Published in final edited form as:

J Cell Physiol. 2017 January ; 232(1): 145–153. doi:10.1002/jcp.25399.

Glucocorticoids Hijack Runx2 to Stimulate *Wif1* for Suppression of Osteoblast Growth and Differentiation

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Abstract

Inhibition of Runx2 is one of many mechanisms that suppress bone formation in glucocorticoid (GC)-induced osteoporosis (GIO). We profiled mRNA expression in ST2/Rx2^{dox} cells after treatment with doxycycline (dox; to induce Runx2) and/or the synthetic GC dexamethasone (dex). As expected, dex typically antagonized Runx2-driven transcription. Select genes, however, were synergistically stimulated and this was confirmed by RT-qPCR. Among the genes synergistically stimulated by GCs and Runx2 was *Wnt inhibitory Factor 1 (Wif1)*, and *Wif1* protein was readily detectable in medium conditioned by cultures co-treated with dox and dex, but neither alone. Cooperation between Runx2 and GCs in stimulating *Wif1* was also observed in primary preosteoblast cultures. GCs strongly inhibited dox-driven alkaline phosphatase (ALP) activity in control ST2/Rx2^{dox} cells, but not in cells in which *Wif1* was silenced. Unlike its antimitogenic activity in committed osteoblasts, induction of Runx2 transiently increased the percentage of cells in S-phase and accelerated proliferation in the ST2 mesenchymal pluripotent cell culture model. Furthermore, like the inhibition of Runx2-driven ALP activity, dex antagonized the transient mitogenic effect of Runx2 in ST2/Rx2^{dox} cultures, and this inhibition eased upon *Wif1* silencing. Plausibly, homeostatic feedback loops that rely on Runx2 activation to compensate for bone loss in GIO are thwarted, exacerbating disease progression through stimulation of *Wif1*.

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Conflict of interests: None.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

The efficacy of pharmacological glucocorticoids (GCs) for the treatment of rheumatoid arthritis and other autoimmune and inflammatory diseases is blemished by metabolic adverse effects, including GC-induced osteoporosis (GIO) (Frenkel et al., 2015). A hallmark of GIO is extreme inhibition of bone formation. Early after commencement of GC administration, the inhibition of bone formation is associated with accelerated bone resorption, and some 10% of bone mass can be lost in the first year of treatment. But even later, when resorption rates drop below physiological levels, bone loss continues due to extremely low osteoblast number and function (Frenkel et al., 2015). Suppression of bone formation persists as long as GCs are administered, and rebounds with treatment cessation (Laan et al., 1993).

The master regulator Runx2 controls osteoblast differentiation and bone formation during both embryogenesis and postnatal life (Komori et al., 1997; Otto et al., 1997; Ducy et al., 1999; Adhami et al., 2015). GCs inhibit the Runx2-driven transcription of myriad target genes (Koromila et al., 2014). Suppression of the *Runx2* gene itself (Chang et al., 1998; Pereira et al., 2002) has also been reported, although it is likely secondary to post-translational inhibition of Runx2 activity (Koromila et al., 2014). Many other mechanisms compromise osteogenesis in GIO, most notably inhibition of the Wnt signaling pathway as well as down-regulation of growth factors and their receptors and downstream kinases (Frenkel et al., 2015). A question remains, however, why adaptive mechanisms that normally respond to bone loss with stimulation of osteoblastogenesis fail to stimulate bone formation in GIO.

Several steroid hormones have been shown to post-translationally inhibit Runx2 in the osteoblast and other cell lineages, potentially attributable to physical interaction with their respective nuclear receptors (Chang et al., 1998; Kawate et al., 2007; Khalid et al., 2008; Baniwal et al., 2009). Many exceptions, however, have been reported, including cases where androgens, estrogens, as well as 1,25 dihydroxyvitamin D3, cooperate with Runx2 in stimulating specific target genes (Paredes et al., 2004; Baniwal et al., 2012b). Using a prostate cancer cell model, we have recently demonstrated locus-dependent interaction between Runx2 and the androgen receptor (AR). While Runx2 and AR inhibit each other at most of their target genes, a minority of these genes is synergistically stimulated (Little et al., 2014). Here, we tested this concept for the case of the interaction between Runx2 and GCs in mesenchymal pluripotent cells. Among the minority of genes synergistically stimulated by Runx2 and GCs is *Wnt inhibitory factor 1 (Wif1)*, which may block biomechanical feedback loops triggered by bone loss in GIO and exacerbate disease progression.

The pivotal role of Wnt signaling in osteoblastogenesis and bone formation has been demonstrated by skeletal phenotypes associated with naturally occurring, as well as experimental mutations of various components of the pathway (Monroe et al., 2012; Baron and Kneissel, 2013). In the canonical Wnt pathway, Wnt ligands bind co-receptors composed of frizzled and low-density lipoprotein receptor-related protein (Lrp) 5/6 (Bhanot et al., 1996; Tamai et al., 2000). Receptor activation leads to the escape of β -catenin from a destruction complex that includes adenomatous polyposis coli (Apc), glycogen synthase kinase (Gsk3), and Axin. β -catenin then accumulates and translocates to the cell nucleus,

where it co-activates LEF/TCF target genes (Monroe et al., 2012; Baron and Kneissel, 2013). Activation of frizzled receptors by Wnt ligands can also affect LEF/TCF-independent cellular functions through activation of the non-canonical planar cell polarity and the calcium pathways (Liu et al., 1999). A variety of Wnt inhibitors that control canonical and/or non-canonical Wnt signaling have been implicated in bone mass control (Monroe et al., 2012; Baron and Kneissel, 2013), and several of them have been specifically implicated in GIO (Frenkel et al., 2015). These include Dkk1, which inhibits canonical Wnt signaling by targeting the Lrp co-receptor (Ohnaka et al., 2004), as well as the decoy Frizzled receptor *Sfrp1* (Wang et al., 2005). Like *Sfrp1*, *Wif1* functions as a decoy receptor and inhibits both canonical and non-canonical Wnt signaling (Hsieh et al., 1999). Our finding that Runx2 and GCs synergistically stimulate *Wif1* in pluripotent mesenchymal progenitor cells therefore provide a novel insight to GIO: feedback loops otherwise activated to promote Runx2-driven bone formation in response to skeletal weakening would now block osteoblastogenesis instead.

Materials and Methods

Cell culture

Construction of the murine ST2/Rx2^{dox} cell line with the doxycycline (dox)-inducible Runx2 cassette has been described (Baniwal et al., 2012a). Cells were cultured in RPMI-1640 (Mediatech, Manassas, VA) supplemented with 10% FBS (Gemini Bio-Products, West Sacramento, CA). For osteoblast differentiation, cultures at 90% confluency were treated with 50 µg/ml L-ascorbic acid (Sigma-Aldrich, St. Louis, MO) and 10 mM β-Glycerolphosphate (Sigma-Aldrich). Newborn mouse calvarial osteoblast (NeMCO) cultures were prepared from 2 days old newborn mice at the University of Tennessee Health Science Center (Memphis, TN) under an approved IACUC protocol and in accordance with the guidelines of the NIH (Guide for the Care and Use of Laboratory Animals, 1996). Parietal bones were excised, free of sutures, and then digested as previously described (Krum et al., 2008). Cells were maintained in alpha minimal essential medium supplemented with 10% FBS. Cells were transfected with pCMV-OSF2 (RUNX2, a gift from Dr. Gerard Karsenty) using the Nucleofector Kit (Lonza). Dox (Calbiochem, La Jolla, CA) and dexamethasone (dex, from Sigma-Aldrich) were added to culture media at final concentrations of 200 ng/ml and 1 µM, respectively, unless otherwise indicated.

Microarray analysis

ST2/Rx2^{dox} cells were treated with dox and/or dex for 48 h, and total RNA from quadruplicate experiments was extracted using AurumTM Total RNA mini-kit (Bio-Rad Laboratories, Inc., Hercules, CA) and subjected to expression microarray analysis using MouseRef-8 v2.0 Expression BeadChips (Illumina, San Diego, CA). Gene expression raw data processing was performed using GenomeStudio (Illumina). Differential gene expression was analyzed using Partek Genomics SuiteTM 6.4 (Partek, Inc., St. Louis, MO).

Quantitative RT-qPCR

Between 0.7 and 1 µg RNA, extracted as above, was reverse-transcribed using qScriptTM cDNA SuperMix synthesis kit (Quanta BioSciences, Inc., Gaithersburg, MD). Quantitative

Real Time PCR was carried out in triplicates using a CFX96 instrument (Bio-Rad Laboratories, Inc., Hercules, CA), the Maxima SYBR Green/Fluorescein Master Mix (Thermo Fisher Scientific, Waltham, MA), and the primers listed in Supplemental Table S1. Relative mRNA expression values were normalized to those of either 18S RNA (ST2 cells) or Gapdh mRNA (NeMCO), which themselves varied minimally across samples.

Western blot

Cell layers were washed with PBS and proteins were extracted using a 50 mM Tris-HCl lysis buffer (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and fresh protease inhibitor cocktail (Sigma-Aldrich). For detection of secreted Wif1, proteins were precipitated from 72-h conditioned medium using 20% trichloroacetic acid and suspended in the same lysis buffer. Protein concentrations were determined using Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Inc., Hercules, CA) and aliquots diluted in Laemmli buffer were subjected to 8% SDS-PAGE. Proteins were transferred to Amersham Hybond™-P PVDF membranes (GE Healthcare, Piscataway, NJ), and hybridized with either rabbit monoclonal antibodies against Wif1 (ab155101; Abcam, Cambridge, MA), rabbit polyclonal antibodies against RUNX2 (M-70; Santa Cruz Biotechnology, Inc., Dallas, TX) or goat antibodies against β -Actin (sc-1616; Santa Cruz Biotechnology, Inc., Dallas, TX). Proteins were visualized using HRP-conjugated donkey anti-goat (sc-2033) and goat anti-rabbit (sc-2004) antibodies from Santa Cruz Biotechnology and the Pierce ECL2 western blotting detection system (Thermo Scientific, Rockford, IL).

Gene silencing

For Wif1 silencing, specific Mission-shRNA lentiviral plasmids (TRCN0000089190 and TRCN0000089191) and a negative control shRNA plasmid (SHC002) were purchased from Sigma-Aldrich. For packaging, the plasmids were co-transfected into HEK293T cells along with helper plasmids pMD.G1 and pCMV. R8.91 using jetPRIME transfection reagent (Polyplus-transfection S.A., Illkirch-Graffenstaden, France). ST2/Rx2^{dox} cells were transduced with the lentiviral particles followed by selection with 5 μ g/ml Puromycin (Invitrogen, Carlsbad, CA) for at least 1 week.

Alkaline phosphatase (ALP) staining

ST2/Rx2^{dox} cells were treated as indicated for 6 days in 12-well plates and fixed in 4% paraformaldehyde for 2 min. Staining was performed using ALP detection kit (EMD Millipore Corporation, Billerica, MA) following the manufacturer's instructions and the ALP-positive area was quantified using the ImageJ64 software package.

Cell proliferation and cell cycle analysis

Cell proliferation was assessed using a colorimetric assay based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO). Cells were grown to 90% confluency in 48-well plates, treated as indicated, and then incubated with 0.5 mg/ml of MTT for 4h prior to lysis with DMSO. Absorbance was measured at a 595 nm wavelength using Victor3V™ plate reader (PerkinElmer, Shelton, CT). For cell cycle profiling, cells were stained with propidium iodide and percentage of

cells in G1, S, and G2/M was determined by flow cytometry using a BD LSRII flow cytometer (BD Biosciences, San Jose, CA) and the Modfit LT software package (Verity Software House).

Results

GCs synergize with Runx2 in stimulating a select gene set

In a previous study investigating modulation of several handpicked Runx2 target genes by GCs, dex attenuated to various extents dox-(Runx2)-driven gene expression in ST2/Rx2^{dox} cells undergoing osteoblast differentiation (Koromila et al., 2014). Taking a genome-wide approach, the present study confirms that GCs globally attenuate responses to Runx2 in the ST2/Rx2^{dox} model system. This is demonstrated by the volcano plot in Figure 1A, describing effects of dex on genes that were either stimulated or inhibited in response to dox alone. Specifically, genes stimulated by Runx2^{dox} alone (*red* in Fig. 1A), were generally inhibited in response to dex (in the presence of dox). Reciprocally, genes repressed by Runx2 alone were generally less repressed in the presence of dex (*blue* in Fig. 1A). The volcano plot also highlights a unique group of eight genes (Fig. 1A, *top right* and Supplemental Table S2), which were strongly stimulated by dex in the presence of dox (8-fold, $P < 1e-08$), even though most of them were not significantly affected by dox alone (indicated by *gray* color in Fig. 1A). While two of them (*Ehd3* and *BC022765*) were merely dex-responsive (Supplemental Table S2), the remaining were synergistically stimulated by the co-treatment with dex and dox (Supplemental Table S2). Thus, although GCs most commonly interact with Runx2 to attenuate its effects on target genes, our microarray analysis disclosed unusual cases of synergism, where Runx2 and GCs together strongly stimulate genes that respond to each of them relatively weakly or not at all.

We performed an independent, validation experiment by treating ST2/Rx2^{dox} cells with dox (to induce Runx2) and/or dex, followed by RT-qPCR analysis of genes that displayed in the microarray study the strongest Runx2-dependent dex-mediated stimulation. To further characterize the combinatorial regulation of these genes by Runx2 and dex, cells were treated with either 1 μ M (as in the microarray experiment) or 0.1 μ M dex for either 24 h, 48 h (as in the microarray experiment), or 72 h. As shown in Figure 1B–G, the RT-qPCR analysis confirmed the synergistic stimulation between Runx2 and dex. In some cases, such as *PscA* (Fig. 1B) and *Serpina3h* (Fig. 1D), dex synergized with Runx2 even though it had an inhibitory effect on its own. In other cases, for example, *C3*, the RT-qPCR analysis disclosed dex-mediated stimulation of gene expression in the absence of dox, but the stimulation was much stronger in the presence of dox (Fig. 1F). For almost every gene and every condition, with only two exceptions (Fig. 1C, 72 h and Fig. 1D, 24 h), the highest expression levels were observed in cultures co-treated with dox and dex. Thus, while generally antagonizing Runx2 (Fig. 1A), GCs synergize with Runx2 in stimulating select genes.

Robust synergistic stimulation of *Wif1* by Runx2 and GCs is unique among Wnt antagonists

Wif1 is one of the genes most strongly stimulated by cotreatment with dox (to induce Runx2) and dex (Fig. 1G). Thus, in the presence of pharmacological GCs in vivo,

stimulation of Runx2 may result in *Wif1*-mediated blockade of Wnt signaling, a pathway critical for cell replication, differentiation, and survival in the osteoblast lineage (Monroe et al., 2012; Baron and Kneissel, 2013). We therefore tested the co-regulation of *Wif1* by GCs and Runx2 at the protein level. ST2/Rx2^{dox} cells were treated with dox and/or dex for 72 h and *Wif1* was measured by Western blot analysis of the cell lysates and the conditioned media. *Wif1* protein was either undetectable or hardly detectable in the untreated cultures or after treatment with dox or dex alone, but was readily detectable in both the cell layer and the conditioned medium obtained from cultures co-treated with dox and dex (Fig. 2A). We next tested by RT-qPCR whether GCs and Runx2 cooperated in stimulating other Wnt antagonists previously implicated in GIO. *Dkk1*, which is stimulated by GCs in human and rat osteoblasts (Ohnaka et al., 2004; Mak et al., 2009), was not significantly stimulated by dex in ST2 mesenchymal progenitor cells (Fig. 2B). *Dkk2*, which is stimulated by GCs in primary NeMCO cultures (Gabet et al., 2011), was stimulated only transiently in ST2 cells and only at the 0.1 μ M dex concentration (Fig. 2C). Interestingly, Runx2 stimulated both *Dkk1* (11-fold at 48 h; Fig. 2B) and *Dkk2* (21fold at 48 h; Fig. 2C). However, dex did not augment, but in fact antagonized these Runx2 effects in ST2 cells. At the 72-h time point, expression of both *Dkk1* and *Dkk2* in the presence of dox returned to baseline. At this later time point, there was some evidence for cooperation between GCs and Runx2 (Fig. 2B and C), but not nearly at the magnitude observed with *Wif1* (Fig. 1G). GC-mediated stimulation of *Sfrp1*, which was observed in both rat and mouse osteoblast cultures (Wang et al., 2005; Mak et al., 2009), was absent or weak in ST2 cells, and the presence of Runx2, either alone or with dex, did not increase *Sfrp1* expression (Fig. 2D).

We further investigated the regulation of *Wif1*, *Dkk1*, *Dkk2*, and *Sfrp1* in Day-3 newborn mouse calvarial osteoblast (NeMCO) cultures. Cells were transiently transfected with a Runx2-expressing or a control vector and treated for 72 h with either dex or vehicle. As shown in Figure 3A–B by RT-qPCR and Western blot analysis, dex and Runx2 cooperated in stimulating *Wif1*. As with the ST2 culture model, RT-qPCR analysis demonstrates that such cooperativity is shared by neither *Dkk1*, nor *Dkk2*, nor *Sfrp1* (Fig. 3C–E).

We next examined the dependence of GC-mediated *Wif1* stimulation on Runx2 by determining the effect of dex on *Wif1* expression in more mature NeMCO cultures, in which endogenous Runx2 is turned on. Similar to previous reports describing inhibition of endogenous Runx2 by GCs (Chang et al., 1998; Pereira et al., 2002), dex down-regulated Runx2 protein levels in day-7 NeMCO cultures (Fig. 3F). However, consistent with a demonstrated anabolic effect that transient GC administration has on osteoblast cultures prior to their commitment to terminally differentiate (Leclerc et al., 2004), dex stimulated Runx2 expression in earlier, day-5 NeMCO cultures (Fig. 3F). Supporting the role of Runx2 in GC-mediated *Wif1* stimulation, dex no longer stimulated *Wif1* when Runx2 was down-regulated on day 7 (Fig. 3G). These results are in line with our conclusion from the ST2 culture system that GCs cooperate with Runx2 to stimulate *Wif1*.

GCs inhibit expression of neither *Axin2* nor *Ccnd1* in ST2 pluripotent mesenchymal cells

To investigate the role of *Wif1* in GC-mediated inhibition of Runx2-driven osteoblast differentiation, we engineered three ST2/Rx2^{dox} sub-lines constitutively expressing either a

nonspecific shRNA (*shNS*) or hairpin RNAs that target *Wif1* (*shWif1¹* or *shWif1²*). First, we documented by RT-qPCR analysis >90% decrease in *Wif1* mRNA in ST2/Rx2^{dox}/*shWif1¹* and ST2/Rx2^{dox}/*shWif1²* compared to ST2/Rx2^{dox}/*shNS* cells (Fig. 4A). As shown in Figure 4B, western blot analysis confirmed *Wif1* knockdown at the protein level.

We treated the three ST2/Rx2^{dox} sub-lines with dox and/or dex and measured expression of two canonical Wnt target genes, *Axin2* and *Ccnd1*, using RT-qPCR. As expected, *Wif1* silencing stimulated *Axin2* expression (Fig. 4C). Interestingly, dox stimulated *Axin2* expression in all three sub-lines (Fig. 4C). Consistent with the general attenuation of the Runx2 response (Fig. 1A and [Koromila et al., 2014]), dex attenuated, albeit weakly, the dox-mediated stimulation of *Axin2*, but surprisingly evidence for such attenuation was present only in the *Wif1*-depleted cells (Fig. 4C). These results suggest that GCs may employ pathways other than canonical Wnt signaling when inhibiting Runx2-driven osteoblast differentiation in the ST2 mesenchymal progenitor cells (Koromila et al., 2014). Further supporting this notion, dex did not attenuate dox-mediated stimulation of *Ccnd1* expression in this culture model (Fig. 4D). In fact, dex on its own stimulated *Ccnd1* expression, but interestingly this occurred only in *Wif1*-depleted cultures (Fig. 4D). The non-responsiveness to *Wif1* silencing may reflect Wnt-unrelated regulation of *Ccnd1* in ST2 cells (Fig. 4D).

***Wif1* is required for GC-mediated antagonism of Runx2-driven alkaline phosphatase (ALP) stimulation in ST2 cells**

To directly determine whether *Wif1* plays any role in GC-mediated inhibition of Runx2-driven osteoblast differentiation in the ST2 culture model (Koromila et al., 2014), we investigated the effect of dex on Runx2-induced ALP activity in ST2/Rx2^{dox}/*shWif1¹* versus ST2/Rx2^{dox}/*shNS* cells. Consistent with the reported effect of GCs in the parent ST2/Rx2^{dox} cell line (Koromila et al., 2014), dex inhibited ALP activity in the dox-treated ST2/Rx2^{dox}/*shNS* control sub-line by almost 70% compared to vehicle-treated cultures (Fig. 5). However, this inhibition amounted to less than 20% in the respective *Wif1*-depleted cultures (Fig. 5). Thus, despite the questionable effects of *Wif1* and of GCs on canonical Wnt signaling in ST2 cells (Fig. 4C and D), *Wif1* is clearly necessary for GC-inhibition of Runx2-driven osteoblast differentiation in this culture model.

***Wif1* silencing mitigates GC-mediated suppression of Runx2-driven proliferation in ST2 cells undergoing osteoblast differentiation**

Reflecting reciprocity between cell proliferation and differentiation, the osteoblast master regulator Runx2 has been shown to promote cell cycle exit (Pratap et al., 2003; Lucero et al., 2013). Because Runx2-mediated stimulation of *Ccnd1* expression (Fig. 4D) is inconsistent with this notion, we wondered whether it reflected a true mitogenic effect, and how it may be related to GIO and to *Wif1*. We treated control ST2/Rx2^{dox}/*shNS* cultures and the *Wif1*-depleted ST2/Rx2^{dox}/*shWif1¹* cultures with dox and/or dex and initially analyzed their cell cycle profiles. As shown in Figure 6A and Supplemental Table S3, dox treatment almost doubled the percentage of ST2 cells in S-phase. Although dex decreased neither basal nor Runx2-stimulated *Ccnd1* expression (Fig. 4D), it decreased the percentage of S-phase cells both at the baseline and in the presence of dox (Fig. 6A). Interestingly, the mitogenic and

anti-mitogenic effects of Runx2 and GCs, respectively, were observed in cultures harvested on day 3, but not on day 6 (Fig. 6A), consistent with developmental stage-dependent regulation of osteoblast proliferation and differentiation (Smith et al., 2000; Gabet et al., 2011; Mikami et al., 2011). Thus, Runx2 and GCs appear to play opposing, mitogenic, and antimitogenic roles, respectively, as mesenchymal progenitor cells initiate differentiation toward the osteoblast phenotype.

The mitogenic effect of Runx2 in day-3ST2 cell cultures was almost completely independent of *Wif1*, indicated by similar results obtained with the ST2/Rx2^{dox}/shNS and the ST2/Rx2^{dox}/sh*Wif1* sub-lines (Fig. 6A). The same was true for the antimitogenic effect of dex in the absence of Runx2 (Fig. 6A). However, whereas the percentage of S-phase cells in dox-treated ST2/Rx2^{dox}/shNS cells returned to near-control levels in response to dex, dex did not antagonize Runx2 with regard to cell cycle control in the *Wif1*-depleted cells (Fig. 6A). Consistent with the cell cycle profiling, MTT assays of ST2/Rx2^{dox} cells within the first few days of culture demonstrated that dex inhibited proliferation both in the presence and absence of dox, and that the inhibition in the presence of dox was partially mitigated in the ST2/Rx2^{dox}/sh*Wif1* compared to the ST2/Rx2^{dox}/shNS sub-lines (Fig. 6B). Thus, the synergistic stimulation of *Wif1* by Runx2 and GCs (Fig. 1G) appears necessary for GC-mediated inhibition both cell proliferation and differentiation in mesenchymal pluripotent cells undergoing commitment to the osteoblast phenotype.

Discussion

Attenuation of Runx2 activity is one of many mechanisms potentially contributing to GIO (Frenkel et al., 2015). At first glance, our finding of synergism with Runx2 seems inconsistent with previously reported effects of GCs (7–9). We show, however, that GCs modulate the transcriptional activity of Runx2 in a locus-specific manner. While GCs generally attenuate effects of Runx2 on gene expression (Fig. 1A), exceptions to this general trend are not uncommon (Fig. 1A). In fact, we submit that genes synergistically stimulated by Runx2 and GC signaling (Fig. 1B–G), in particular *Wif1*, may play a critical role in GIO. Diverse effects of GCs on Runx2 is not unlike the recently reported locus-specific modulation of Runx2 activity by estrogens (Chimge et al., 2012), androgens (Little et al., 2014), and 1,25-dihydroxyvitamin D3 (Stephens and Morrison, 2014), and is attributable in part to genomic distribution of their respective nuclear receptors relative to Runx2-occupied regions (Little et al., 2014).

Rapid bone loss that follows high-dose GC administration is due to both accelerated resorption and diminished formation (Frenkel et al., 2015). Importantly, adaption mechanisms normally activated by the ensuing mechanical overload seem to fail in GIO. Unlike postmenopausal osteoporosis, GIO is characterized by a blockade of bone formation, which can resume upon GC withdrawal (Laan et al., 1993). Extremely low bone formation rates despite bone loss, even in GC-treated children (Hansen et al., 2014), may be explained by synergism between Runx2 and GCs in stimulating anti-osteoblastogenic genes(s). Such synergism may constitute a vicious cycle, which prevents compensation for bone loss through Runx2-driven osteoblast differentiation (Fig. 7A). Other GIO mechanisms that inhibit osteoblast differentiation and function can be partially overcome, at least in principle,

by stimulation of Runx2 and recruitment of osteoblasts to compensate for their compromised function. In the presence of pharmacological GCs, however, the coupling of such mechanisms to *Wif1* stimulation would divert Runx2-driven osteoblastogenesis into a Runx2-driven blockade of Wnt signaling and thus exacerbation of GIO (Fig. 7A).

Antagonism of *Wif1* may therefore offer novel therapeutic approaches for the management of GIO. Although *Wif1* is normally expressed during osteoblast differentiation (Baker et al., 2015), no overt phenotype, either skeletal or otherwise, has been reported in *Wif1* knockout mice (Kansara et al., 2009). This normal baseline phenotype predicts little or no adverse reaction to in vivo administration of anti-*Wif1* reagents. On the other hand, implementation of such putative therapeutic approaches must proceed with caution given the demonstrated protective role of *Wif1* against osteosarcoma (Kansara et al., 2009; Baker et al., 2015).

GCs engage multiple mechanisms to inhibit Wnt signaling in the osteoblast lineage (Frenkel et al., 2015). Among them, canonical Wnt signaling is inhibited by direct stimulation of *Dkk1* expression (Ohnaka et al., 2004), indirect stimulation GSK3 β (Smith et al., 2002), and induction of transcription factors that interfere with β -catenin-mediated LEF/TCF activation (Smith and Frenkel, 2005; Almeida et al., 2007). It is conceivable that adaptive activation of Runx2 in pluripotent mesenchymal cells could have potentially compensated for compromised Wnt signaling. Such an adaptive mechanism could also ameliorate bone loss due to GC-mediated stimulation of *Sfrp1* (Wang et al., 2005), even though *Sfrp1* inhibits both canonical and non-canonical Wnt signaling. Like *Sfrp1*, GC-mediated *Wif1* induction is expected to block both canonical and non-canonical Wnt signaling (Hsieh et al., 1999). We envision at least two deleterious outcomes of *Wif1* secretion from stromal pluripotent cells initiating osteoblast differentiation in the bone marrow space. First, secreted *Wif1* might target in a paracrine manner dendritic processes projected from matrix-embedded osteocytes, the cells responsible for the bone anabolic effect of canonical Wnt signaling (Tu et al., 2015). This mechanism, however, is not unlike many others that could be at least partially overcome by Runx2-mediated stimulation of osteoblastogenesis. Through a second putative mechanism, however, secreted *Wif1* might target its producing pre-osteoblasts in a paracrine manner, blocking both canonical and non-canonical Wnt signaling. Compensatory stimulation of Runx2 in the producing cell would fail to mount an osteogenic response because a vicious cycle is now turned on to further increase *Wif1* biosynthesis and ablate both canonical and non-canonical Wnt-driven signals critical for osteoblast differentiation (Baron and Kneissel, 2013) (Fig. 7A).

Wif1 was among many genes previously reported to respond to GCs in vitro and in vivo (Yao et al., 2008; Naito et al., 2012). The present study assigns to *Wif1* a critically unique role in GIO based on its synergistic stimulation by GCs and RUNX2 and the resistance of *Wif1*-depleted cells to GC-mediated inhibition of Runx2-driven osteoblast differentiation (Fig. 5). *Wif1* also plays a role in GC-mediated antagonism of Runx2-driven cell proliferation (Fig. 6). Although Runx2 is traditionally considered an inhibitor of cell proliferation (Pratap et al., 2003; Galindo et al., 2005), its mitogenic property demonstrated here in ST2 cells is not without precedence (Chen et al., 2014). Mechanisms underlying the transient mitogenic effect of Runx2 in mesenchymal pluripotent cells initiating the osteoblast differentiation program remain to be explored. In particular, it would be important to clarify the potential role of the Wnt pathway. On one hand, a role for canonical Wnt

signaling is suggested by the stimulation of *Axin2* (Fig. 4C) and *Ccnd1* (Fig. 4D). Stimulation of these genes, however, is insensitive to GCs and the associated *Wif1* biosynthesis (Fig. 2A). Still, GCs antagonize the transient mitogenic effect of Runx2 in a *Wif1*-dependent manner (Fig. 6A), similar to their effect on Runx2-mediated induction of ALP (Fig. 5). Linkage between GC-mediated inhibition of osteoblast differentiation and a differentiation-related cell cycle has been noted previously (Smith et al., 2002). Furthermore, in order for GCs to inhibit osteoblast differentiation and the associated cell cycle, as well as to promote the linked adipogenesis in vitro, they must be administered early during phenotype development (Gabet et al., 2011; Smith et al., 2002; Mikami et al., 2011) (Fig. 7C). We speculate that the requirement for early GC administration is related to the transient nature of Runx2 expression/activity (Komori, 2010; Meyer et al., 2014; Yu et al., 2015); commencement of GC treatment at later time points does not inhibit development of the osteoblast phenotype possibly because Runx2 is down regulated (Fig. 7B).

In summary, this study validates previous observations of GC-mediated inhibition of Runx2, but more importantly it highlights exceptions where GCs do not inhibit, and even synergize with Runx2 in stimulating specific genes, including *Wif1*. Thus, induction of Runx2 in mesenchymal progenitor cells, intended to mount an osteogenic response to bone loss, would instead result in a Wnt blockade and exacerbation of GIO. Targeting *Wif1* and/or the interaction between Runx2 and GCs at multiple co-stimulated genes may lead to novel therapeutic approaches for the management of GIO.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

BF holds the J. Harold and Edna L. LaBriola Chair in Genetic Orthopaedic Research at the University of Southern California. The bioinformatics software and computing resources used in the analysis are funded by the USC Office of Research and the Norris Medical Library.

Contract grant sponsor: National Institutes of Health; Contract grant numbers: DK071122, DK071122S, AR064354.

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Fig. 1.

GCs synergize with Runx2 in stimulating a select gene set. (A) ST2/Rx2^{dox} mesenchymal pluripotent cells were treated for 48 h in quadruplicate with vehicle *control* (C), *dox* (to induce Runx2; D), the synthetic GC dex (1 μ M; G), or dox and dex together (DG). Global gene expression was profiled using the Illumina BeadChip platform and the response of genes to dex in the presence of dox is illustrated as a volcano plot, with red and blue symbols representing genes that were stimulated or repressed, respectively, by dox alone (D/C ≥ 2 or D/C ≤ -2 , respectively; FDR $P < 0.05$). Data representing genes that did not significantly respond to dox alone by these criteria are in gray. NC, no change. Triangles represent genes that were strongly stimulated by dex in the absence of dox (G/C ≥ 5 , FDR $P < 0.05$). Dashed lines highlight a set of 8 genes at the top right that were strongly stimulated (≥ 8 fold; $P < 1e-08$) by dex in the presence of dox. (B–G) ST2/Rx2^{dox} cells were treated for 24, 48, or 72 h with dox and/or dex at the depicted concentrations, and expression of the indicated genes was measured by RT-qPCR. Data are means and SDs ($n = 3$) relative to the control values at 24 h, defined as 1. [Color figure can be viewed at wileyonlinelibrary.com].

Fig. 2.
Robust synergistic stimulation of Wif1 by Runx2 and GCs is unique among Wnt antagonists. (A) ST2/Rx2^{dox} cells were treated for 72 h as indicated and Wif1 protein levels were assessed by Western blot analysis of either whole cell extracts (WCE) or conditioned media (CM). Western blot analysis of β -actin in the cell layer was carried out as control. (B–D) Expression of mRNAs for the indicated Wnt antagonists was measured as in Figure 1B–G. Data represent the means and SDs (n = 3) relative to the control values at 48 h (defined as 1).

Fig. 3.

Synergistic stimulation of *Wif1* by Runx2 and GCs in primary osteoblast cultures. Pre-osteoblasts isolated from newborn mouse calvariae were transiently transfected with RUNX2 or a control plasmid and then treated for 72 h with 1.0 μ M dex or vehicle. Expression of the indicated genes was assessed by either Western blot analysis of whole cell extracts (A) or (B–E). (F, G) Untransfected cells were treated with differentiation medium on day 2 of culture. Dex (1 μ M) or vehicle was added for 72 h before lysis, followed by Western blot analysis of Runx2 (F) and RT-qPCR analysis of *Wif1* (G) on days 5 and 7. Bars represent mean $\pm$ SD (n = 3).

Fig. 4.

Effect of *Wif1* silencing on Wnt target gene expression in ST2 cells expressing Runx2 and treated with GCs. *A–B*. ST2/Rx2^{dox} cells were transduced with lentiviruses encoding a nonspecific hairpin RNA (*shNS*) or either of two hairpins (*shWif1*¹ or *shWif1*²) targeting distinct region in *Wif1* mRNA. The derived shRNA-expressing sub-lines were treated for 72 h with dox and/or dex as indicated and *Wif1* expression was measured by RT-qPCR (*A*, Mean $\pm$ SD; *n* = 3) and by Western blotting (*B*). +/- signs indicate presence or absence of dox and dex in both *A* and *B*. The RNA data in *A* are corrected for 18S RNA and Coomassie blue-stained proteins that remained in the SDS-PAGE gels after transfer are shown as a loading control in *B*. (*C*, *D*) The RNAs from Figure 4A were subjected to RT-qPCR analysis of *Axin2* and *Ccnd1* (mean $\pm$ SD, *n* = 3).

Fig. 5.

Wif1 silencing mitigates GC-mediated suppression of Runx2-driven osteoblast differentiation. (A) ST2/Rx2^{dox} cells expressing a non-specific shRNA (*shNS*) or one that targets *Wif1* mRNA (*shWif1*) were treated for 6 days by dox and/or dex as indicated, and then subjected to ALP staining. (B) ALP activity was assessed as in A, and the stained area was quantified using the ImageJ software. Bars represent Mean $\pm$ SD values from three independent experiments, where the average stained area in the dox-treated cultures is defined as 100. * $P < 0.05$.

Fig. 6.

Wif1 silencing mitigates GC-mediated suppression of Runx2-driven proliferation in mesenchymal pluripotent cells undergoing osteoblast differentiation. Quadruplicate ST2/Rx2^{dox} cells expressing either *shNS* or *shWif1* as in Figure 4 were treated as indicated commencing one day after plating (defined as *Day 0*). (A) Percentage of S-phase cells was determined by propidium iodide staining and flow cytometry. (B) Cell growth rates were calculated based on MTT assays as the differences between the day 4 values and the respective day 0 values (just prior to commencement of treatment), or between the day 7 values and the respective day 4 values. * $P < 0.05$. Complete raw data from the FACS and the MTT assays are presented in Supplemental Tables S3 and S4, respectively.

Fig. 7.

GCs hijack Runx2. (A) Working model. *Black arrow* depicts adaptive circuits that enhance osteoblast-driven bone formation in response to bone loss (by any mechanism). GCs cause bone loss through a myriad of reported mechanisms (1), and at the same time synergize with Runx2 (2) to stimulate *Wif1*. Stimulation of Runx2 by homeostatic mechanisms is now hijacked to exacerbate GIO. (B) Schematic depiction of the transient expression of Runx2 at the initiation of osteoblast differentiation and the downregulation of Runx2 expression and/or genomic occupancy as the osteogenic program progresses (Komori, 2010; Meyer et al., 2014; Yu et al., 2015). C, Illustrative representation of results (Smith et al., 2000; Gabet et al., 2011; Mikami et al., 2011) suggesting that GCs do not inhibit osteoblast differentiation in vitro when administered at or after a time point that coincides with Runx2 down-regulation.