. 2003). These effects are probably mediated through interactions with members of the Axl family of kinases, including C-mer (Varnum et al. 1995, McCloskey et al. 1997). Because Gas6/Axl signaling has been shown to inhibit osteogenic differentiation of vascular pericytes (Collett et al. 2003), it is possible that the DEX-induction of Gas6 and C-mer mRNA levels plays a role in GC-induced osteoporosis.
DEX increased the level of Fgfr2 mRNA by 3.0-fold (Table 4). Fgfr1–4 binds fibroblast growth factor (FGF), leading to cell growth, differentiation, migration and chemotaxis (reviewed by Galzie et al. 1997) and the Fgfr2 gene is expressed during osteogenesis in vivo (Eswarakumar et al. 2002). However, increased Fgfr2-mediated signaling could be detrimental to bone through mechanisms similar to those operative in patients with Crouzon, Apert and Pfeiffer syndromes, who have constitutively active mutant forms of Fgfr2 (Mansukhani et al. 2000).

**Small G proteins and their regulators**

Of the 91 genes that responded to DEX by \( \geq 2.5 \)-fold, 22 are involved in intracellular signaling, and of these, five are small G proteins or their regulators. Small G proteins (also called small GTP binding proteins or small GTPases) are divided into five families: Ras, Rho/Rac/Cdc42, Rab, Sar1/Arf, and Ran (reviewed by Takai et al. 2001). By transducing upstream signals to downstream effectors, small G proteins serve as molecular switches that regulate processes as diverse as cytoskeletal reorganization, intracellular vesicle trafficking, nucleocytoplasmic transport and gene expression. In the absence of activating signals, small G proteins are found in their inactive, GDP-bound form. Upon stimulation, GDP is exchanged for GTP, a process controlled by specific guanine exchange factors (GEFs), leading to activation of the downstream effector(s). The signal transduction is terminated by the intrinsic GTPase activity of the small G proteins, which is under the control of specific GTPase activating proteins (GAPs).

**p21 Rasgap3**

DEX increased p21 Rasgap3 mRNA level by 2.7-fold (Table 4), which can be expected to inhibit Ras signaling and the downstream MAPK and phosphatidylinositol 3-kinase pathways (reviewed by Takai et al. 2001). As mentioned above, MAPKs contribute to osteoblast differentiation by supporting the actions of BMPs, PTH, PTHrP and Runx2 (Xiao et al. 2000, Carpio et al. 2001, Miao et al. 2001, Suzawa et al. 2002). The DEX-mediated inhibition of the MAPK pathway, both via MKP-1 (see above) and the up-regulation of p21 Rasgap3, could severely compromise osteoblast function.

**RhoB and Rho regulators**

DEX increased RhoB mRNA level by 2.7-fold (Table 4). Despite this increase, our results suggest that overall Rho signaling is probably decreased in DEX-treated cells, because the Rhogef5 mRNA level was decreased by 2.7-fold and the Rhogap8 mRNA level was increased by 3.2-fold. The increase in Rhogap8 expression was confirmed by RT-PCR (Fig. 4). Rho belongs to the Rho/Rac/Cdc42 family, which regulates the organization of the actin cytoskeleton at focal adhesions, and in filipodia and lamellipodia. Therefore, the decrease in Rho signaling may be related to the morphological arrest of DEX-treated MC3T3-E1 cultures in the so-called cobblestone stage (Fig. 2B).

**EST C79050 (Cdc42 gef9-like gene)**

Unlike the inhibition of Rhogef5, DEX stimulated the expression of a Cdc42 gef9-like gene by 4.6-fold (Table 4). Thus, whereas Rho and Ras signaling are probably attenuated in DEX-treated cells, Cdc42-dependent pathways might in fact be stimulated. This result is somewhat surprising because another Cdc42 gef gene, Fgd1, is specifically expressed in calcified tissues (Gorski et al. 2000), where it might contribute to bone cell differentiation through activation of c-jun N terminal kinase (Zheng et al. 1996).

**Dexamethasone-responsive genes encoding metabolic enzymes**

**Prostaglandin-endoperoxide synthase 1 (aka cyclooxygenase 1, Cox1)**

The two Cox enzymes, Cox1 and Cox2, are integral membrane proteins which localize primarily to the endoplasmic reticulum, and function in PG biosynthesis. Cox1 is constitutively expressed and is thought to be involved in cell signaling and tissue homeostasis. Cox2 is expressed in a limited number of cell types and is probably responsible for prostanooid biosynthesis in response to mitogens and pro-inflammatory factors (reviewed by Smith et al. 1996). The microarray analysis indicated a 2.8-fold DEX-mediated decrease in Cox1 mRNA level (Table 5), and this result was confirmed by
RT-PCR (Fig. 4). Cox2 mRNA level was decreased by 2·1-fold, less than our 2·5-fold cutoff (Table S2; http://hacialab.usc.edu/supplement/frenkel). Both Cox1 and Cox2 genes are known to be expressed in osteoblastic cells, in association with osteogenesis (Sato et al. 1997), and PGs are known to contribute to BMP signaling (Chikazu et al. 2002) and bone formation (as well as resorption) (reviewed by Raisz 1999 and by Pilbeam et al. 2002). Thus, down-regulation of Cox genes in osteoblasts could contribute to the GC-mediated inhibition of osteoblast function and bone formation.

Steroidogenic enzymes

DEX induced the expression of three genes involved in the synthesis of steroid hormones: cytochrome P450 1b1 (2·6-fold), Ferredoxin reductase (2·8-fold), and Hydroxysteroid 17-β dehydrogenase 11 (3·4-fold), (Table 5). Cytochrome P450 1b1 (Cyp1B1) is involved in the metabolism of diverse substrates including steroids, fatty acids and xenobiotics. The gene is broadly expressed in bone (Sasano et al. 1997, Eyre et al. 1998), where it is responsible for the local production of estrogens (Nawata et al. 1995). The up-regulation of Cyp1B1 is consistent with the reported increase in its mRNA and activity in GC-treated cultures of normal human osteoblast-like and osteosarcoma cells (Nawata et al. 1995). Ferredoxin reductase, a component of the mitochondrial electron transport chain, is involved in the first step of steroid synthesis. Hydroxysteroid 17-β dehydrogenase is also expressed in bone cells (Sasano et al. 1997, Eyre et al. 1998), where it converts estrone produced by Cyp1B1 to estradiol. Given the well-established anti-apoptotic properties of estrogens in osteoblasts (Gohel et al. 1999, Manolagas et al. 2002), the putative stimulation of estradiol synthesis may help explain the unexpected inhibition of apoptosis in GC-treated osteoblast cultures (Fig. 2G–J and Zalavras et al. 2003). In addition, GC-induced estradiol synthesis may contribute to the attenuation of bone resorption and the low bone turnover seen after long-term GC treatment.

Table 5 Metabolic enzyme genes altered by DEX. Data are means±S.E.M. (n=3). Only genes altered by ≥2·5-fold are listed

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>Affymetrix identification</th>
<th>Control</th>
<th>DEX</th>
<th>Fold induction/repression by DEX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldehyde dehydrogenase family 1, subfamily A1</td>
<td>M74570</td>
<td>100068_at</td>
<td>100±0</td>
<td>578±97</td>
<td>5·8</td>
</tr>
<tr>
<td>Glutamine synthetase</td>
<td>U09114</td>
<td>94852_at</td>
<td>158±29</td>
<td>753±164</td>
<td>4·8</td>
</tr>
<tr>
<td>Glutamate-cysteine ligase, catalytic subunit</td>
<td>U85414</td>
<td>99649_at</td>
<td>269±28</td>
<td>959±62</td>
<td>3·6</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase kinase 4</td>
<td>AJ001418</td>
<td>102049_at</td>
<td>718±66</td>
<td>2541±132</td>
<td>3·5</td>
</tr>
<tr>
<td>Hydroxysteroid 17-β dehydrogenase 11</td>
<td>AA822174</td>
<td>102370_at</td>
<td>930±40</td>
<td>3181±88</td>
<td>3·4</td>
</tr>
<tr>
<td>Xanthine dehydrogenase</td>
<td>X75129</td>
<td>97950_at</td>
<td>558±95</td>
<td>1898±60</td>
<td>3·4</td>
</tr>
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<td>Galactokinase</td>
<td>AV217354</td>
<td>161363_r_at</td>
<td>210±55</td>
<td>595±42</td>
<td>2·8</td>
</tr>
<tr>
<td>Ferredoxin reductase</td>
<td>D49920</td>
<td>92754_at</td>
<td>376±50</td>
<td>1061±76</td>
<td>2·8</td>
</tr>
<tr>
<td>Cytochrome P450 1b1, benz[a]anthracene inducible</td>
<td>X78445</td>
<td>99979_at</td>
<td>1128±346</td>
<td>2871±315</td>
<td>2·5</td>
</tr>
<tr>
<td>Carnitine palmitoyltransferase 1, liver</td>
<td>AF017175</td>
<td>93320_at</td>
<td>1018±116</td>
<td>395±66</td>
<td>−2·6</td>
</tr>
<tr>
<td>Prostaglandin-endoperoxide synthase 1</td>
<td>M34141</td>
<td>95597_at</td>
<td>338±31</td>
<td>123±19</td>
<td>−2·8</td>
</tr>
<tr>
<td>RIKEN cDNA 2610002H19 gene (cytochrome c-1)</td>
<td>AV069997</td>
<td>162469_at</td>
<td>4556±310</td>
<td>1508±436</td>
<td>−3·0</td>
</tr>
<tr>
<td>Platelet-activating factor acetylhydrolase, isofrom 1b, α1</td>
<td>U57746</td>
<td>100576_at</td>
<td>828±130</td>
<td>250±36</td>
<td>−3·3</td>
</tr>
</tbody>
</table>

RT-PCR (Fig. 4). Cox2 mRNA level was decreased by 2·1-fold, less than our 2·5-fold cutoff (Table S2; http://hacialab.usc.edu/supplement/frenkel). Both Cox1 and Cox2 genes are known to be expressed in osteoblastic cells, in association with osteogenesis (Sato et al. 1997), and PGs are known to contribute to BMP signaling (Chikazu et al. 2002) and bone formation (as well as resorption) (reviewed by Raisz 1999 and by Pilbeam et al. 2002). Thus, down-regulation of Cox genes in osteoblasts could contribute to the GC-mediated inhibition of osteoblast function and bone formation.

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Additional genes altered by DEX

**FK506 binding protein 5 (aka Fkbp51)**

The transcript encoding Fkbp51 was induced 7·0-fold after DEX treatment, and this result was confirmed by RT-PCR (Fig. 4). Fkbp51 is a
member of the immunophilin protein family and is involved in protein folding and trafficking, including the nuclear transport of the GC receptor following ligand binding (Davies et al. 2002). Fkbp51 mRNA is abundant in T cells and thymus, and increases during GC-induced apoptosis of murine thymoma cells (Baughman et al. 1995). The gene product also selectively accumulates during adipogenesis (Yeh et al. 1995). Noteworthy, the decrease in bone volume in GC-induced osteoporosis is accompanied by increased marrow fat (Meunier et al. 1971, Burkhardt et al. 1987, Nuttall & Gimble 2000). Thus, the increase in Fkbp51 mRNA level may reflect a GC-induced switch from an osteoblastic to an adipocytic phenotype (Wang et al. 1977, Nuttall et al. 1998).

Discussion

This study provides an extensive profile of the alterations in gene expression that occur when GCs block the progression of MC3T3-E1 osteoblast cultures through a commitment stage around the time of confluency. While some of the disclosed genes have previously been described as GC-responsive (e.g. Mmp13, Serum/GC-regulated kinase, Tieg, Fkbp51), the response of many others, such as Krox20/Egr2, Thrombospondin 1 and 2, and Follistatin, is described here for the first time. Based on these data, several potential mechanisms by which GCs impair osteoblast function and induce osteoporosis can be proposed.

The strong repression of Krox20/Egr2 in GC-treated cells, shown here at both the mRNA and protein levels, most likely contributes to GC-induced osteoporosis, because Krox20 gene ablation in the mouse leads to osteoblast insufficiency and bone loss (Levi et al. 1996). Decreased Krox20 mRNA levels could result in decreased collagen synthesis (Galera et al. 1996) and may also contribute to the observed increase in the mRNA for Follistatin, a BMP antagonist negatively regulated by Krox20 (Seitanidou et al. 1997).

BMP signaling plays a key role in regulating bone mass and skeletal integrity (Styrkarsdottir et al. 2003). Taken together, a number of changes observed following DEX treatment can be expected to ultimately inhibit BMP signaling. Most directly, DEX increased the expression of genes encoding the secreted BMP antagonists, Follistatin and Dan. DEX could also have inhibited BMP action through parallel pathways known to interact with BMP signaling. For example, DEX increased the expression of the genes encoding p21 Rasgap3 and the PTPs Ptpn16 and RptpD. These effects probably result in the inhibition of the MAPK pathway, which is known to synergize with Smad-mediated BMP signaling and to transmit Smad-independent signals (Lou et al. 2000, Gallea et al. 2001, Lai & Cheng 2002, Suzawa et al. 2002, Hassel et al. 2003). In addition, the DEX-induced decrease in Cox mRNA levels can be expected to inhibit BMP signaling because PGs, the products of Cox activity, support BMP action (Chikazu et al. 2002). Evidence for abrogated BMP signaling in the GC-treated cultures is provided by the 5-8-fold decreased expression of Tieg, a BMP-responsive gene (Hefferan et al. 2000).

Along with the alterations that may contribute to osteoblast dysfunction, the expression of several genes was inconsistent with the overall adverse effect of GCs. For example, DEX stimulated the expression of genes involved in the synthesis of estrogens, known to promote osteoblast differentiation and preserve bone mass (Qu et al. 1998, Gohel et al. 1999, Manolagas et al. 2002). Cyr61, an extracellular matrix-associated protein that promotes cell proliferation (Kireeva et al. 1996), was also induced by DEX, as was Bsp, a late marker for osteoblast differentiation (Mintz et al. 1993, Zhou et al. 1995). Indeed, GCs can either stimulate or suppress osteoblast function in vitro, depending on their concentration, the specific culture system and the administration protocol. We performed our microarray analysis under conditions where the adverse effects apparently override the stimulatory ones.

This study employed a cell line, MC3T3-E1, a novel culture system in which GCs inhibit commitment to the ultimate osteoblast phenotype, i.e. elaboration of mineralized extracellular matrix. This inhibitory effect is associated with alterations in pathways, which critically contribute to bone health in vivo, including the Wnt (Smith et al. 2002) and the BMP signaling pathways (Luppen et al. 2003b). Furthermore, some GC-responsive genes identified in the present study (e.g. Krox 20/Egr2 and thrombospondin 2) have been directly linked to bone health in vivo. However, extrapolation of observations made in the MC3T3-E1 cell line to GC-induced osteoporosis will require careful studies with other osteoblast culture systems,
animal models and/or human bone biopsies, which will have to demonstrate, for example, similar alterations in gene expression in vivo and a role for these alterations in mediating the adverse effects of GCs on bone mass and quality.

Analysis of gene expression using microarrays suffers a number of inherent limitations. First, by setting our cutoff to a 2.5-fold change, we filtered out some smaller, yet possibly important effects. Such appears to be the case for the mRNAs encoding the type I collagen chains, which were down-regulated by only 1.7- to 1.9-fold in the DEX-treated cultures. A second obvious limitation is that the microarray analysis is confined to the RNA level. This limitation too is well demonstrated by the inhibition of type I collagen gene expression, which reaches a factor of 4- to 10-fold when measured in the same cultures at the protein level (Luppen et al. 2003b). Thirdly, we only reported robust effects (≥ 2.5-fold, P ≤ 0.05) for genes whose expression levels were above a predetermined threshold (see Materials and methods). Along with many potentially misleading differences within the noise range, this approach could have filtered out important effects of DEX on rare transcripts.

Our RT-PCR analyses generally confirmed the microarray results. Most of the primers used for RT-PCR amplified protein-coding sequences spanning adjacent exons, in order to disclose possible contamination of the cDNA-derived signal by amplification of genomic DNA. In contrast, the sequences represented on the microarrays usually interrogate the 3′-UTRs of mRNAs. The general agreement between the microarray and the RT-PCR results, despite the difference in sequences targeted by the two methods, increases our confidence in the disclosed effects of DEX. However, the difference between the sequences targeted by the microarray versus the RT-PCR analysis can lead to disagreement between the two methods in cases where DEX affects, for example, alternative polyadenylation or alternative splicing. This could have been the case for four of the twenty genes tested, where the RT-PCR results were different from those of the microarray analysis. These four genes are denoted by asterisks in Table S1 (http://hacialab.usc.edu/supplement/frenkel).

In summary, we used oligonucleotide microarray analysis to identify DEX-responsive genes in MC3T3-E1 osteoblast cultures and highlight potential mechanisms of GC-induced osteoporosis. Two of the most attractive potential mechanisms emerging from this study are the suppression of Krox20/Egr2 and the abrogation of BMP signaling.

### Supplemental material

Supplemental material, including the complete set of raw microarray data and the list of the 91 genes most responsive to DEX, is available online at http://hacialab.usc.edu/supplement/frenkel.

### Acknowledgements

The authors thank Dr Jack Turman (University of Southern California) for generously providing the Krox20 antibodies. The authors are also grateful to Vijaya Rao, Sara Martinez and Jack Chen for their contribution to the calcium assays and the RT-PCR analyses. This study was supported by grants from the National Institutes of Health (AR47052), the Arthritis Foundation (Atlanta, GA, USA) and the Robert E and May R Wright Foundation (Los Angeles, CA, USA). N L is the recipient of an Arthritis Foundation postdoctoral training grant.

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Received in final form 24 March 2004

Accepted 30 March 2004

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