Glucocorticoid receptor-mediated cis-repression of osteogenic genes requires BRM-SWI/SNF☆

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Abstract
Glucocorticoids are an effective therapy for a variety of severe inflammatory and autoimmune disorders; however, the therapeutic use of glucocorticoids is severely limited by their negative side effects, particularly on osteogenesis. Glucocorticoids regulate transcription by binding to the glucocorticoid receptor (GR), which then binds the promoters of target genes to induce either activation or repression. The gene activation effects of nuclear hormone receptors broadly require the cooperation of the chromatin remodeling complex known as SWI/SNF, which is powered by an ATPase core. The well-studied SWI/SNF ATPase, BRG1, is required for gene activation by a spectrum of nuclear hormone receptors including GR. However, glucocorticoid-induced side effects specifically related to impaired osteogenesis are mostly linked with GR-mediated repression. We have considered whether cis-repression of osteogenic genes by GR may be mediated by a distinct subclass of SWI/SNF powered by the alternative ATPase, BRM. BRM does not have an essential role in mammalian development, but plays a repressor role in osteoblast differentiation and favors adipogenic lineage selection over osteoblast commitment, effects that mirror the repressor effects of GR. The studies reported here examine three key GR cis-repression gene targets, and show that GR association with these promoters is sharply reduced in BRM deficient cells. Each of these GR-targeted genes act in a different way. Bglap encodes osteocalcin, which contributes to normal maturation of osteoblasts from committed pre-osteoblasts. The Per3 gene product acts in uncommitted mesenchymal stem cells to influence the osteoblast/adipocyte lineage selection point. Fas ligand, encoded by FasL, is a means by which osteoblasts can modulate bone degradation by osteoclasts. Repression of each of these genes by glucocorticoids favors bone loss. The essential role of BRM in cooperation with GR at each of these control points offers a novel mechanistic understanding of the role of GR in bone loss.

1. Introduction
Glucocorticoids (GCs) are an effective therapy for a variety of severe inflammatory and autoimmune disorders; however, the use of glucocorticoids at pharmacological doses is severely limited by their negative side effects, particularly on osteogenesis (for recent reviews, see Moutsatsou et al., 2012; Manolagas 2013; Henneicke et al., 2014; Frenkel et al., 2015; Hartmann et al., 2016). Physiological levels of glucocorticoids promote skeletal growth and development, but pharmacological doses can cause osteoporosis, with fractures occurring in 30–50% of patients receiving extended GC therapy (reviewed in Hartmann et al., 2016). Glucocorticoids affect multiple cell types that impinge on bone formation and remodeling, but disruption of GC signaling in a cell-specific manner indicates that direct action of GCs on osteoblasts is the main basis for glucocorticoid–induced osteoporosis (O’Brien et al., 2004; reviewed in Henneicke et al., 2014 and Hartmann et al., 2016).

Glucocorticoids regulate expression of target genes by binding to the glucocorticoid receptor (GR). Upon binding, the GR translocates from the cytoplasm to the nucleus, where it homodimerizes and can bind to glucocorticoid response elements (GREs) in the promoters of target genes. Binding directly to positive GREs leads to upregulation of gene expression, whereas binding to negative GREs (nGREs) leads to transcriptional repression (reviewed in Moutsatsou et al., 2012). The glucocorticoid receptor may also influence gene expression indirectly by binding to other DNA-bound transcription factors. By means of these
effects on gene expression – direct and indirect, positive and negative – the glucocorticoid receptor mediates increased transcription of anti-inflammatory genes and decreased expression of pro-inflammatory genes. The anti-inflammatory effects are believed to arise from indirect repression of transcription factors that promote inflammation, as well as direct activation of anti-inflammatory gene expression.

In contrast, the side effects that specifically relate to impaired differentiation and maturation of osteoblasts are mostly linked with GR-mediated repression (Moutsatsou et al., 2012). In particular, the glucocorticoid receptor has been shown to target nCREs directly in the promoter of the osteogenic gene, Bglap (Aslam et al., 1995; Shallhoub et al., 1998), which encodes osteocalcin, a key component of bone extracellular matrix and a physiological marker of osteoblast activity. In addition, pharmacological doses of glucocorticoids favor diversion of mesenchymal stem cells to the adipocyte lineage rather than the osteoblast lineage (reviewed in Hennicke et al., 2014; Hartmann et al., 2016). This includes transactivation effects on pro-adipogenic genes, but glucocorticoid-mediated repression plays an important role as well by targeting inhibitors of adipogenesis. A well-characterized example is the Per3 gene product, which forms an inhibitory complex with the pro-adipogenic transcription factor PPARγ, and is repressed (directly or indirectly) with glucocorticoid treatment (Costa et al., 2011).

We have considered whether cis-repression by GR may be mediated by a specific subclass of the SWI/SNF chromatin-remodeling complex. Mammalian SWI/SNF is recognized as a necessary effector for the general transcription-activating effects of the nuclear hormone receptor family including GR (King et al., 2012). The complex uses the energy of ATP hydrolysis to remodel chromatin to permit or preclude promoter access for various transcription-regulating factors. Mammalian SWI/SNF exists as subclasses, powered by either of two core ATPases. One is the BRM ATPase, which was named as an analog of the Dro sophila protein, brhma. The second is BRG1 (the product of BRM-related gene-1). Though the two ATPases are closely related, their requirement in development is very different. Mice lacking BRG1 die early in embryogenesis (Bultman et al., 2000), while BRM-null mice are viable and fertile and live full life spans (Reyes et al., 1998). Consequently, BRG1 has been widely studied, and among other findings, appears to be essential for the transcription activation effects of nuclear hormone receptors, including the glucocorticoid receptor (Clapier and Cairns 2009, McKenna et al., 1999). On the other hand, the involvement of SWI/SNF in nuclear hormone receptor mediated repression has not been broadly addressed, but evidence outlined below suggests that the BRM ATPase may play a role in GR-mediated repression.

Despite the limited role of BRM in development, conservation of this alternative ATPase across all higher eukaryotes argues that BRM enacts biologically significant functions. Evidence that BRM has an important influence on lineage fate comes from studies of gene expression specific to osteoblasts and adipocytes. BRM is not essential for development of either lineage, but BRM depletion in mesenchymal stem cell precursors impairs adipogenic gene expression and favors commitment to the osteoblast lineage. This manifests in BRM-null mice as resistance to age-related osteoporosis with reduced bone marrow adiposity (Nguyen et al., 2015). BRM depletion releases repression of key osteoblastic genes, including the osteocalcin gene Bglap, whose promoter is occupied directly by BRM-SWI/SNF prior to induction (Flowers et al., 2009). BRM-depleted cells are unable to maintain effective co-occupation of the Bglap promoter by repressor factors that include repressor members of the E2F transcription factor family and their binding partner p130, as well as histone deacetylase-1 (HDAC1) (Flowers et al., 2011). There is a striking correlation between the effects of BRM and the biological effects of glucocorticoids on bone, including the evidence that GR and BRM both target the osteocalcin promoter directly to repress transactivation. Moreover, a recent ChIP-seq approach linked BRM with GR-mediated repression, in direct contrast to the general co-activating role of BRG1 (Engel and Yamamoto, 2011). This analysis was performed in tumor cells, but is likely to be more widely applicable. Given this background, we have investigated the possibility that BRM plays a specific role in GR-mediated repression of osteogenesis.

2. Results and discussion

2.1. BRM depletion blocks glucocorticoid-mediated repression of osteocalcin (Bglap) gene expression

The MC3T3-E1 murine calvarial cell model (Kodama et al., 1981; Sudo et al., 1983; Kartsogiannia and Ng, 2004) was used here to assess the role of BRM in glucocorticoid-mediated repression of gene expression. As a chromatin-remodeling complex, BRM-SWI/SNF acts directly by promoter association. The best-characterized gene target of glucocorticoid-mediated repression in osteoblasts is osteocalcin, a key marker of late-stage osteoblast differentiation. Expression of the osteocalcin-encoding gene (Bglap) is negatively affected by glucocorticoid treatment in vitro (Strömstedt et al., 1991). We showed previously that induction of osteocalcin gene expression is accelerated in BRM-deficient osteoblasts (Flowers et al., 2009), raising the possibility that glucocorticoid-mediated repression of osteocalcin expression may be dependent on BRM. Analysis by quantitative RT-PCR (qRT-PCR) in parental cells shows typical robust induction of osteocalcin expression at days 7 and 14 post-induction with differentiation medium (Fig. 1A). Treatment with dexamethasone blocks induction almost completely in the parental pre-osteoblasts. However, the BRM-depleted cells show accelerated induction of osteocalcin gene expression that is largely resistant to the suppressive effect of dexamethasone. A parallel experiment comparing cells depleted for the BRG1 ATPase shows BRG1 required for normal transactivation of the osteocalcin gene as expected. Notably, repression of the osteocalcin gene by dexamethasone remains effective in the BRG1-depleted cells, emphasizing that repression via BRM in cooperation with GR is distinct from the activation effect of BRG1.

2.2. BRM is required for efficient GR promoter access in the regulation of osteocalcin expression

Very little is known about specific gene targets of GR repression in osteoblasts, but DNase protection assays indicate GR acts on Bglap by direct cis-repression (Strömstedt et al., 1991; reviewed in Moutsatsou et al., 2012). The osteocalcin gene is also a direct target of BRM during repression (Flowers et al., 2009), but it is not known whether GR targeting depends on BRM. We addressed this question using chromatin immunoprecipitation (ChIP) analysis (Fig. 1B). The results show that GR targets the osteocalcin promoter directly in non-induced MC3T3-E1 pre-osteoblasts when osteocalcin expression is repressed; however, in BRM-depleted cells incubated in the same conditions, GR is undetectable at the promoter. The agarose gel images demonstrate the end-point PCR products directly, and quantitative analysis confirms the loss of GR and BRM. As end-point PCR is more informative in this context, this was used in subsequent assays.

In pre-osteoblasts, osteocalcin gene expression is normally repressed, and the action of GR in Fig. 1B is supported by endogenous ligand. The promoter association patterns of GR and BRM were also examined in cells induced to osteoblast differentiation in the presence or absence of exogenous dexamethasone at pharmacological levels (Fig. 1C). By day 14 post-induction in parental cells in the absence of dexamethasone, GR and BRM have both dissociated from the promoter. In the presence of exogenous dexamethasone, GR remains on the promoter at day 14 despite the differentiation-induction signals, and BRM remains present as well, implying that BRM is recruited by GR. In cells depleted of BRM, GR is not detected at the promoter prior to induction, and even exogenously added dexamethasone is not sufficient to establish GR at the promoter. Because BRM-SWI/SNF acts at the level of transcription, there is a possibility that the failure to detect GR at the promoter in BRM-depleted cells might be caused by loss of expression of the GR-
encoding gene (Nr3c1). However, qRT-PCR analysis shows that expression of Nr3c1 is not significantly affected by depletion of BRM or by induction of dexamethasone (Fig. 1D). Similar results were shown previously for BRM and BRG1 (Nguyen et al., 2015). The requirement for BRM-SWI/SNF to mediate GR access to the promoter provides a direct biochemical mechanism for the gene expression results in Fig. 1A.

2.3. BRM effects are coupled with GR during lineage determination in a mesenchymal stem cell model

BRM and glucocorticoids both play important roles in fate determination in multipotent stem cells. The C3H10T1/2 cell model of mesenchymal stem cells permits examination of whether the roles of GR and BRM overlap during lineage determination, and we have previously characterized a stable BRM-depleted derivative of this cell line (Nguyen et al., 2015). Glucocorticoids cause mesenchymal stem cells to favor adipogenesis over differentiation along the osteoblast lineage (reviewed by Henneicke et al., 2014; Hartmann et al., 2016). This often involves transactivation of adipogenic genes, but an essential step in advancing mesenchymal stem cells to the adipogenic fate is glucocorticoid-mediated repression of the Per3 gene, which encodes a strong inhibitor of adipogenesis. (Period 3 was originally classified as a circadian rhythm gene product, but has only minimal effects on circadian rhythmicity; Shearman et al., 2000). Following microarray identification of Per3 as differentially expressed in C3H10T1/2 cells exposed to glucocorticoids (So et al., 2008), moderate overexpression of Per3 (circa 5-fold) was found to potently inhibit adipocyte differentiation in mesenchymal stem cells, while Per3 knockout mice showed increased adipogenesis (Costa et al., 2011). These authors showed further that the Period 3 protein forms a complex with PPARγ and inhibits PPARγ-mediated transcriptional activation. Thus, glucocorticoids repress Per3 expression, and repression of Per3 favors selection of the adipocyte lineage in mesenchymal stem cells. Whether Per3 is a direct target of GR has not yet been determined.

We used the C3H10T1/2 cell model to ask whether Per3 is a direct target of GR and/or BRM. ChIP analysis (Fig. 1E) shows that both proteins occupy the promoter, and depletion of BRM shows that BRM is required to maintain GR access to the promoter. To address whether the
effects of BRM deficiency are sufficient to de-repress Per3, expression of Per3 was assessed by qRT-PCR. The results show Per3 expression elevated nearly 6-fold in the BRM depleted background (Fig. 1F), an expression level similar to that which inhibited adipocyte differentiation in the experiments discussed above (Costa et al., 2011). In summary, glucocorticoids cause mesenchymal stem cells to favor adipogenesis over differentiation to an osteoblast phenotype (Henneicke et al., 2014; Hartmann et al., 2016), in part by mediating repression of Per3 (Costa et al., 2011). The current results show GR targets the Per3 promoter directly, and neither GR targeting of Per3, nor repression of Per3, is maintained in conditions of BRM deficiency.

2.4. GR promoter access to Fasl and inhibition of Fasl expression is BRM-dependent

Direct action of glucocorticoids on osteoblasts appears to be the main basis for GC-induced osteoporosis (O’Brien et al., 2004; reviewed in Henneicke et al., 2014), but osteoblasts also have indirect effects on osteoclasts (the cells that resorb bone). Among the signaling molecules expressed by osteoblasts is the Fas ligand, encoded by the Fasl gene. Binding of secreted Fas ligand to receptor-bearing cells activates apoptotic functions. Fasl expression has mostly been studied in (activated) T cells, where expression is strongly inhibited by glucocorticoids and the Fasl promoter is targeted directly by GR for repression (Baumann et al., 2005; Novac et al., 2006). Fasl is also expressed by osteoblasts, and because osteoclasts express Fas ligand receptors and are susceptible to Fas ligand-induced apoptosis, expression of Fasl by osteoblasts is a means by which osteoblasts can decrease osteoclast numbers (Krum et al., 2008). Glucocorticoid-mediated repression of Fasl expression in osteoblasts would permit increased osteoclast numbers and excessive bone resorption (Fig. 2A).

We examined Fasl expression in differentiating MC3T3-E1 osteoblasts, asking whether the level of expression is affected by BRM depletion (Fig. 2B). This analysis shows that Fasl expression increases dramatically during osteoblast differentiation, and that induction is sharply inhibited when the cells are grown in the presence of dexamethasone. Parallel analysis in BRM-depleted cells shows robust induction of Fasl expression, and strong resistance to the inhibitory effect of dexamethasone. ChIP assays were performed to assess promoter occupation (Fig. 2C). These assays show the presence of GR on the Fasl promoter in differentiating parental cells treated with pharmacological levels of dexamethasone, but no detectable presence of GR on the promoter in BRM-deficient cells treated in parallel. Together these results indicate that GR, in the presence of exogenous glucocorticoid signals, acts directly in cis to inhibit expression of Fasl. This disruption to a normal feedback mechanism by which osteoblasts can potentially limit the activity of osteoclasts is highly dependent on BRM.

2.5. BRM depletion blocks the negative effect of glucocorticoids on osteoblast differentiation to a mineralization phenotype

These studies identify BRM as a required factor for direct GR-mediated cis-repression at promoters whose gene products favor osteoblastogenesis. GR presumably has additional direct and indirect repression targets in osteoblasts and osteoblast precursors that are not yet known. If repression of any critical glucocorticoid target were independent of BRM, glucocorticoids would be expected to prevent osteoblast maturation even in BRM-deficient cells. Therefore, to assess the overall biological significance of BRM with respect to the glucocorticoid effect in osteoblasts we asked whether BRM-depleted cells are able to achieve the extracellular matrix mineralization phenotype of a mature osteoblast in the presence of dexamethasone. The mineralization end-point can be detected by biological staining with Alizarin Red. This assay (Fig. 2D) verifies that dexamethasone at the pharmacological concentrations used here impairs progression of pre-osteoblasts to the mineralization phenotype. Alizarin Red staining in parental cells not exposed...
to dexamethasone is readily observable by day 21; however, the dexamethasone-treated cells show only weak staining even at day 28. BRM-depleted MC3T3-E1 cells were previously shown to undergo enhanced mineralization (Flowers et al., 2009). Results here show that this enhanced mineralization phenotype is maintained or even heightened in the presence of dexamethasone. The robust mineralization phenotype implies that the critical repressive effect of glucocorticoids on osteoblast maturation are largely relieved when BRM is depleted.

2.6. Conclusions

Pharmacological levels of glucocorticoids exert their negative effects on skeletal integrity through multiple pathways. The three direct GR targets examined here each act in a different way. Osteocalcin functions in normal maturation of osteoblasts from committed pre-osteoblasts. The Per3 gene product acts in uncommitted mesenchymal stem cells to influence the osteoblast/adipocyte lineage selection point. Fas ligand is a means by which osteoblasts can modulate bone degradation by osteoclasts. Repression of these genes by glucocorticoid signals would favor bone loss because pre-osteoblasts would not mature properly, while fewer mesenchymal stem cells would commit to the osteoblast lineage, and rising numbers of osteoclasts would degrade more bone. The essential role of BRM in promoter targeting by GR at each of these control points reveals an intimate functional association between GR-mediated repression and BRM-SWI/SNF in osteogenesis. BRM may be a potential therapeutic target for blocking the negative effects of glucocorticoids on bone. In addition, because BRM-SWI/SNF acts at the level of chromatin remodeling, BRM deficiency releases multiple repressors other than GR from osteogenic genes (Flowers et al., 2009, 2011) and reduces age-related osteoporosis (Nguyen et al., 2015), so targeting BRM could be beneficial in helping to replace bone loss in general, even subsequent to glucocorticoid treatment.

3. Materials and methods

3.1. Materials

Puromycin, Alizarin Red S and dexamethasone were obtained from Sigma Chemical Co. (St. Louis, MO); fetal bovine serum (FBS) from Atlanta Biologicals; Trizol from Thermo Fisher (Waltham MA).

3.2. Cell lines and culture

Culture and differentiation of low passage MC3T3-E1 pre-osteoblasts by exposure to differentiation-induction medium containing ascorbic acid and β-glycerol phosphate were described previously (Beck et al., 2001). Generation of the MC3T3-E1-derived BRM-deleted cell line BRM-Seq1.GG5 and the BRG1-depleted cell line BRG1.D16, as well as staining with 0.1% Alizarin Red to reveal mineralization were also described previously (Flowers et al., 2009). For glucocorticoid treatment, cell medium was supplemented with dexamethasone at a concentration of 1 μM per ml; the medium was changed every 3–4 days and supplemented with fresh dexamethasone at each change for the duration of the experiments. Propagation of C3H10T1/2 cells and characterization of the BRM-depleted line CD606 derived from the C3H10T1/2 cell line were reported earlier (Nguyen et al., 2015).

3.3. RNA expression

RNA expression was evaluated by quantitative reverse transcription and PCR (qRT-PCR). Each assay was performed in triplicate and the results shown are an average of at least three independent trials. The qRT-PCR methodology and the primers for Gapdh and Bglap (osteocalcin) were described previously (Flowers et al., 2009). The FasL and Nr3c1 (GR) primer sequences were designed by Origene (Rockville, MD); the Per3 primer sequences were taken from Costa et al. (2011); the sequences are:

| Gene | Forward Primer | Reverse Primer |
|------|----------------|----------------|
| FasL | 5′-GAAG ACT GCC CAC GAG ACT CCG T-3′ | 5′-GCC ACA TTC CTC GGC TCT TTT T-3′ |
| Nr3c1 (GR) | 5′-TGG AGA GGA CAC CTT GAC TTC C-3′ | 5′-ACG GAG GAG AAC CAT CAC G-3′ |
| Per3 | 5′-CTG ACA CCA GAG TCC CAT GAA G-3′ | 5′-AGT GCC CTG GAA CAC AGG-3′ |

3.4. Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed as described previously (Flowers et al., 2014). Individual protein associations were typically analyzed at least twice in independent experiments, and at two discrete time points in differentiating cells. Differentiation was monitored with parallel biological assays. Pre- and post-induction samples were always assayed in parallel. The knockdown lines provide additional controls, particularly for contrasting results. Quantification was performed in triplicate on three independent samples and normalized using the percent input method. The following antibodies were obtained from Santa Cruz Biotechnology: BRM (sc-6450), BRG1 (sc-10768), and glucocorticoid receptor (sc-1002). Normal mouse IgG is a component of the EZ ChIP system (Upstate Cell Signaling Solutions: Lake Placid, New York). The primers for Bglap (osteocalcin) were described previously (Flowers et al., 2009). The FasL primer sequences were taken from Baumann et al. (2005); the sequences of the FasL and Per3 primers used here are:

| Gene | Forward Primer | Reverse Primer |
|------|----------------|----------------|
| FasL | 5′-CTG AAC TCA AAT CCA ACG CAG AG-3′ | 5′-AAC TTG GAC AGG AGT TT-3′ |
| Per3 | 5′-TGGG GAG AAA CCA ACA AGT TT-3′ | 5′-AGT GCC CTG GAA CAC AG-3′ |

Potential conflict of interest

S. Flowers and E. Moran are authors on US Patent 8415316, whose value could be affected by publication.

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