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Glucocorticoids Antagonize RUNX2 During Osteoblast Differentiation in Cultures of ST2 Pluripotent Mesenchymal Cells

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Abstract

The efficacy of glucocorticoids (GCs) in treating a wide range of autoimmune and inflammatory conditions is blemished by severe side effects, including osteoporosis. The chief mechanism leading to GC-induced osteoporosis is inhibition of bone formation, but the role of RUNX2, a master regulator of osteoblast differentiation and bone formation, has not been well studied. We assessed effects of the synthetic GC dexamethasone (dex) on transcription of RUNX2-stimulated genes during the differentiation of mesenchymal pluripotent cells into osteoblasts. Dex inhibited a RUNX2 reporter gene and attenuated locus-dependently RUNX2-driven expression of several endogenous target genes. The anti-RUNX2 activity of dex was not attributable to decreased RUNX2 expression, but rather to physical interaction between RUNX2 and the GC receptor (GR), demonstrated by co-immunoprecipitation assays and co-immunofluorescence imaging. Investigation of the RUNX2/GR interaction may lead to the development of bone-sparing GC treatment modalities for the management of autoimmune and inflammatory diseases.

Keywords

GLUCOCORTICOID-INDUCED OSTEOPOROSIS; OSTEObLAST DIFFERENTIATION; TRANSCRIPTION

More than half a century after their introduction for the treatment of rheumatoid arthritis, glucocorticoids (GCs) are still abundantly and effectively used in the management of a wide range of autoimmune, inflammatory, neoplastic, and post-transplant conditions [Canalis et al., 2007; Baschant et al., 2012; Weinstein, 2012]. One of the chief side effects, though, is severe bone loss observed in men and women, young and old, especially those treated with high dose GCs and those treated for prolonged time periods [Canalis et al., 2007; Baschant et al., 2012; Weinstein, 2012]. Furthermore, beyond their roles in bone turnover, bone cells also regulate energy metabolism [Lee et al., 2007], and osteoblasts contribute to

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extraskeletal side effects such as insulin resistance and glucose intolerance observed in GC-treated patients [Brennan-Speranza et al., 2012]. Improved understanding of molecular mechanisms of action of GCs in osteoblasts may therefore lead to the development of therapeutic modalities that facilitate the use of GCs as immune suppressants while avoiding GC-induced osteoporosis (GIO), aberrant energy metabolism and possibly other side effects.

Even though exaggerated bone resorption contributes to loss of bone mass and strength during early disease stages, the chief mechanism underlying GIO is inhibition of osteoblast proliferation and function [Canalis et al., 2007; Baschant et al., 2012; Weinstein, 2012]. Exposure of osteoblasts to GCs at pharmacological concentrations results in both attenuation of cell cycle progression and acceleration of apoptosis [Canalis, 1984; Weinstein et al., 1998; Smith et al., 2000]. Mechanisms underlying these anti-proliferative effects, as well as abrogation of mineralized extracellular matrix synthesis have been extensively reviewed [Canalis et al., 2007; Baschant et al., 2012; Weinstein, 2012] and include interference with the function of growth factors, their receptors and critical downstream effectors such as insulin-like growth factors and the Wnt signaling pathway. Reports on GC-mediated inhibition of RUNX2 [Chang et al., 1998; Pereira et al., 2001], a master regulator of osteoblast differentiation and bone formation, and on direct physical interaction between the GC receptor (GR) and RUNX2 [Ning and Robins, 1999], have attracted only limited attention to date.

The critical role of RUNX2 in osteoblast (and chondrocyte) differentiation has been elucidated through gene knockout studies in mice [Komori et al., 1997; Otto et al., 1997] and by experiments manipulating RUNX2 in cell culture models [Banerjee et al., 1997; Ducy et al., 1997; Komori, 2011]. Because osteoblast-mediated bone formation is needed to balance the resorptive activity of osteoclasts at bone multicellular units (BMUs) throughout life, post-natal inhibition of RUNX2 can be expected to result in bone loss [Ducy et al., 1999; Estrada et al., 2012]. Thus, inhibition of RUNX2 by GCs may constitute a pivotal mechanism of GIO. Given that the work on the physical interaction between the GR and RUNX2 has been limited [Ning and Robins, 1999] and that only *Tgfb β 1* has been investigated to date as a RUNX2-target gene affected by GCs [Chang et al., 1998], we revisited the issue and addressed the interaction between GC signaling and RUNX2 during the induction of osteoblast differentiation in cultures of ST2 bone marrow-derived pluripotent mesenchymal cells. We find that the GR and RUNX2 colocalize and physically interact in ST2 cells, and that GCs antagonize RUNX2-mediated stimulation of target genes to various degrees.

MATERIALS AND METHODS

CELLS

Construction of the ST2/Rx2^{dox} cells line was recently described [Baniwal et al., 2012]. Cells were cultured in RPMI-1640 supplemented with non-essential amino acids and L-glutamine (Gibco, Grand Island, NY) as well as 10% fetal calf serum (FCS; Clontech, CA). Doxycycline (dox; from Calbiochem, La Jolla, CA) was dissolved in distilled water (5 mg/ml stock solution) and dexamethasone (dex; Sigma, St Louis, MO) was dissolved in ethanol (10 mM stock solution). Final dox concentration was 500 ng/ml and dex was used at

the indicated final concentrations. Equal volume of distilled water and 0.01% ethanol were used as vehicle.

ALKALINE PHOSPHATASE AND CELL PROLIFERATION ASSAYS

Cells were seeded in 24-well plates (30,000 cells/well), treated as indicated and fixed for 2 min in 4% paraformaldehyde. For histochemical staining of alkaline phosphatase (ALP), we used the ALP detection kit from EMD Millipore Corporation, Billerica, MA. Images were captured using a dissecting microscope and ALP-positive nodules were enumerated using the ImageJ64 software package. For assessment of cell proliferation, cells were treated as indicated for the depicted periods of time and then incubated with PBS-dissolved 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) for 2 h at 37°C. Cells were then lysed using DMSO and absorbance at 595 nM was measured using a Victor3V™ spectrophotometer (PerkinElmer, Shelton, CT).

LUCIFERASE ASSAY

ST2/Rx2^{dox} cells were plated in 24-well plates at 10,000 cells/well and transiently transfected using Lipofectamine® LTX & Plus Reagent (Invitrogen, Carlsbad, CA) with 1 µg of p6XOSE2-luc, a kind gift from Dr. Gerard Karsenty (20). After 48 h of treatment with Dox and/or dex, cells were harvested in 200 µl of passive lysis buffer (Promega, Madison, WI) and luminescence was measured using the Luciferase assay kit from Promega in a Victor3V™ plate reader (PerkinElmer).

QUANTITATIVE RT-PCR

RNA was extracted from cells using Aurum Total RNA kit (Bio-Rad Laboratories, Inc., Hercules, CA) following the manufacturer's recommendations. For cDNA synthesis, 1 µg of RNA was reverse-transcribed using qScript™ cDNA SuperMix as per the manufacturer's instructions (Quanta BioSciences, Inc., Gaithersburg, MD). The cDNA was diluted 10-fold with distilled water and subjected to real-time PCR amplification using Maxima® SYBR Green/Fluorescein qPCR Master Mix (Fermentas Inc., Glen Burnie, MD), gene-specific primers (Table S1) and a CFX96™ real-time PCR system from Bio-Rad. Expression levels of mRNAs of interest were normalized for the respective 18S RNA levels, which themselves were not significantly influenced by any treatment.

CO-IMMUNOPRECIPITATION

Cells were plated with culture medium supplemented with 10% complete serum in six-well plates at a density of 300,000 per well. After 24 h, cells were treated for 48 h as indicated and then lysed in a 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and fresh Protease Inhibitors Cocktail (1%; Sigma). After homogenization by passing 10 times through a 1 cm³ microfine insulin syringe, lysates were cleared by centrifugation at 14,000 rpm for 5 min in a bench-top microfuge, and 15% of the lysate solution was set aside and used as input. The remaining lysate was immunoprecipitated with approximately 3 µg of the specified antibody and 30 µl protein-G beads (Amersham Biosciences, Freiburg, Germany) and washed three times for 5 min each with the same buffer, followed by centrifugation at 4,000 rpm for 1 min. Thirty microliters

of the immunoprecipitate/bead suspension were mixed with 20 μ l of 2.5 $\times$ “sample buffer” (50% Glycerol, 10% SDS, 1 M Tris-HCl pH 6.8, 25% beta β -Mercaptoethanol, 0.5% Bromopenol blue) and boiled for 5 min. Input and immunoprecipitates were subjected to Western blot analysis with either anti-FLAG antibodies (Sigma) to detect FLAG-RUNX2 or anti-GR antibodies cat. no. sc-1004 (M20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

CONFOCAL IMMUNOFLUORESCENCE MICROSCOPY

Cells were grown on 18 mm² cover-slips in six-well plates for 24 h in growth medium with 10% FCS. Following 48-h treatment, the cells were fixed with 95% methanol for 15 min and incubated with anti-GR or anti-FLAG antibodies (1:500) followed by a rhodamine- or fluorescein-conjugated secondary antibody, respectively. Cells were mounted using Vectashield Hard Set mounting medium with DAPI (Burlingame, CA) and viewed using an LSM 510 Zeiss confocal microscope at 60 $\times$ magnification. Images were processed using the software program Image J.

STATISTICAL ANALYSIS

Results are expressed as the mean $\pm$ SD. Each assay was performed in triplicate and repeated at least twice. Differences between groups were considered significant when $P < 0.05$ using Student's *t*-test.

RESULTS AND DISCUSSION

Manipulation of RUNX2 in various mesenchymal cell culture models strongly impacts expression of osteoblast marker genes and related phenotypes such as ALP activity [Banerjee et al., 1997; Ducy et al., 1997]. We recently engineered ST2 bone marrow stroma-derived cells with a doxycycline (dox)-regulated RUNX2 lentiviral vector to yield the ST2/Rx2^{dox} sub-line. Dox treatment of these cells resulted in robust induction of RUNX2, ALP activity, and expression of genes related to both osteoblastogenesis and osteoblast-driven osteoclastogenesis [Baniwal et al., 2011]. To test whether GCs inhibit RUNX2-dependent ALP activity in ST2/Rx2^{dox} cultures, we treated cells with dox along with increasing concentrations of dexamethasone (dex; 1 nM to 1 μ M). As shown in Figure 1A, dex antagonized RUNX2-mediated formation of ALP-positive osteoblast-like nodules in a dose-dependent manner. As little as 1 nM dex inhibited the number of ALP-positive nodules by 13% and 24% on Day 2 and Day 5, respectively (Fig. 1B). Maximum inhibition in this experiment, approximately 50%, was observed after 5 days of treatment with 64 nM-1 μ M dex (Fig. 1B). Because GCs are anti-mitogenic in many cell types, including osteoblasts [Canalis, 1984; Smith et al., 2000], we tested by MTT assays whether the compromised formation of ALP-positive nodules (Fig. 1) was attributable to decreased cell proliferation. As shown in Figure 1C, dex only minimally affected cell proliferation in the ST2/Rx2^{dox} cultures, suggesting that it specifically inhibited processes related to osteoblast differentiation.

To test whether inhibition of ALP activity was associated with decreased RUNX2 activity, we transiently transfected ST2/Rx2^{dox} cells with the 6XOSE2-luc plasmid, in which the luciferase reporter gene is controlled by six copies of the RUNX2-binding site from the

bone-specific mouse osteocalcin gene promoter [Ducy and Karsenty, 1995]. After transfection, cells were treated with dox to induce RUNX2 along with 10 nM, 100 nM, or 1 μ M dex. Treatment with dox robustly stimulated activity of the RUNX2 luciferase reporter, and dex antagonized this stimulation in a dose-dependent manner, with nearly 50% inhibition observed with 1 μ M dex (Fig. 2A). Notably, the low basal luciferase activity measured without dox was unaffected by dex (Fig. 2A).

Antagonism of RUNX2 by dex could be mediated by inhibition of RUNX2 expression as previously demonstrated by Northern and Western analyses of GC-treated rat primary osteoblast cultures [Chang et al., 1998; Pereira et al., 2001]. In the ST2/Rx2^{dox} culture model, however, RUNX2 expression is less likely sensitive to GCs because it is coded by a virally introduced DNA sequence, not by the endogenous gene [Baniwal et al., 2012]. We measured the effects of dex at 1–1,000 nM on expression of the virally introduced RUNX2 cDNA, initially at the mRNA level. RT-qPCR analysis demonstrated that dex did not significantly affect either basal or dox-induced RUNX2 mRNA levels in ST2/Rx2^{dox} cells (Fig. 2B). Furthermore, data from our Western (Fig. 2C, *input*) and immunofluorescence analyses (Fig. 3) demonstrated that RUNX2 protein levels did not decrease, and possibly even increased, when dox treatment was accompanied by 1 μ M dex. Therefore, the inhibition of RUNX2 activity by dex in ST2/Rx2^{dox} cells appears to be post-translational.

Based on a GST pull-down result described by Ning and Robins, [1999], we reasoned that GCs could have inhibited RUNX2 by means of direct interaction between the activated GR and RUNX2. We examined whether such interaction could occur in ST2/Rx2^{dox} cells by co-immunoprecipitation assays. As shown in Figure 2C, the GR was readily detectable in RUNX2 immunoprecipitates obtained with FLAG antibodies, but not in non-specific precipitates obtained with IgG control antibodies. The physical interaction between the GR and RUNX2 in ST2/Rx2^{dox} cells may contribute to the observed dex-mediated inhibition of RUNX2 activity and ALP-positive osteoblast-like nodule formation.

In order to physically interact in living cells, GR and RUNX2 must reside in the same subcellular compartment, and this was next examined by immunofluorescence (Fig. 3). Indeed, RUNX2 (red) and GR (green) were both localized in the nucleus and appeared to share characteristic sub-nuclear domains in dox-treated ST2/Rx2^{dox} cells. Furthermore, physical interaction between RUNX2 and the GR within these domains was suggested by a dramatic increase in colocalization of the two transcription factors, as indicated by yellow color, in response to treatment with dex (Fig. 3). Thus, the GR and RUNX2 can potentially interact even in the absence of added GCs (Fig. 2C, top), but treatment of living cells with dex stimulated the interaction (Fig. 3), probably by inducing in the GR changes that facilitated closer proximity to RUNX2.

Interactions between RUNX2 and steroid hormone receptors usually result in mutual transcriptional inhibition [Kawate et al., 2007; Khalid et al., 2008; Baniwal et al., 2009] although some exceptions have been reported [McCarthy et al., 2003; Paredes et al., 2004; Baniwal et al., 2012]. Our recent genome-wide analysis of the response of RUNX2 to estradiol in breast cancer cells [Chimge et al., 2012] and to dihydrotestosterone in prostate cancer cells (Little and Frenkel, unpublished) suggest that steroid hormones influence

RUNX2, presumably through interaction with the respective nuclear receptors, in a locus-dependent manner. Whereas the predominant outcome of these interactions is inhibitory, that inhibition may be minimal or absent for some RUNX2 target genes, and a few of these may even be co-stimulated by RUNX2 and steroid hormone signaling. With such diverse outcomes in mind, we assessed by RT-qPCR the influence of increasing dex concentrations on twelve RUNX2-responsive genes from our recent genome-wide analysis of dox-treated ST2/Rx2^{dox} cells [Baniwal et al., 2012]. The results demonstrate various degrees of antagonism of RUNX2 by dex-activated GR (Fig. 4). RUNX2-dependent expression of *Bglap* (osteocalcin), *Ibsp*, *Efnb1*, and *Cdk5rp* mRNAs was inhibited by 1–4 nM dex only weakly or not at all, but dex concentrations 16 nM resulted in approximately 50% repression of these genes (Fig. 4A). In other cases, the anti-RUNX2 activity of dex was very weak at all concentrations tested, up to 1 μ M (Fig. 4B). In sharp contrast, dex strongly antagonized RUNX2-driven expression of *Ltc4s*, *Tnc*, and *Sema7a* even at low concentrations of 1–4 nM (Fig. 4C). Finally, dex did not significantly influence RUNX2-driven transcriptional repression (Fig. 4D). Notably, none of the 12 genes tested was cooperatively regulated by RUNX2 and dex (Fig. 4).

CONCLUSIONS

Previous work with osteoblasts, including from our laboratory, documented physical and functional interactions between RUNX2 and several steroid hormone receptors including the vitamin D receptor, estrogen receptor α (ER α) and the androgen receptor [Paredes et al., 2004; Kawate et al., 2007; Khalid et al., 2008; Baniwal et al., 2009]. Data obtained here by co-immunoprecipitation and co-immunofluorescence assays suggest physical interaction between RUNX2 and the GR in ST2 mesenchymal pluripotent cells undergoing osteoblast differentiation. As a likely outcome of this interaction, dex predominantly inhibited RUNX2, demonstrated by both a reporter assay and mRNA analysis of several RUNX2 target genes. Variable degrees of anti-RUNX2 activity were recorded for different genes, potentially reflecting different organization of genomic sites occupied by RUNX2, the GR, and possibly other transcription factors that shape the ultimate outcome of the interaction. Because RUNX2 activity in osteoblasts plays pivotal roles in regulating bone turnover—both formation and resorption [Frenkel et al., 2010], its inhibition by GCs likely contributes to GIO. Improved GC therapy for the management of autoimmune and inflammatory diseases may be developed by identification of novel GC immunosuppressive compounds that spare RUNX2 activity in the osteoblast lineage or by developing molecules that intercept the anti-RUNX2 activity of conventional GC drugs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Dex inhibits RUNX2-driven formation of ALP-positive osteoblast-like nodules. A: Representative images from Day-2 (top) and Day-5 (bottom) ST2/Rx2^{dox} cultures treated with dox and/or dex as indicated and stained for ALP activity. B: ALP-positive nodules in ST2/Rx2^{dox} cultures treated as in A were enumerated using the ImageJ64 software. Data are Mean±SD (n = 3) after adjustment to the number of ALP-positive nodules in the dex-free cultures, defined as 100%. Continuous and dashed lines represent Days 2 and 5, respectively and thin lines represent the number of ALP-positive nodules that formed in the absence of dox or dex. C: ST2/Rx2^{dox} cells were plated and treated as in A and their proliferation was assessed by performing MTT assays on Days 0, 2, and 5 (Mean±SD; n = 3). The error bars in C are smaller than the symbols.

Fig. 2. GR physically interacts with and inhibits RUNX2. A: ST2/Rx2^{dox} cells were transiently transfected with the RUNX2 reporter p6XOSE2-luc and luciferase activity was measured after 48 h of treatment with dox (filled circles) or vehicle (open circles) along with dex at the indicated concentrations (mean±SD, n = 3). B: ST2/Rx2^{dox} cells were treated for 48 h with dox (filled circles) or vehicle (open circles) along with dex at the indicated concentrations, and RUNX2 mRNA levels were measured by RT-qPCR (mean±SD, n = 3). C: ST2/Rx2^{dox} cells were treated with dox in the presence of dex or vehicle. RUNX2 complexes were immunoprecipitated using FLAG antibodies, or non-specific IgG antibodies were used as control. Presence of GR and RUNX2 in the precipitates or in 15% of the input (in) was assessed by Western analysis with anti-GR and anti-FLAG antibodies, respectively.

Fig. 3. RUNX2 interacts with GR in living cells. ST2/Rx2^{dox} cells grown on cover slips were treated with dox and/or 1 μ M dex as indicated and subjected to confocal microscopy to image FLAG-RUNX2 (red), GR (green) and colocalization of both (yellow). Cells were stained with DAPI to visualize their nuclei.

Fig. 4. Dex inhibits RUNX2-stimulated genes to various degrees. ST2/Rx2^{dox} cells were treated for 48 h with dox and/or dex as depicted, and the mRNA levels of the indicated genes were measured by RT-qPCR (mean±SD, n = 3).