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Chapter 3

Mechanism of Glucocorticoid-Induced Osteoporosis: An Update

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1. Introduction

Synthetic oral steroids were initially developed in the 1940-1950's. Although their use was initially limited by their high cost, as they became more affordable and began to be used for treatment of a wide variety of conditions, side effects associated with their use became much more prevalent. In fact, glucocorticoid-induced osteoporosis is now the most common secondary cause of osteoporosis. Until relatively recently, the mechanism of action of these drugs and the mechanisms involved in the development of side effects such as osteoporosis and the higher incidence of bone fractures was not known. Although steroids are widely viewed as mainly catabolic for bone a distinction needs to be made between physiologic and pharmacologic doses of steroids. Recent evidence demonstrates that steroids can clearly be anabolic for bone. In this chapter we review recent findings and mechanisms of glucocorticoid action on bone and some of the clinical consequences of pharmacologic doses of these compounds on bone.

2. Glucocorticoids and osteoporosis

Glucocorticoids are among the most potent anti-inflammatory and immunosuppressive agents and are key therapeutic agents for the management of chronic inflammatory diseases, including rheumatic diseases [1-4], pulmonary disease [5;6], asthma [7-11] and post transplantation immunotherapy [12]. However, long-term glucocorticoid therapy (>3 months) causes bone loss resulting in osteoporosis (glucocorticoid-induced osteoporosis or GIOP) [3;4;13-15], a severe-side effect that occurs in 30 – 50% of patients [16-18]. The incidence of GIOP is indiscriminate of race, age and gender [19;20]. Children, as young as 4 years of age, and adolescents who are on glucocorticoid therapy for various pediatric disorders, including asthma [20-22], juvenile rheumatoid arthritis [23;24], Crohn's disease [25], systemic lupus erythematosus [26;27], and inflammatory bowel disease [28;29] have

been reported to endure significant bone density decrease. There is no clearly defined threshold for safe use of glucocorticoids. In practice, a dose equal to or greater than 5mg/day of prednisone is considered as low, and 10mg/day or more is high. The severity of bone loss in GIOP is both time- and dose-dependent. GIOP occurs in two phases: a rapid, early phase in which bone mineral density is reduced, within the first 5 to 7 months of therapy, possibly as a result of excessive bone resorption, and a slower, progressive phase in which bone mineral density declines because of impaired bone formation [30]. Bone loss continues as long as treatment is maintained.

3. Glucocorticoid mechanism of action as anti-inflammatory and immunosuppressant drugs

Glucocorticoids exert their actions via intracellular glucocorticoid receptors (GRs) [31;32]. The GR belongs to the ligand-regulated nuclear receptor superfamily [33]. Like other members in this superfamily, GR contains three major functional domains: a N-terminal activation domain required for transcriptional activation and association with basal transcription factors; a central DNA-binding domain (DBD) consisting of two highly conserved zinc finger regions that are critical for dimerization, DNA binding, transcriptional activation and repression; and a C-terminal ligand-binding domain (LBD) that serves as the binding site for glucocorticoids, chaperone proteins, and coactivators [34;35]. In the absence of ligand, GR is predominantly retained in the cytoplasm as an inactive multi-protein complex consisting of heat shock protein (hsp90) and a number of other proteins, including the immunophilins. The binding of glucocorticoid triggers a conformational change in the GR and leads to dissociation of the multi-protein complex and exposure of a nuclear localization sequence resulting in its nuclear translocation. Once in the nucleus, GR, in the form of a homodimer, binds to a palindromic glucocorticoid-response element (GRE) in the target gene promoter and activates transcription (*e.g.*, of the tyrosine amino transferase gene), or it can bind to a negative GRE (nGRE) to repress transcription (*e.g.*, of the osteocalcin gene) [36].

Glucocorticoids suppress the expression of a panel of inflammatory-relevant genes including cytokines [interleukins (IL) and tumor necrosis factors (TNF-α,β], chemokines (Regulated upon Activation Normal T-cell Expressed and Secreted or RANTES, Macrophage Inflammatory Protein-1-alpha or MIP-1α, Monocyte Chemotactic Protein or MCP-1, -3, and -4], inflammatory enzymes (COX-2, iNOS), and adhesion molecules (Intercellular Adhesion Molecule 1 or ICAM-1, E-selectin) that play a key role in the recruitment of inflammatory cells to the inflammation sites [37-39]. However, most of these genes do not have negative GREs in their promoter regions, and therefore, they are not directly regulated by the binding of GRs to such regulatory elements. These genes do contain NF-κB- and/or AP-1-binding sites and are activated through these sites by NF-κB and/or AP-1 in response to stimuli (cytokines). Thus, one mechanism by which glucocorticoids could regulate transcription would be modulation of NF-κB or AP-1 DNA-binding activity. In 1990, three independent groups found cross-talk between GR and AP-1 [40-42]. In these studies, it was found that activated GR can interact with c-Jun/AP-1 and that the formation of a GR-c-Jun complex prevents c-Jun/AP-1 DNA-binding, resulting in

the inhibition of gene expression. Later, it was found that the activated GR can associate with the p65 subunit of NF-κB and inhibit gene activation mediated by NF-κB [43;44]. These findings led to the establishment of the **protein-protein interaction model**.

In 1995, it was found that glucocorticoids induce the expression of a cytoplasmic inhibitor of NF-κB, the IκB-α [45;46]. These studies led to the establishment of a second model, **the IκB-α upregulation model**. This model proposes that glucocorticoids induce the expression of IκB-α and that the newly synthesized IκB-α sequesters the p65 subunit of the NF-κB in the cytoplasm and thereby inhibits NF-κB nuclear functions. However, this mechanism has been challenged by a number of studies. It has now been established that the effect of glucocorticoids on IκB-α expression, and subsequently NF-κB nuclear translocation, is cell-type specific. In some cell types glucocorticoid inhibition of proinflammatory stimuli-induced p65 nuclear translocation is coupled with the induction of IκB-α [45-48]. In other cell types, however, these two events are uncoupled [49;50]. Moreover, a GR mutant that does not enhance IκB-α expression, is still able to repress NF-κB activity [51].

4. Glucocorticoid effects on bone cells

Glucocorticoids have both anabolic and catabolic effects on bone. However, the outcome of glucocorticoid therapy is a net loss of bone [4;52;53]. Corticosteroid 11β-hydroxysteroid dehydrogenase 2 [11β-HSD2] is an enzyme that oxidizes the active form of glucocorticoid cortisol to the inactive metabolite cortisone, thus the levels of expression and activity of this enzyme is critical for glucocorticoid signaling. *In vivo* studies show that bone-specific transgenic overexpression of 11β-HSD2, under the control of type I collagen promoter, impairs osteoblast differentiation and bone acquisition [54-56]. These studies demonstrate that the endogenous glucocorticoid signaling is essential for normal skeletal development. However, glucocorticoid in excess such as patients with Cushing's syndrome [22] or the patients on glucocorticoid therapy rapidly lose bone mass resulting in osteoporosis. The direct effects of glucocorticoids on bone cells are illustrated in Figure 1.

5. Glucocorticoid effects on bone marrow Mesenchymal Stem Cells (MSCs)

Bone marrow MSCs are multipotent cells that can give rise to several distinct cell lineages, including osteoblasts, adipocytes, and chondrocytes [57-60]. Patients on glucocorticoid therapy not only lose bone but also accumulate large amounts of marrow fat (fatty marrow), indicating that glucocorticoid has altered lineage commitment of MSC to adipocytes at the expense of osteoblasts because these two pathways have a reciprocal relationship [61-64]. Thus, one possible mechanism by which glucocorticoids alter MSC fate determination is through the induction of the master adipogenic regulator peroxisome proliferator-activated receptor gamma (PPARγ) [65;66], which is transcriptionally activated by the CCAAT/enhancer binding protein (C/EBP) family transcription factors in response to glucocorticoid [67-70] (Figure 2). Indeed, Weinstein and colleagues showed that administration of glucocorticoids to mice reduces the numbers of osteoprogenitor cells [71].

This could be achieved through induction of PPARγ since under the same condition bone marrow adipogenesis is enhanced [72], and that a reduction in PPARγ dosage (haploinsufficiency) in mice results in reduced adipogenesis and enhanced osteogenesis from bone marrow progenitors [73].

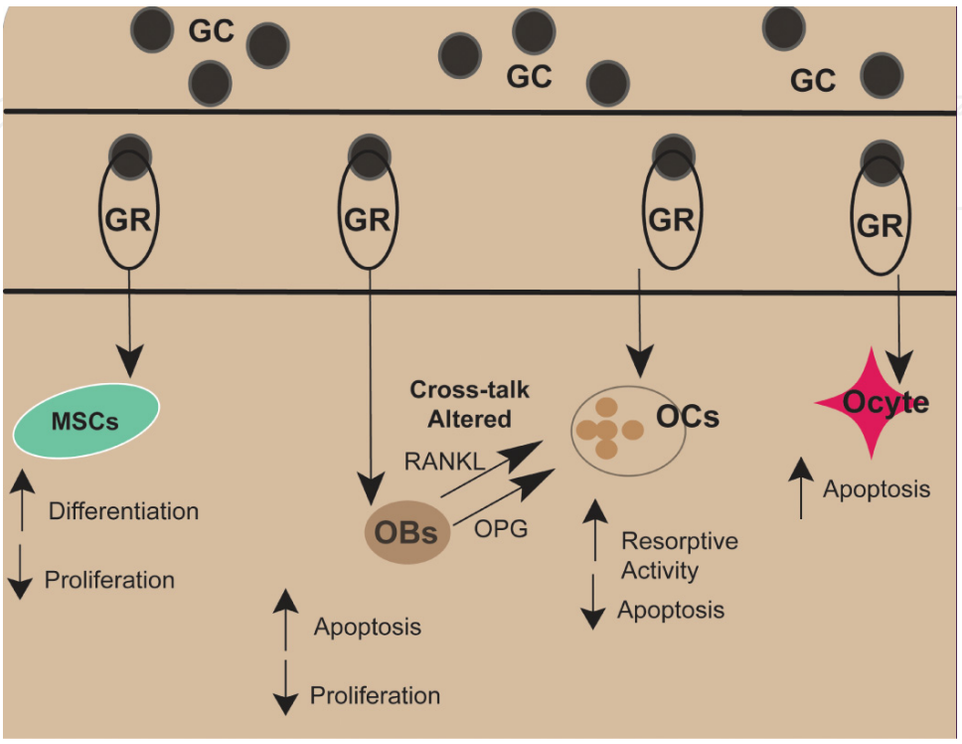

Figure 1. Glucocorticoids bind to the glucocorticoid receptor (GR) and affect mesenchymal stem cell (MSC), osteoblast (OB), osteoclast (OC) and osteocyte (Ocyte) function. The net result is decreased bone formation and increased bone resorption. ↑increase; ↓decrease.

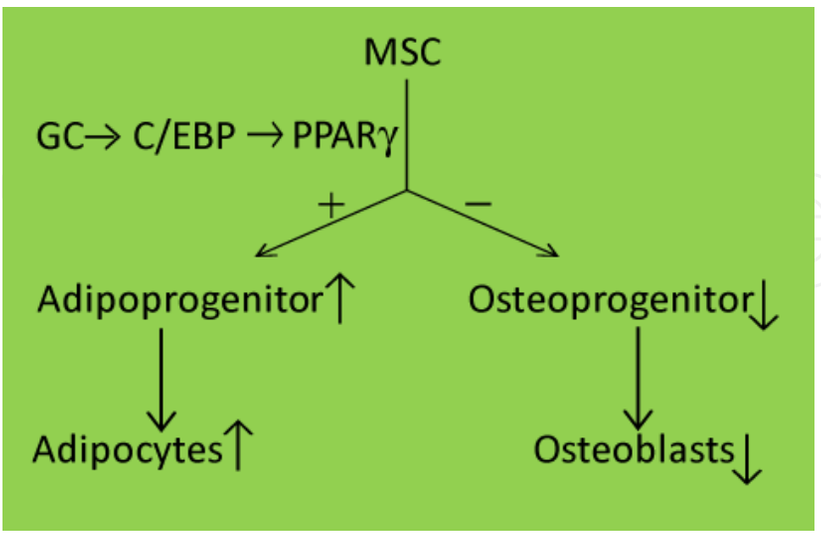

Figure 2. Glucocorticoid reduces the number of osteoprogenitors from MSC by promoting adipogenic differentiation pathway. Glucocorticoid induces the expression of C/EBP family transcription factors that directly activate the transcription of PPARγ, the master regulator of adipogenesis, and shifts the lineage commitment of MSCs to adipocyte pathway, thus reducing the number of osteoprogenitor cells.

6. Glucocorticoid effects on osteoblasts and osteocytes

It has been known for decades that glucocorticoid inhibits bone formation [52;74], but only recently have we realized that glucocorticoids directly target bone cells. By administering a high dose of prednisolone to mice, Weinstein and colleagues found that glucocorticoid induces the death of mature osteoblasts and osteocytes [71;75]. In the same study, the authors also showed that the same is true in bone biopsy samples obtained from patients with glucocorticoid-induced osteoporosis. These results were further strengthened in a transgenic mouse model, in which the glucocorticoid signaling is disrupted by overexpression of 11β-HSD2 specifically in osteoblasts. The study showed that the 11β-HSD2 transgenic mice are protected from glucocorticoid-induced osteoblasts and osteocytes apoptosis and suppression of bone formation [76]. These studies demonstrate that glucocorticoids cause bone loss by restricting the supply of bone building cells, the osteoblasts, and by interfering with the communication network within bone environment via osteocyte death. The osteocytes are the mechanosensory cells that detect and send signals for bone formation in response to damages caused by mechanical loading and unloading [77;78].

7. Glucocorticoid effects on osteoclasts

Osteoclasts are bone resorbing cells and play a key role in the maintenance of bone homeostasis through bone remodeling. In patients, glucocorticoid-induced osteoporosis features a rapid early phase increase in bone resorption, followed by a slow progressive decrease in bone formation [52]. Earlier studies showed that glucocorticoids stimulate osteoclast differentiation and increase their activity [72;79;80]. It is now recognized that glucocorticoids increase the longevity of osteoclasts but may inhibit their bone resorptive activity [81;82]. Moreover, a recent study suggests that glucocorticoids do not inhibit, but modify osteoclast resorptive behavior, making osteoclasts erode bone surfaces over long distances without interruption [83].

8. Glucocorticoid-induced Leucine Zipper (GILZ): A new glucocorticoid anti-inflammatory effect mediator

The protein-protein interaction and the IκB-α upregulation models described earlier in this chapter were established prior to the discovery of a glucocorticoid-inducible protein named glucocorticoid-induced leucine zipper (GILZ), which was identified in 1997 [84]. GILZ is a member of the leucine zipper protein family [84;85] and belongs to the transforming growth factor-beta (TGF-β)-stimulated clone-22 (TSC-22d3) family of transcription factors [86;87]. Members of this family of proteins contain three distinct domains; an N-terminal domain containing a TSC box (N-Ter), a middle leucine zipper domain (LZ), and a C-terminal poly-proline rich domain (PRR).

Unlike IκB-α, which is induced by glucocorticoids in certain cell types [49;50;88], GILZ is induced by glucocorticoids virtually in all cell types examined so far, including bone

marrow mesenchymal stem cells, osteoblasts, adipocytes, macrophages and epithelial cells [89]. *In vitro* studies show that overexpression of GILZ protects T-cells from apoptosis induced by anti-CD3 monoclonal antibody, but not other apoptosis-inducing agents such as dexamethasone, ultraviolet irradiation, starvation, or triggered by cross-linked anti-Fas mAb [84]. T-cell-specific transgenic overexpression of GILZ results in thymocyte apoptosis *ex vivo*, possibly through down-regulation of Bcl-xL [90]. The *in vitro* actions of GILZ have been shown to be mediated through direct protein-protein interactions between GILZ and NF-κB, and between GILZ and AP-1 [86;91;92]. The interaction between GILZ and NF-κB blocks NF-κB nuclear translocation and DNA-binding, and the interaction with AP-1 inhibits the binding of AP-1 to its DNA elements [91;92]. GILZ also interacts directly with the mitogen-activated protein kinase (MAPK) family members, Ras and Raf-1, resulting in inhibition of Raf-1 phosphorylation and subsequently, inhibition of MEK/ERK-1/2 phosphorylation and AP-1-dependent transcription [86;93]. Moreover, GILZ can deactivate macrophages [94], inhibit proinflammatory cytokine-induced inflammatory enzymes such as cyclooxygenase-2 [95], inhibit IL-2/IL-2 receptor and IL-5 expression [91;96], and stimulate the production of anti-inflammatory IL-10 by immature dendritic cells, thereby, preventing the production of inflammatory chemokines by CD40L-activated dendritic cells [97]. These studies demonstrate that GILZ is a glucocorticoid anti-inflammatory effect mediator and utilizes very similar mechanisms, to those GR uses [98].

9. GILZ mediates the anabolic effect of glucocorticoids

GILZ is a direct GR target gene with several GREs present in its promoter region [99]. In the absence of glucocorticoid stimulation GILZ is expressed at a very low basal level. However, in the presence of glucocorticoid, GILZ expression is rapidly induced (Figure 3] but GR is also activated, and the activated GR negatively impacts bone, both directly (i.e., inhibits osteocalcin gene transcription) and indirectly through other pathways as illustrated (Figure 4]. Because of that, it is impossible to determine the role of GILZ in osteoblast differentiation and bone formation without the influence of GR, which plays a negative role and may override GILZ actions. To further study this problem, a retrovirus-mediated GILZ overexpression system was established in bone marrow MSCs/osteoprogenitor cells. Studies carried out in this system showed that GILZ has potent pro-osteogenic activity as demonstrated by significantly increased alkaline phosphatase activity, enhanced mineralized bone nodule formation, and the expression of osteoblast-associated genes such as Runx2, type I collagen, alkaline phosphatase, and osteocalcin [100]. Furthermore, our recent studies have shown that overexpression of GILZ can antagonize the inhibitory effect of TNF-α on MSC osteogenic differentiation [101]. Possible mechanisms underlying this antagonism may include GILZ inhibition of TNF-α-induced ERK/MAP kinase activation, which has been shown to be responsible for TNF-α down-regulation of a key osteogenic factor Osx [102;103], and inhibition of TNF-α-induced expression of E3 ubiquitin ligase Smurf proteins, which have been shown to accelerate the degradation of Runx2 protein [104-106].

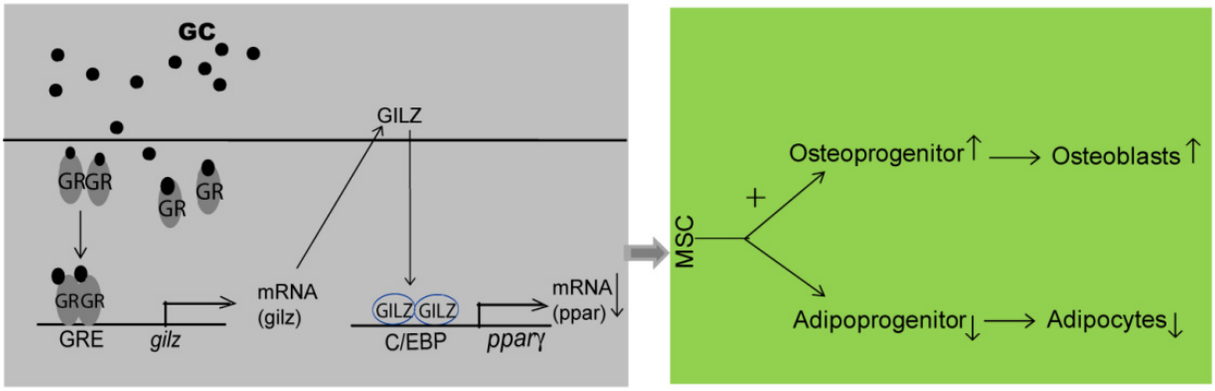

Figure 3. GILZ enhances MSC osteogenic differentiation by shifting MSC lineage preference to osteogenic pathway.

10. GR mediates the catabolic effects of glucocorticoids

There are many glucocorticoid effectors involved in the regulation of bone development or metabolism through different pathways. However, it was only recently demonstrated that the GR was directly responsible for glucocorticoid-induced bone loss *in vivo*. Using a bone-specific GR knockout mouse model, Rauch et al showed that glucocorticoids are unable to induce bone loss or to inhibit bone formation in these mice because the GR-deficient osteoblasts become refractory to glucocorticoid-induced apoptosis, inhibition of proliferation, and differentiation [107]. Interestingly, data from this study also demonstrated that GR-deficiency results in a low bone mass phenotype, confirming the previous studies that the endogenous glucocorticoid signaling is critical for normal bone acquisition [54-56]. Other evidence supporting the role of GR in glucocorticoid-induced bone loss includes: 1] the glucocorticoid-activated GR binds directly to the negative glucocorticoid response elements (nGREs) in the promoter region of the osteocalcin (*Ocn*) gene, an osteoblast-specific gene that plays an important role in bone mineralization, and inhibit its transcription [36;108]; 2] GR transcriptionally activates the expression of MAP kinase phosphatase-1 (MKP-1] [109], which inactivates MAP kinase and thus inhibits osteogenic differentiation [64;110;111]; and 3] GR can physically interact with and inhibit the transcriptional functions of Smad3, an intracellular signaling mediator of transforming growth factor-beta (TGF-β) [112] (Figure 4]. Glucocorticoids have been known to antagonize TGF-β action in bone [113-115] and TGF-β stimulates osteoprogenitor cell proliferation [116-119] and attract osteoprogenitor cells to the remodeling sites during bone remodeling [120]. It is important to note that while the catabolic effects of glucocorticoids are often associated with long-term glucocorticoid excess [1-4;7], a short term exposure to glucocorticoid seems beneficial; for example, treatment of bone marrow stromal cells or osteoblasts with dexamethasone enhances, rather than inhibits, alkaline phosphatase (ALP) activity. The ALP is expressed at the early stage of osteoblast differentiation program and the increase of ALP expression or activity marks the entry of cells into the osteoblast lineage.

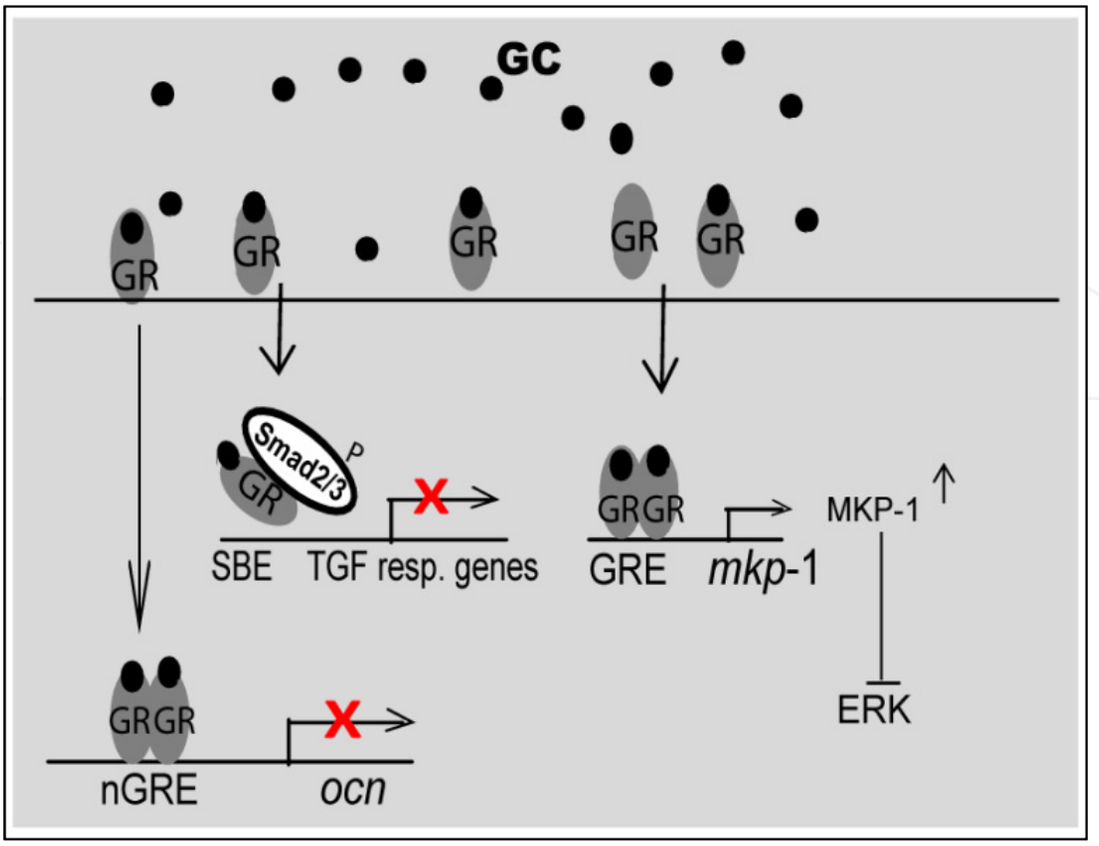

Figure 4. GR inhibits MSC proliferation, ERK activation and Ocn expression. Ligand-bound GR physically interacts with: 1] TGF-β signaling mediator Smad3 and disrupts its transcriptional activity; 2] Activates mkp-1 transcription, by binding to GRE in the mkp-1 promoter region, resulting inhibition of ERK activation; and 3] Suppresses Ocn expression by binding to nGRE in the Ocn promoter region.

11. Glucocorticoid-Induced Osteoporosis (GIOP)

Although glucocorticoids are an essential hormone for survival and normal function, when present in excess (pharmacologic doses) lead to a number of serious side effects including bone loss and fractures. In fact, it is estimated that 30-50% of patients chronically exposed to high levels of glucocorticoids will develop a bone fracture [121]. Glucocorticoid excess can result from either endogenous (Cushing's) or exogenous (iatrogenic) sources. Glucocorticoids are widely used for the treatment of a variety of inflammatory and autoimmune conditions. It is estimated that 0.5% of the population receives steroid therapy and exogenous steroids are thus the most common cause of secondary osteoporosis [122]. There has been a lot of discussion on the dose, duration and mode of administration of steroids and the impact on the development of osteoporosis. There has been a lot of debate on a "safe" dose for glucocorticoid replacement. Doses as low as 2.5 mg of prednisolone have been reported to result in osteoporosis [122]. Even patients on "physiologic" glucocorticoid replacement for Addison's disease have been reported to have lower bone density than controls, although clearly many of these patients were overreplaced with steroid therapy [123]. Further, steroids even when given in an intermittent, rather than continuous fashion, or in an inhaled rather than oral fashion, are still associated with an

increased risk of fracture. In treatment guidelines by the American College of Rheumatology in 2010, it was recommended that for patients with low fracture risk receiving more than 7.5 mg of prednisolone equivalents for more than 3 months receive some form of therapy for fracture prevention. In contrast for patients at high fracture risk it was recommended that they receive some form of therapy even at glucocorticoid doses lower than 5 mg and even for periods for less than one month [124].

12. Mechanism

Glucocorticoids have multiple effects on bone and bone cells. In addition, in cases where the glucocorticoids are given to treat systemic inflammatory conditions (e.g. rheumatoid arthritis), the underlying condition also contributes to bone loss. Glucocorticoids also inhibit endogenous production of sex steroids (testosterone and estrogen) in addition to production of adrenal androgens, all of which may have protective effects against bone loss [125]. Further, prolonged high dose glucocorticoid use results in both muscle weakness thus predisposing to an increased number of falls and muscle wasting. Bone-muscle interactions may also contribute to maintaining bone health. Glucocorticoids also decrease intestinal calcium absorption thus further predisposing to osteoporosis. Recently, effects of glucocorticoids on decreasing bone vasculature, has also been implicated as a potential mechanism for glucocorticoid effects on bone [126]. There also seems to be an age-dependence of glucocorticoid effects on bone. The likelihood of fractures with glucocorticoids appears to increase with increasing patient age. Glucocorticoid-induced bone loss appears to be biphasic with an initial rapid phase of bone loss of 5-15% /year followed by a more sustained bone loss rate of 2% [121].

Glucocorticoids affect all bone cells, they result in osteocytic and osteoblastic apoptosis and decreased function of both osteoclasts and osteoblasts. However, they decrease osteoclastic apoptosis. Thus, the net effect is reduced bone formation and increased bone breakdown. Trabecular bone seems to be particularly sensitive to the detrimental effects of steroids resulting in a higher incidence of vertebral and femoral neck fractures [121]. Vertebral compression fractures are commonly missed since only about 30% of them are symptomatic. A study by Angeli et al [127] which examined the prevalence of vertebral fractures in patients receiving glucocorticoids for a variety of autoimmune conditions determined that over 37% of patients had at least one asymptomatic vertebral compression fracture and more than 14% had two or more asymptomatic fractures.

Glucocorticoid effects on bone appear to be generally reversible and once therapy is stopped bone repair occurs over the year following drug cessation. Thus, if feasible, steroid cessation may be the therapy of choice for GIOP.

13. Diagnosis

Determining fracture risk for patients on steroids is difficult since even patients with normal bone densitometry on steroids have a higher fracture risk. Current use of steroids is one of the risk factors used in the calculation of the FRAX (Fracture Risk Assessment) score.

However a recent joint position statement by the International Society for Clinical Densitometry and the International Osteoporosis Foundation concluded that when using the FRAX tool there probably was an underestimation of fracture risk with daily prednisone doses greater than 7.5 mg and an overestimation of fracture risk with daily prednisone doses of less than 2.5 mg. In addition, FRAX probably underestimated fracture risk when high dose inhaled steroids were used. Finally, it was concluded that for patients with adrenal insufficiency receiving appropriate replacement steroid doses this not be included in the FRAX calculation [128].

The American College of Rheumatology recommends that some form of therapy be considered for all patients receiving prolonged steroid therapy and that for those who have a bone densitometry test (Dual energy x-ray absorptiometry or DXA), a T-score of less than -1.0 be considered abnormal [125].

14. Therapy

Since glucocorticoids interfere with intestinal calcium absorption, all patients about to start glucocorticoid therapy should be placed on calcium and vitamin D replacement. Antiresorptive agents such as bisphosphonates (both oral and IV) have been used for the therapy of GIOP, are effective in decreasing the increased fracture risk associated with steroids and are approved for this indication. However, as discussed by Teitelbaum et al. [129], although initial use of steroids is associated with increased bone resorption (osteoclast mediated and related to decreased osteoclastic apoptosis and a situation in which antiresorptive use makes sense), more prolonged steroid use is associated with decreased bone formation and antiresorptive agents have the theoretical possibility of making things worse by further suppressing a low bone turnover state. Thus, use of an anabolic agent such as teriparatide (synthetic parathyroid hormone) for treatment of GIOP would appear more appropriate. In fact in a clinical trial, comparing alendronate vs. teriparatide for 18 months in 428 men/women with established osteoporosis and who had received at least 5mg of prednisone for at least 3 months, teriparatide was significantly more effective in both increasing bone mineral density at the spine [7.2 vs 3.4%) and in decreasing new vertebral fractures [0.6% vs 6.1%) [130]. Of note, this was a secondary instead of a primary osteoporosis prevention trial and there was a greater incidence of side effects associated with teriparatide use as compared to controls [131]. In addition, use of teriparatide by patients is currently limited to two years, thus alternative and better forms of therapy for GIOP need to be developed.

15. Conclusion

Although adverse side effects of glucocorticoids on bone have been long recognized, both from endogenous sources as described by Harvey Cushing in the 1930's or from exogenous sources after development of glucocorticoids in the 1950's the mechanisms involved in this process have only recently began to be understood. It is clear that although physiologic levels of glucocorticoids are important in normal bone development, pharmacologic doses

result in a high level of fractures, particularly of vertebral bone. Thus, it would seem that glucocorticoids have both anabolic and catabolic actions on bone. Data from our labs and from others suggest that GILZ may be an important mediator of GR's anabolic actions and thus may be an attractive therapeutic target for drug development.

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