Lessons from Glucocorticoid Receptor Action in Bone: New Ways to Avoid Side Effects of Steroid Therapy

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1 Introduction

Glucocorticoids (GCs) as anti-inflammatory agents proved successful for the first time in patients suffering from rheumatoid arthritis (RA) in 1948 [1] and this finding was awarded with the Nobel prize in 1950. RA is currently one of the most investigated diseases in the emerging research area "osteoimmunology" addressing the interactions between bone and hematopoietic tissues. RA is still treated with GCs in combination with disease modifying antirheumatic drugs (DMARDs) [2] to ameliorate the most pro-inflammatory boosts. Soon it became evident that steroid therapy is hampered by a multitude of side effects acting on metabolism, cardiovascular system, and tissue integrity. A general loss of bone at long-term GC treatment is considered to be one of the major complications. To improve steroid therapy and avoiding bone loss in RA, a detailed understanding of the cellular and molecular mechanisms is required. Work from our laboratory and others shed some light into the molecular mechanisms implicated in GC action in suppression of inflammation and GC-induced bone loss. We review here these recent advances and also define new criteria for selective acting GCs that avoid GC-induced bone loss, but may retain therapeutic potential.

1.1 The Glucocorticoid Receptor

GCs, such as the endogenous secreted hydrocortisol or corticosterol as well the synthetic GCs prednisolone, dexamethasone, and others bind to a member of the

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nuclear receptor family, the glucocorticoid receptor (GR). The GR is composed of an N-terminal transcriptional activation domain (AF-1) followed by a two Zn finger containing DNA binding domain (DBD), a short hinge region, the ligand binding domain (LBD), and a C-terminal transactivation domain (AF-2) [3]. The GR resides in the absence of ligands in the cytoplasm and is associated with a complex of chaperonic molecules composed of heat shock proteins and so called immunophillins. Hypothalamo–pituitary–adrenal axis triggered or pharmacologically applied GCs diffuse through the cell membrane. Within the cells 11-β-hydroxysteroid dehydrogenase type 1 (11-β-HSD1) converts the inactive GCs, such as cortisone and corticosterone to their active forms cortisol and corticosterol, respectively. Active GCs bind to the high affinity GR-heat shock protein complex [4]. Upon binding this complex disrupts and allows the released GR molecule to interact in the cytoplasm with signal transduction components, such as JNK, PI3K, 14-3-3 proteins or in T cells with the T cell receptor associated kinases lck and fyn [3]. The majority of the GR molecules translocate to the nucleus, facilitated by HSP90, the co-chaperone immunopholin FKBP52 [5], importin alpha/beta, and importin 7 [6]. Within the nucleus the GR executes two major activities: binding as a homodimer to palindromic response elements (GRE) of GC-induced genes and associating to sites of proinflammatory transcription factors. Homodimerization of the GR at the DNA is mediated by dimerization motifs within the 2nd Zn finger of the DBD [7], dimerization interfaces at the LBDs and presumably by LBD/DBD interaction as shown for other nuclear receptors [8]. The transactivation domains AF-1 and AF-2 of DNA bound GR serve as platforms for the recruitment of coactivators. Chromatin remodeling complexes of the SWI/SNF/Brg1 family are interacting with AF-1 in a ligand independent manner. In contrast AF-2 recruits proteins of the p160 family and subsequently coactivators such as CBP/p300 only in the presence of ligand. The kind of coactivators are recruited further depends on the conformational change of the GR influenced by the palindromic DNA binding sequence itself. Different GRE sequences lead to different transactivation capacities [9]. Recent global chromatin-immunoprecipitation sequencing combined with studies of fluorescence-tagged GR molecules at a high time resolution shows a complex dynamic behavior of GR DNA occupancy [10]. The binding of the GR seems to follow the oscillating GC release during the day, which is different from permanent nuclear residing GR by high affine synthetic GCs. Thus a comprehensive picture of GR DNA binding activity—so far only defined for a limited amount of cell lines—is just being unraveled. These experiments once performed in mesenchymal and hematopoietic cells will give fundamental insights into gene regulation of GC effects in osteoimmunological processes.

The second mode of GR activity, the interaction with already DNA bound transcription factors had attracted much attention over the last two decades as one of the major mechanisms of immune suppression by the GR [3]. In particular the GR represses proinflammatory molecules based on tethering to AP-1 [11], NF-κB [12], or IRF-3 [13]. These interactions occur in the presence of integrator proteins like thyroid hormone receptor interactor 6 (TRIP6) [14] or SRC1 and TIF2-associated binding protein (STAMP) [15]. The GR is supposed to prevent the recruitment of coactivators to NF-κB [16] or to inhibiting RNA-polymerase II phosphorylation on

the C-terminal domain by recruitment of phosphatases [17]. In addition the GR inhibits Toll-like receptor signaling via sequestration of the p160 protein GRIP1 from IRF3 and STAT1 sites, thereby interfering with their transactivating activity [13, 18, 19]. Due to the plethora of pro-inflammatory mediators, such as cytokines, enzymes and adhesion molecules that are under the control of the aforementioned transcription factors it became almost a dogma in the field that immune suppression of GCs solely depends on this tethering mode of nuclear GR action. To which extend this dogma holds and has to be modified will be discussed below.

1.2 Selective Glucocorticoid Receptor Modulators

Based on the two major nuclear mechanisms of GR action—binding as a homodimer and tethering as a monomer towards pro-inflammatory transcription factors—pharmaceutical companies started an intensive search for dissociating GR ligands that exclusively address the monomer function of the GR [20]. Such ligands should maintain anti-inflammatory efficacy, but avoid side effects which were attributed to the GR dimer. So far only for the regulation of enzymes involved in glucose metabolism the requirement of the GR dimer was clearly demonstrated, at the time these programs were launched. Thus, screening programs were mainly based on GRE-, AP-1-, or NF-κB-dependent reporter gene assays. Compounds were identified that exert a certain GR affinity and failed to induce GRE driven reporters, but maintained transrepression of AP-1- and/or NF-κB-dependent reporter genes. Whereas the first compounds did not maintain their dissociative properties in vivo [21], other substances, including AL-438 [22], "compound A" [23], LGD-5552 [24], ZK 216348 [25], and ZK 245286 [26] continued to be successful in some inflammatory assays in rodents, such as phorbol ester-induced skin irritation, air pouch-induced inflammation, contact allergy, and EAE [27, 28]. CpdA also proved therapeutically successful in an example of inflammatory bone disease, collagen-induced arthritis [29].

Concerning side effects the AL-438 [22], CpdA [23], and ZK 216348 [25] failed to induce glucose levels and to decrease glucose tolerance in rodent models in comparison to the full GR agonist dexamethasone. The investigated selective GR modulators SEGRMs spared effects on thymus weight, adrenal weight, and bone growth [20]. Little has been done on the effects of SERGMs on primary bone cells and bone integrity. Only for AL-438 and LGD-5552 a lack of bone formation inhibition was reported for rats [22, 24]. In tissue culture cells a reduced repression of the RANKL/OPG ratio in comparison to the classical agonists had been observed for AL-438 and ZK 216348 [30]. This might indeed lead to a decreased osteoclastogenesis triggered by osteoblastic cells, which remains to be investigated.

So far, however, the SEGRMs were only defined on a few selected reporter genes and therefore it is not entirely clear, how selective they are in other promoter contexts and whether they act in a tissue selective manner in vivo. To solve this issue, even for classical GR action, target tissues and molecular mechanisms have to be defined.

2 Cell Type Specific Molecular Mechanisms of Anti-inflammatory Actions of the GR

To define which molecular action of the GR, homodimerization or the monomeric activity contributes to anti-inflammation and to GC-induced bone loss as the major side effect we utilized an approach using conditional mutant mice of the GR.

For the identification of cell types critical for anti-inflammatory effects we utilized mice with a conditional GR allele with exon 3 flanked with loxP sites. These mice are suitable for recombination by transgenic cre expression to create a conditional GRnull allele in selected cell types [31, 32]. This strategy has the advantage to overcome the perinatal lethality in complete GR knockout mice [32, 33] to address GR function in adult animals. Using this approach we could first define for GC treatment of contact allergy in the skin using a murine model of contact hypersensitivity (CHS) that antigen presenting dendritic cells, keratinocytes and—interestingly—T cells were not critical for GC therapy. Myeloid cells however, when devoid of the GR in vivo in $GR^{LysMCre}$ mice rendered resistant to GC suppression of the inflammatory response in CHS [34]. Myeloid cells including neutrophils and macrophages turned out to be also critical for the actions of endogenous GCs in septic shock experiments [35]. In contrast in experimental encephalitis the GR in T cells was most critical for GC actions. Mice lacking the GR in T cells in GR^{LckCre} mice had a stronger disease progression due to impaired action of endogenous GCs and a diminished response to GC therapy. $GR^{LysMCre}$ mice were comparable to wild-type animals in their response to GCs [36]. Thus, these data demonstrate that the cell type most important to execute an anti-inflammatory effect of GCs depends on the type of inflammation investigated.

The type of inflammatory response does not only require the GR in different cell types but also dictates whether the monomer GR is sufficient for immune suppression. To discriminate between dimerization dependent and independent action of the GR in vivo, mice with a functional GR mutation abrogating the dimerization of the receptor were generated (GR^{dim}). A458T substitution in the 4th exon encoding the 2nd zinc finger disrupts the GR dimerization interface of the DBD. Interestingly, these mice are viable despite loosing the dimerization dependent DNA binding. Furthermore they preserve tethering of the monomeric GR in particular for the repression of AP-1 and NF-κB activity [37, 38]. As expected from the current dogma that anti-inflammatory effects of GCs rely on the monomer function of the GR, GR^{dim} mice were fully responsive to suppression of phorbol ester-induced inflammation and of AP-1-mediated Mmp13 expression in skin [38, 39]. Surprisingly, in contact hypersensitivity it was shown that GR^{dim} mice were not treatable with glucocorticoids revealing a potent anti-inflammatory action of the dimerized DNA bound GR in vivo [34]. Thus, the classical dogma that GR DNA binding is not involved in immunosuppression cannot be applied to all inflammatory conditions. This is further supported by the identification of GC-induced anti-inflammatory genes such as glucocorticoid-induced leucine zipper (GILZ) [40], annexin A1 [41], or mitogen-activated protein kinase phosphatase 1 (MKP1/DUSP-1) [42, 43].

Indeed, DUSP-1 knockout mice failed to respond to GCs in the zymosan-induced air pouche model [44].

We just recently uncovered the requirement for GR dimerization in a model of rheumatoid arthritis (Baschant and Tuckermann, unpublished). Thus, although so-called dissociating SEGRMs are presumably therapeutically efficative in RA models, we clearly show here by genetic in vivo evidence that GR dimerization is involved in anti-inflammatory activities. This can be possibly explained by recent findings that SEGRMs are capable to induce the GR dimer-dependent anti-inflammatory acting gene *Dusp1* in some inflammatory cell types (Egene Jeanny poster-abstract on BES meeting 2010; http://www.endocrine-abstracts.org/ea/0021/ea0021p361.htm). This strongly suggests that SEGRMs are not fully dissociative concerning DNA dimerization versus non-dimerization in anti-inflammatory aspects, but they rather may induce differential coactivator recruitment and fulfill thereby differential effects in comparison with classical GCs.

Besides the identification of target cells and addressing the mechanism for the beneficial aspects of GC therapy in greater detail, only recently the critical cell types in bone loss as one of the major side effects have been identified. We discuss in the following sections recent advances concerning the mechanisms of GC-induced osteoporosis (GIO) and the consequences for the demands on selective ligands that spare the bone.

3 The Role of the Glucocorticoids Receptor in Bone Homeostasis

3.1 Glucocorticoid-Induced Osteoporosis

Bone homeostasis depends on the balance of bone formation and bone resorption. High levels of glucocorticoids are known to negatively influence bone homeostasis since the early 1930s due to characterization of people with Cushing syndrome [45]. By the time of their clinical use the detrimental effects on bone turned out to be one of the most frequent side effects. Moreover approximately 25% of all clinical relevant osteoporosis in particular with high fracture risk are linked to high GC exposure [46]. People subjected to more than 7.5 mg prednisolone per day over 3 months showed an 50% increase in bone fracture risk. In these patients bone mineral density declines very fast with the onset of steroid therapy during the first 3–6 months [47]. The inhibitory effect on bone formation, osteoid thickness, mineral apposition, and mineralization by GCs is undisputed, whereas differences in bone resorption seems to be controversial [48]. Also to which extend systemic effects of GCs, e.g., on calcium metabolism may contribute to bone loss was until recently not shown due to the lack of respective mouse models. Below we discuss the effects of GCs on systemic physiology, osteoclasts and osteoblasts in detail and focus of recent advances by our laboratory and others.

3.2 Systemic Effects by Glucocorticoids

Glucocorticoid exposure opposes vitamin D actions on serum Ca^{2+} levels by a decrease of intestinal calcium absorption [49] and an increase in renal Ca^{2+} excretion [50]. Decreased calcium levels cause hyperparathyroidism [51]. However bone turnover in glucocorticoid-induced osteoporosis is low [48] in contrast to elevated turnover in hyperparathyroidism [52]. Systemic glucocorticoids may affect bone integrity by the reduction of gonadal hormones. First they blunt pituitary luteinizing hormone secretion [53]. Second they inhibit production of testosterone and estrogen in testes and ovary, respectively [54, 55]. However estrogen deficiency and glucocorticoid excess were described to be additive in rats [56] suggesting a minor role for regulation of gonadal function in glucocorticoid-induced osteoporosis.

3.3 Effects of Glucocorticoids on Osteoclasts

In contrast to systemic effects direct GC actions on bone cells seem to be crucial for GC-induced osteoporosis (GIO). GCs have been shown to act on osteoclasts directly and indirectly via other cells, such as the osteoblasts. Glucocorticoids are potent inducers of osteoclastogenesis-promoting RANKL and suppress the osteoclast-inhibitor OPG [57]. Suppression of OPG expression was described by interfering with JNK activity and transrepression of AP-1 bound to the OPG gene promoter [58]. The mechanism of upregulation of *Tnfsf11* mRNA, encoding for RANKL, is less understood. There is a potential GRE in the *Tnfsf11* promoter [58] implicating a direct transcriptional control, but also evidence for a contribution of enhanced mRNA stability [59]. Cytokines like IL-1α, β, TNF-α [60], and IL-6 [61] are potent promoters of osteoclastogenesis in bone inflammation, but are rather suppressed by GCs, such as IL-6 in osteoblasts [62, 63].

In the presence of RANKL in monocytic cultures GCs stimulate osteoclastogenesis at low concentration, but inhibit osteoclast formation at high concentration [64]. These data might explain the differential effects of GCs observed in vivo.

Indeed findings of GC effects on resorption are controversial. In one study prednisolone treatment increased resorption in Balb/c mice but not in human RANKL knock-in mice. Furthermore osteoclast numbers were not increased significantly in both mouse strains [65]. Also in other studies osteoclast numbers were unaltered in prednisolone treated mice, but resorption even decreased around 20–30% [62, 66].

Although osteoclast numbers seem not to be changed to a large fraction in rodent studies, GCs have been reported to increase osteoclast life span, e.g., by inhibiting caspase-3 activity [66]. Mice lacking glucocorticoid signaling in mature osteoclasts, but not progenitor cells displayed reduced osteoclast numbers upon steroid exposure in contrast to wild-type mice. This finding suggests that GCs enhance life span in mature osteoclasts, whereas osteoclastogenesis from osteoclast progenitors is inhibited [66]. The inhibition of osteoclastogenesis by pharmacological GC

concentrations was observed in cocultures of osteoblast and osteoclast precursors and was dependent on GR expression in both cell types [62]. This observation was in line with the observed decrease of resorption *in vivo* reported by Teitelbaum and colleagues and by our group [62, 67]. Indeed the reduced resorption could depend in part by the GR in osteoblasts, since we found a less severe reduction of resorptive activity in prednisolone treated mice lacking the GR in osteoblasts [62].

Kim et al. explained the cell-autonomouse effects of GCs on osteoclastogenesis by prevention of M-CSF-induced activation of Ras homolog gene family GTPases, in particular RhoA, Rac, and Vav3, which consequently disrupts actin ring formation. Reorganization of cytoskeleton is crucial for formation of so-called ruffled boarders and consequently resorptive activity [67]. Thus, the osteoclasts may lose their potential to degrade bone. Interestingly reduced osteoclast activity by prednisolone depends on the monomeric GR in osteoblasts and osteoclasts [62].

Finally there was a role of the GR in osteoclast suggested to influence bone formation [67]. Despite there is no doubt that osteoclast communicate to osteoblasts in bone remodeling this could not be supported by the analysis of mice with inactivated GC signaling in osteoclasts [66] and in our study using mice lacking the GR in myeloid cells and thus osteoprogenitors [62].

Although as we will discuss below the inhibition of bone formation is the major mechanism of GIO it should be noted that a full suppression of osteoclastogenesis by the monoclonal antibody Desonosumab inhibiting human RANKL in hRANKL knock-in mice ameliorates bone loss [65].

3.4 Effects of Glucocorticoids on Osteoblasts and Osteocytes

A hallmark of GC-induced bone loss is the inhibition of bone formation and thus a suppression of osteoblast function accompanied with a loss of osteoblast and osteocyte number [51]. Using conditional knockout mice ($GR^{Runx2Cre}$ mice) we recently demonstrated that the GR in osteoblasts is not only required to mediate suppression of bone formation, but is also instrumental for GC-induced bone loss [62].

The reduced osteoblast activity and numbers are attributed to inhibition of proliferation, induction of apoptosis, and suppression of differentiation.

3.4.1 Proliferation

Most evidence of GC effects on osteoblast proliferation derives from tissue culture experiments involving primary calvarial cells and immortalized and/or transformed cell lines. For MC3T3-E1 cells there was a postconfluent antiproliferative effect by GCs postulated as a prerequisite for reduced differentiation [68]. The inhibition of proliferation was explained by antagonizing the Wnt pathway, e.g., activating with GSK-3β kinase [69], suppressing PKB/Akt [70], inactivating TCF/LEF or inducing the wnt antagonist DKK-1 [71, 72]. Recently, it was shown that GC-induced

MAPK phosphatase 1/dual-specific phosphatase (DUSP1) is functionally involved in the reduction of mitogenic signaling and thus participates in anti-proliferative effects of GCs [73]. This is in line with our results that GR^{dim} osteoblasts with a GR impaired in dimerization are unable to induce DUSP-1 (Rauch and Tuckermann unpublished) and indeed failed to exhibit a reduction of osteoblast proliferation. Nevertheless GR^{dim} mice have impaired bone formation upon GC exposure, indicating that effects of GCs on proliferation are only to a minor part involved in GC-induced bone loss [62].

3.4.2 Apoptosis

Apoptosis of osteocytes and osteoblasts is a well-described feature in GC exposed rodents and humans [74].

Interestingly dexamethasone increases caspase-3 activity and induce consequently apoptosis in osteoblasts [75] which is opposite to the reduction of caspase-3 observed in osteoclasts [66].

Surprisingly the mechanical activation of osteocytes leads to prevention of apoptosis [76]. Mechanical forces signal via focal adhesion kinas (FAK), SRC and finally activation of ERK [77]. This outside-in survival signaling is compromised by the GC-mediated activation of the proapoptotic proline-rich tyrosine kinase 2 (PYK2) via phosphorylation at Tyr^{402} [78]. Activated PYK2 triggers reorganization of the cytoskeleton, cell detachment by disruption of integrin matrix engagement and finally apoptosis [79]. It is hypothesized that this occurs via mechanisms independent of GR-mediated gene regulation [78]. From our studies we could show that induction of apoptosis in primary osteoblastic cells dependents on GR expression but not binding to DNA in vitro [62]. It remains to be clarified whether direct interaction between the GR and PYK2 at the cell membrane occurs in order to induce apoptosis or whether nuclear effects such as inhibition of transcription are involved.

Nonetheless GCs could cause apoptosis also by elevation of the potent pro-apoptotic protein BAX, that was recently found to be upregulated by dexamethasone in a proteomic study using MC3T3-E1 cells [80].

Of note it has to be stressed that the observation of apoptosis of osteoblasts by dexamethasone affected only a minor fraction of all osteoblasts in vitro (approx. 10%) [78]. The minor role of apoptosis in bone loss is supported by a study from O'Brien and colleagues [81]. Here they used mice overexpressing 11β-HSD2 under the osteocalcin promoter, inactivating GC signaling in terminally differentiated osteoblasts and presumably osteocytes. These mice displayed a reduced apoptotic rate under prednisolone treatment [81]. Nonetheless there was still an overall bone loss observed, indicating that apoptosis is not sufficient to cause bone loss. This was corroborated by the fact that in other mouse strains subjected to GIO an increase of osteoblast/osteocyte apoptosis was hardly to be observed under prednisolone treatment at different time points [62].

3.4.3 Differentiation

Whereas induction of apoptosis take place to a minor degree, inhibition of differentiation in terms of alkaline phosphatase activity and mineralization occurs around 70–90% in primary osteoblasts at high concentration of GCs. This effect of GCs is biphasic. Low and physiological concentrations of GCs promote differentiation of pre-osteoblasts in numerous tissue culture systems [82, 83] and may depend on a specific time window of GC exposure [84]. These so-called anabolic effects of endogenous GCs can be observed in vivo for bone mass. Mice lacking the GR in osteoblasts [62] as well as osteoblast-specific overexpression of 11β-HSD2 thereby disrupting glucocorticoid signaling [85] display a reduced bone mineral density, albeit with no abnormalities in bone growth and architecture [62, 86]. Indeed osteoblasts lacking the GR display a reduced differentiation potential when grown in normal tissue culture medium with vitamin C and β-glycerolphosphate ad differentiation conditions [62]. As GR^{dim} mice, carrying a dimerization deficient GR, have no obvious bone phenotype and a unaltered osteoblast differentiation, anabolic GC actions on bone are independent of dimerized induced DNA binding [62].

Nonetheless it has been generally accepted that the treatment of osteoblastic cells with high-dose glucocorticoids leads to a suppression of osteoblast differentiation, which could be monitored by the reduced expression of Runx2 [87], the master osteoblast transcription factor [88], and of other marker genes of differentiation. Following reduced differentiation osteoblast function in terms of collagen production declines. Transcription of α1-(I)-procollagen was shown to be effected by glucocorticoid treatment [89]. Accordingly we could show that the suppression of *Col1a1* mRNA in vivo as well in vitro depends on the expression of the GR in osteoblasts [62] since mice lacking the GR in osteoblasts had no reduction of *Col1a1* expression upon glucocorticoid exposure. For this reduction the GR monomer was sufficient suggesting that tethering mechanisms of the GR with transcriptional activators of the Col1a1 gene are involved. It is tempting to speculate that the monomeric GR interferes with TGFβ-triggered smad signaling to reduce collagen I expression [90], but remains to be proven. Furthermore $GR^{Runx2Cre}$ mice but not GR^{dim} mice are resistant to glucocorticoid-induced suppression of bone formation. Consequently bone mass was not affected after 2 weeks of prednisolone treatment in $GR^{Runx2Cre}$ mice. From these findings together with the observation of a strong suppression of primary osteoblast differentiation around 70–90% in vitro we conclude that inhibition of differentiation is a major mechanism of GIO.

The underlying mechanisms of suppression of osteoblast differentiation by GCs are not completely understood. A number of evidence was reported from the Smith and Frenkel lab although they performed their experiments almost exclusively in the MC3T3-E1 cell line. Their data suggest that glucocorticoids suppress differentiation by interference with BMP/TGFβ signaling. In particular BMP-2 seems to be a promising target for interference with suppression of differentiation. *Bmp2* expression declines by GC treatment [91] and its exogenous administration rescues mineralization of glucocorticoid treated cells but not collagen deposition in MC3T3-E1 cells [92]. Expression profiling of glucocorticoid-treated MC3T3-E1 cells identified

early growth response 2 (EGR2/Krox20), a zinc finger transcription factor as a glucocorticoid suppressed target gene and a potential mediator of suppression [93]. EGR2/Krox20 is involved in chondrocyte–osteoblast interactions and its ablation severely attenuates bone formation [94]. Intriguingly Krox20 is a transcriptional activator of follistatin [95], an extracellular inhibitor of bone morphogenic proteins (BMPs) [96]. Due to GC prompted downregulation of EGR2 follistatin expression increases and thereby potentially inhibits BMP signaling. Thus, by interfering BMP signaling on several levels GCs can suppress osteoblast differentiation.

Other studies suggest that glucocorticoids might reduce osteoblast differentiation by regulation of insulin like growth factor-1 (IGF-1) action. IGF-1 increases osteoblast lineage expansion, collagen synthesis, and matrix apposition [97, 98]. GCs can directly suppress *Igf1* transcription by upregulation of CAAT/enhancer binding proteins, in particular C/EBPβ and C/EBPδ that are transcriptional inducers of the *Igf1* gene [99]. Furthermore IGF-1 activity can be influenced by the regulation of the IGF activating IGF binding protein 5 (IGFBP-5) that is diminished upon GC treatment of primary osteoblasts [100]. However, the decreased bone formation in IGFBP-5 overexpressing mice [101] questions whether this is a major mechanism.

The induction of C/EBPs as regulators of adipogenic differentiation by GCs is in conformity with the idea that inhibition of osteoblastogenesis leads to a shift towards adipogenesis of mesenchymal progenitor cells [102]. In line with this idea congenic mice with allelic suppression of skeletal and hepatic *Igf1* had low bone mass with fatty infiltration of the bone marrow but no signs of obesity [103]. Furthermore magnetic resonance imaging in humans revealed that the drop in bone mass of osteoporotic men correlates with increased bone marrow adiposity [104]. Importantly, we could recently show GR binding to DNA is instrumental for promotion of adipogenesis, in particular by transcriptional activation of KLF-15 [105]. In the light that adipogenesis by GCs requires GR dimerization [105], which is dispensable for suppression of osteoblast differentiation [62], our findings suggest that adipogenic differentiation by GCs can be uncoupled from suppression of osteoblast differentiation. This also argues against a transdifferentiation of committed osteoblasts towards adipocytes by GCs. Whether the increased bone marrow adiposity in osteoporotic bones originates from switching lineage of osteoblast arrested cells by mechanisms independent of suppression of differentiation or infiltration of mesenchymal progenitor cells is still elusive and requires lineage tracing studies.

Using osteoblasts from GR^{dim} mice that are still capable to undergo GC suppression of osteoblast differentiation we were able to dissect a GR monomer-dependent osteoblast gene expression program (Rauch, unpublished). From this analysis we identified genes encoding members of the IL-6 family like *Il6*, *Lif*, and *Il11* being suppressed in a GR dimer-independent manner. Interestingly, exogenous supplementation of Il11 in GC-treated cultures reversed GC suppression of differentiation. Collagen 1a1 expression, alkaline phosphatase activity and mineralization were at normal levels despite the presence of GCs [62]. We further demonstrated that c-Jun-dependent *Il11* transcriptional upregulation is targeted by the GR, whereas NF-κB interactions were dispensable [62]. IL-11 act in an autocrine manner via the IL11 receptor and the common receptor gp130 which leads to STAT3 phosphorylation, that is reduced in the presence of Dex [63].

Likewise IL-11 itself is a potent inducer of osteoblastogenesis [63] and prevents adipogenesis in culture [106]. The importance of IL-11 in osteoblast function is underscored by the analysis of mice expressing a human *IL11* transgene [106] and mice with activated STAT3 signaling by a *Gp130*$^{F759/F759}$ knock in [107] that both display increased bone formation. Complementary, targeted deletion of the Il-11 receptor results in decreased osteoblast numbers and bone formation in vivo [108] similar as in mice with osteoblast-specific ablation of *Stat3* [107].

Thus, interference with IL-11, an active player in bone formation, is one of the mechanisms how GC suppress bone formation.

4 Novel Criteria for Selective GR Modulators for Therapeutic Efficacy and Avoidance of Osteoporosis

Our approach to dissect the molecular mechanisms of GC action on bone in vivo by the analysis of conditional and function selective GR mutant mice can be summarized as followed (Fig. 1). In GC-induced bone loss the GR in osteoblasts reduces osteoblast activity and numbers mainly by suppression of differentiation. The decreased differentiation of osteoblasts engages the monomer GR without interfering with NF-κB, but interacting with AP-1 bound at promoters, e.g., the *Il11* gene. Suppression of IL-11 release leads to impaired Jak-Stat signaling via gp130/IL11receptors and reduced active phospho-STAT3, important for osteoblast differentiation.

The requirement of the GR monomer for this side effect of GC action might be on the first glance disappointing, since selective ligands had been designed that should maintain the monomeric function, but omit dimerized induced binding of DNA by the GR. Due to our results those compounds would still harm the bone. However, our finding that NF-κB is not involved in suppression of osteoblast differentiation would allow a new profile required for a dissociating GR ligand. A dissociating ligand that should preserve the bone should not induce GR dimerization, in order to avoid anti-proliferation of osteoblasts, and not induce GR interaction with AP-1 to spare suppression of osteoblast differentiation. However this compound should still be able to reduce NF-κB-controlled cytokine expression.

Our analysis of the activity of the GR ligand Compound A (CpdA) on bone cells demonstrates that these criteria can be met [63].

CpdA displays potent anti-inflammatory actions in collagen-induced arthritis [29]. CpdA is in addition capable to suppress pro-inflammatory cytokines, such as CXCL10 and IL-6, and does not influence RANKL/OPG ratio in osteoblastic cell lines and primary cells [63, 109]. Most importantly expression of *Il11* and subsequently osteoblast differentiation are unaffected by CpdA in contrast to classical GCs. Finally mice receiving compound A at the immunosuppressive dose of collagen-induced arthritis have strikingly higher serum osteocalcin levels compared to dexamethasone-treated animals [63]. Unpublished results from De Bosscher and colleagues indeed demonstrate that CpdA does not favor an AP-1/GR interaction in mesenchymal cells in contrast to inflammatory cells. Future work will explain this cell type-specific differential activity of this dissociating ligand.

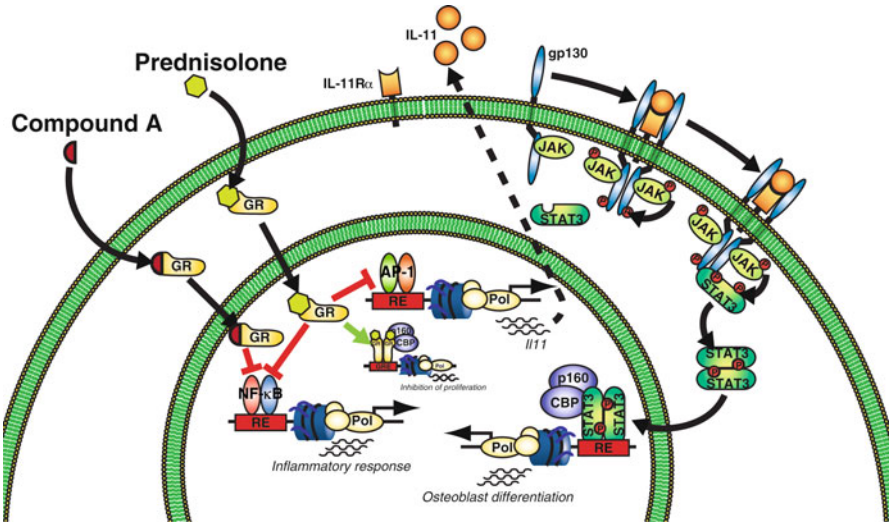

Fig. 1 Selective GR agonists avoiding GR dimerization and GR-AP-1 interaction and subsequent suppression of IL-11 signaling, preserve osteoblast differentiation and maintain anti-inflammatory capacities. Classical GCs such as prednisolone activate the GR in osteoblasts that translocates to the nucleus where it dimerize on DNA elements important for inhibition of proliferation, interferes with NF-κB activity leading to suppression of inflammatory cytokines and suppress AP-1-dependent IL-11 expression. IL-11 itself acts in an autocrine manner on osteoblasts through IL-11 receptor α and gp130 to mainly induce STAT3 activation. In line activated STAT3 and induction of its target genes is important for proper osteoblast differentiation. The dissociating GR ligand CpdA activates only partially the GR by omitting dimerization and GR-AP-1 interaction, but maintains suppression of NF-κB activity in osteoblasts. This allows autocrine IL11 signaling and osteoblast differentiation by keeping anti-inflammatory action

Although CpdA has a narrow therapeutic window we demonstrate here that such substances with optimized pharmacology could be of help in future to suppress inflammatory bone diseases and maintain bone integrity.

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