I. INTRODUCTION

Glucocorticoid hormones are primary mediators of the stress response and circadian rhythm. This is due, in part, to their unique capability to rapidly mobilize glucose, an important fuel for the brain. Glucocorticoids (GCs) also influence whole body homeostasis, modulate the immune response and functions of the brain, affect tissue integrity, and have an impact on the skeletal system.

GCs are secreted by the adrenal glands in response to a hierarchical signaling cascade initiated by the hypothalamic corticotropin-releasing factor which induces the secretion of adrenocorticotropic hormone from the anterior pituitary. Activation of this cascade results in the production of key molecules and rate-limiting enzymes for corticosteroid synthesis in the adrenal cortex (FIGURE 1). The diurnal and ultradian rhythms of cortisol (in humans) and corticosterone (in rodents) secretion are regulated by negative-feedback loops (FIGURE 1). For example, a high GC level suppresses the expression of the adrenocorticotropic hormone precursor proopiomelanocortin and corticotropin-releasing factor, thus shutting down central synthesis of GCs. This tight control of systemic GC secretion allows well-regulated mobilization of required energy (e.g., elevation of glucose levels) in a stress situation or at the beginning of a day. Concomitantly, it prevents adverse effects due to sustained GC exposure.

In addition to adrenal GC synthesis, production of minimal GC amounts can be observed in other tissues such as the brain, gastrointestinal tract, skin, and thymus (322). However, how GC synthesis in these tissues is controlled, is largely unexplored.

The pharmacological application of GCs was established in the late 1940s when it was recognized that GCs were among the most potent immunosuppressant agents for the treatment of chronic inflammatory disorders such as allergic...
shock, asthma, inflammatory bowel disease, and arthritis. Prolonged treatment with GCs (20% of all GC medications are long-term) (226), as well as disturbed negative-feedback loops (as seen in Cushing’s syndrome) or exposure to chronic stress causes severe complications. These include insulin resistance, atrophies of muscle and skin, depression, and severe effects on the skeletal system.

The skeleton, in contrast to traditional views, is a highly dynamic organ. In fact, bone is the only organ that is actively degraded. In cases of strong physiological demands such as low calcium levels, less weight loading on the bone, or pregnancy and lactation, bone matrix is demineralized and bone matrix tissue is degraded. This phenomenon of bone resorption is carried out by hematopoietic-derived multinuclear osteoclasts. These specialized multinucleated cells generate an acidic environment at their sealed basolateral membrane, thereby decalcifying the bone matrix. However, under physiological conditions, the resorptive activity of osteoclasts is strongly coupled to the activity of mesenchyme-derived bone-forming osteoblasts (341).

These cells can give rise to osteocytes embedded in bone matrix. Osteoblasts and osteocytes control the formation of osteoclasts by modulating the expression of osteoclast-inducing factors such as macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL). Under normal physiological conditions in the healthy adult, this bone remodeling is in balance. If resorption exceeds bone formation, bone mineral density decreases, fracture risk increases, and as a result osteoporosis develops. GC excess as a result of long-term high-dose corticosteroid therapy induces immune suppression, GC-induced osteoporosis, adiposity, and insulin resistance.
growth plates and cartilage components have been proposed as underlying causes for GC effects on growth.

Although the multiple clinical effects associated with GC administration have been known for decades, the cell type-specific molecular actions in the context of either therapeutic use or action of endogenous hormones are still far away from being comprehensively understood. The recent discoveries concerning the molecular mechanisms of GC actions are mainly a result of genome-wide analyses on transcriptional control by the GC receptor (GR), a GC-activated transcription factor. The GR interacts with chromatin and numerous other transcription factors in a cell type-specific manner. Furthermore, selective inactivation of GC signaling in mouse models either by misexpressing the GC activating/deactivating 11β-hydroxysteroid dehydrogenases 1 and 2 (11β-HSD1, 11β-HSD2) or by conditional and functional inactivation of the GR itself revealed fundamental insights into GC actions in cartilage and bone. Immense progress has been made in the last 15 years in addressing the cellular processes controlled by GCs and their receptor, occasionally leading to conflicting data caused by the complexity of the different experimental models used.

Therefore, in this review, we discuss in depth the recent advancements to understand the versatile functions of the GR in general. We particularly focus on GR protein structure, different mechanisms of gene regulation, and cell type-specific crosstalk of the GR with chromatin and its implications in gene regulation. We then describe the current understanding of how GCs influence the lineage decisions of mesenchymal stem cells (MSCs) to generate cartilaginous cells, osteoblasts, and adipocytes. The ability of GCs to change the cell fate are suggested to have dramatic impact on the skeletal system. Although GC effects on MSCs have been intensively studied in cell culture, the impact of GCs on mesenchymal precursor cells in vivo remains unclear. With the recent identification of skeletal stem cells in mice by lineage tracing (417), a new chapter of research on GR function in mesenchymal cells in vivo is imminent.

We subsequently provide an update on the current models of GC effects on chondrocytes and the cartilaginous growth plate as an underlying mechanism of decelerated growth in children. Since GIO is one of the major complications of long-term GC treatment, we intensively review the current knowledge of GC effects on bone integrity. Furthermore, we focus on the possible mechanisms of GC-mediated anti-inflammatory effects in inflammatory bone diseases, since GCs are still among the most frequently used therapies (often in combination therapy with, e.g., methotrexate) of rheumatoid arthritis (RA) (NCT02000336; Refs. 4, 276). Recognizing a major role of the bone in overall glucose and energy homeostasis, a provocative model has recently been proposed implicating that one of the other major side effects of GC therapy, namely, insulin resistance, might be mediated directly by the actions of GCs on osteoblasts. Finally, we present an outlook on how our current understanding of GC action in bone tissues and bone diseases can be translated into new strategies in the future to avoid adverse effects on cartilage and bone.

II. GLUCOCORTICOIDS REGULATE GENE EXPRESSION VIA MULTIPLE MECHANISMS

A. The Glucocorticoid Receptor

The cellular response to GCs is generally mediated by the GR (NR3C1), which belongs to the nuclear receptor superfamily of ligand-dependent transcription factors (94, 221). The GR is ubiquitously expressed and is crucial for organ maturation during embryonic development as revealed by a complete GR knockout mouse model (67). The GR controls transcription, often dependent on the physiological state and the specific cell type, to modulate diverse processes such as cell proliferation, apoptosis, and cellular differentiation. Moreover, transcription of genes is either positively or negatively regulated by the GR, which refers to the transcription or transrepression function of the GR, respectively. Apart from transcriptional activity, nongenomic actions have been described; however, their relevance has been less investigated. The multiple domains of the receptor involved in DNA binding, ligand binding, and transcriptional regulation constitute the molecular basis of GC actions via the GR. In this section, the molecular functions of the GR as a multidomain protein are discussed in general to lay out the molecular underpinnings of the specific role of the GR in cartilage and bone cells.

B. Structural Organization of the Glucocorticoid Receptor and Its Isoforms

The GR is composed of four different domains. These encompass the NH2-terminal transactivation domain or activation function 1 (AF-1), the DNA-binding domain (DBD), the hinge region, and the ligand-binding domain (LBD) (FIGURE 2) (184). The AF-1 domain is a hub for co-regulators (co-repressors and co-activators, see below), chromatin-remodeling enzymes, RNA polymerase II, and the basal transcription machinery (9). The DNA-binding domain of the GR recognizes and binds to specific DNA sequences in the promoter region of its target genes. Historically, these DNA sequences were considered to be located relatively close to the vicinity of the regulated gene. However, there is now increasing evidence from genomic approaches that the DNA response elements are often located far away, even on separate chromosomes (130)). The hinge region allows the translocation of the receptor protein to the nucleus. The main function of the LBD is the binding of naturally occurring (cortisol/corticosterone) or synthesized GCs (dexe-
methasone/prednisolone). Furthermore, the LBD contains several dimerization interfaces, indicating that multiple domains are involved in the dimerization of the receptor protein (251, 286). It is likely that not all dimerization interfaces are known, since an X-ray or cryo-electron microscopy structure of the full-length GR bound to DNA as a homodimer has yet to be reported. However, the solved structures of full-length proteins of the nuclear receptor heterodimers peroxisome proliferator-activated receptor gamma (PPARγ)/retinoid X receptor (RXR) and vitamin D receptor/RXR for example have revealed multiple unexpected dimerization interfaces between both partner molecules (57, 262).

Multiple isoforms of the GR are generated by alternative splicing and alternative translation initiation (FIGURE 2). Alternative splicing of the GR primary transcript generates the isoforms GRα, GRβ, GRγ, GR-A, and GR-P (FIGURE 2) (16, 249, 258, 293). The “classical” and most abundant GR protein is the GRα isoform. The GRβ isoform, expressed by many cell types including osteoclasts, osteoblasts, osteocytes, epithelial cells, and neutrophils (25, 239), can regulate the expression of many genes not sensitive to GRα regulation (170, 172, 197). Furthermore, the GRβ isoform has been proposed to attenuate the transcriptional activity of GRα and therefore act (at high GC concentrations) as a dominant negative inhibitor, which may contribute to GC resistance (193). However, this has recently been challenged by overexpression of GRβ in zebrafish, which did not reduce GRα-mediated gene regulation as revealed by microarray analysis of the mRNA expression profile (59).

Insertion of the amino acid arginine between the two zinc fingers of the GR DNA-binding domain (Arg-452) results in the GRγ isoform (293, 303). This isoform binds DNA and GCs to the same extent as GRα. However, the expression of GRγ is associated with GC resistance, since amplification of this isoform in acute lymphoblastic leukemia cells rendered cells more resistant to GC-induced apoptosis (26). In addition, expression profiling with GRγ overexpression revealed that GRγ regulates genes not regulated by GRα (320). In contrast to GRγ, the two isoforms GR-A and GR-P are unable to bind GCs due to missing sequences in their LBD. The isoform GR-P, among others, is highly expressed in GC-sensitive myeloma cells (314) while the iso-

![FIGURE 2. Domain structure of splice variants and isoforms of the glucocorticoid receptor. A: the primary transcript of the GR is made up of nine exons. Exon 1 forms the 5'-untranslated region (5' UTR), whereas exons 2 to 9 form the protein-coding region. In this process, exon 2 encodes the A/B-domain or NH₂-terminal transactivation domain (NTD) which contains the ligand-independent activation function 1 (AF-1); exons 3 and 4 encode the C-domain with the DNA-binding domain (DBD); exon 5 encodes the D-domain, the hinge region involved in nuclear localization; while the other exons (up to exon 9) encode the E-domain containing the ligand-binding domain (LBD) and a nuclear localization signal, involved in the dimerization and Hsp90 binding. Alternative splicing of the GR primary transcript (inter alia in exon 9) generates the isoforms GRα, GRβ, GRγ, GR-A, and GR-P, which differ in the length and sequence of their hinge region and/or their ligand-binding domain. B: alternative translation initiation in exon 2 of the GRα mRNA generates the isoforms GRα-A, GRα-B, GRα-C1, GRα-C2, GRα-C3, GRα-D1, GRα-D2, and GRα-D3, which differ in the length of their A/B-domain and possess shorter NTDs (reviewed in Oakley and Cidlowski, Ref. 256).]
form GR-A is mainly expressed in mature dendritic cells, which probably mediates GC sensitivity towards apoptosis in these cells (54).

In addition to alternative splicing, alternative translation initiation of the GR generates multiple isoforms. The GRα mRNA contains eight different translation initiation codons in exon 2, which generate eight NH2-terminal-truncated GRα isoforms named GRα-A, GRα-B, GRα-C1, GRα-C2, GRα-C3, GRα-D1, GRα-D2, and GRα-D3 (Figure 2). GRα-A refers to the full-length receptor with 777 amino acids, whereas all the other isoforms have up to 335 amino acids shorter NH2-terminal transactivation domains. Furthermore, the mRNA molecules of the GR splice variants GRβ, GRγ, GR-A, and GR-P exhibit the same eight translation initiation codons as GRα, resulting in the ability of these variants to generate additional subsets of GR isoforms (174, 256). Signal transduction is modulated by the expression level, stability, and activity of these GRα isoforms. All eight isoforms can bind to DNA in the same manner and exhibit a similar ligand affinity (257). Nonetheless, they show obvious differences in other functions such as subcellular localization and regulation of gene expression (212, 213), thus exhibiting only a minor functional redundancy (32).

Currently, overexpression studies of the different isoforms in human osteosarcoma cells (U2OS) have demonstrated selective functions in the context of gene regulation and induction of apoptosis (213). However, the extent of the contribution of endogenous isoforms in cartilage and bone cells to GC effects remains completely unexplored.

C. Glucocorticoid Signaling and Glucocorticoid Receptor Activation

In the absence of ligands, the GR is predominantly localized in the cytoplasm, bound to immunophilins, like FK506-binding protein 51 (FKBP51) and FK506-binding protein 52 (FKBP52), heat shock/chaperone proteins (e.g., Hsp70, Hsp90, and p23), as well as to other proteins (120, 282, 284, 285) (Figure 3A). Hsp70 recognizes newly synthesized GR molecules (347) and binds to the LBD of the GR (319). Co-chaperone protein Hsp40 facilitates the binding of Hsp70 to the GR to form a complex of low binding affinity to GCs. However, this complex allows binding of Hsp90 dimers, which increase the affinity of the GR for the ligand (299). On the one hand, the chaperone complex facilitates ligand binding and the stability of the receptor, participating in the proper folding of the GR as well as in its translocation into the nucleus. On the other hand, the complex modulates the transcriptional activation or repression of target genes (120). Furthermore, Hsp90 involved in the formation of a hormone-dependent activation state of the GR controls the final maturation of the GR and permits co-regulators and ligands to act as allosteric effectors (79, 312). Inhibition of Hsp90 and its activity/function influences the GR on diverse levels and leads to, for example, a reduced transactivation and an enhanced degradation of the GR (42, 182, 308).

The chaperonin-acting immunophilin proteins FKBP51 and FKBP52 are considered as negative and positive regulators of the transcriptional activity of the GR, respectively (301, 394). FKBP51 inhibits the translocation of the GR into the nucleus, whereas FKBP52 enhances the nuclear translocation of the GR via enhanced binding to dynein (74, 415). Being a rapidly induced target gene of the GR, FKBP5 (encoding FKBP51) acts in a negative-feedback loop of GR activity to limit the excess of GC responses (149). The increased expression of FKBP51 in neuronal cells in humans has been linked to an altered GR activity that contributes to GC resistance, which might lead to stress-related psychiatric disorders like major depression or bipolar disorders (35, 107, 177).

Endogenous GCs, like cortisol in humans and corticosterone in rodents, are predominantly bound to transcortin, the corticosteroid-binding globulin, which allows the transport of the bound hormones into the blood. Transcortin plays an important role in the distribution and release of the hormones to target cells and tissues. Upon entering the cell, the bioavailability and activity of the GCs are controlled by the enzymes 11β-HSD1 and 11β-HSD2, which act in an opposing manner (326). 11β-HSD1 converts the inactive hormone cortisone into the active form cortisol, whereas 11β-HSD2 oxidizes cortisol into cortisone. Thus altered expression levels and activities of 11β-HSD1 and 11β-HSD2 contribute to the diverse GC sensitivity of different cell types and may have an influence on insulin resistance, metabolic syndrome, and obesity (397a). In contrast to the endogenous cortisol, synthetic GCs (like dexamethasone) are not extensively metabolized by 11β-HSD2. The ineffective inactivation and high affinity of dexamethasone to the GR could lead to a prolonged activity of the GR. In addition, A-ring reductases, in particular 5α-reductase in hepatocytes, can inactive GCs (246), and their reduced activity leads to nonalcoholic fatty liver disease, presumably by augmenting cortisol action.

The binding of its ligand leads to a conformational change of the GR. Consequently, this promotes its translocation into the nucleus and subsequent binding to the corresponding DNA sequences as well as the recruitment of co-factor (co-repressor or co-activator) complexes for regulation of gene transcription (76).

Instead of being dissociated from the chaperone complex after ligand binding as shown previously (229, 313), translocation into the nucleus essentially requires the recruitment of FKBP52 to the Hsp90-p23-GR protein complex
**A**

- Fraction of membrane receptors
- High affinity Cortisone (inactive)
- Low affinity Cortisone (active)
- Non-genomic action
- Cytoplasm
- Nucleus
- Membrane
- Mitochondrion

**B**

- Pioneering TFs
- Closed chromatin densely packed with nucleosomes
- Open chromatin

**C**

- Redistribution of monomer towards dimer upon pharmacological GCs (Lim et al. 2015)
- Lineage specific TFs
- NF-kB/AP-1 binding sites

**D**

<table>
<thead>
<tr>
<th>GR-Monomer Dependent Processes</th>
<th>GR-Dimer Dependent Processes</th>
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<tbody>
<tr>
<td><strong>MSC Differentiation</strong></td>
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<tr>
<td>Inhibition of bone formation (Rauch et al. 2010)</td>
<td>Adipogenic differentiation (Asada et al. 2011)</td>
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<td><strong>Metabolism</strong></td>
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<td>Metabolic liver gene expression in liver (Frijters et al. 2010)</td>
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<td><strong>Inflammation</strong></td>
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<tr>
<td>Inhibition of T cell migration (Schweingruber et al. 2014)</td>
<td>Suppression of arthritis (Baschant et al. 2011)</td>
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<tr>
<td>Inhibition of irritant skin inflammation (Reichardt et al. 2001)</td>
<td>Suppression of lung injury (Vettaroazzi et al. 2015)</td>
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<td>Suppression of contact allergy (Tuckermann et al. 2007)</td>
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**References:**
- Lim et al. 2015
- Rauch et al. 2010
- Asada et al. 2011
- Frijters et al. 2010
- Schweingruber et al. 2014
- Reichardt et al. 2001
- Vettaroazzi et al. 2015
- Kleiman et al. 2012
- Silverman et al. 2013
- Tuckermann et al. 2007
indicating that the integrity of the chaperone complex appears to be crucial for the translocation of the GR (90, 390).

The nuclear localization sequences of the GR bind distinct importins (alpha 7 and 8), which allows the GR chaperone complex to interact with elements of the cytoskeleton and to move along the microtubules into the nucleus (106, 283, 415).

The GR undergoes multiple posttranslational modifications including phosphorylation, acetylation, and sumoylation. Some of them are directly linked with ligand binding, such as phosphorylation on residues S203 and S211. These modifications are linked to transcriptional activity, the determination of the stability of the protein, and possible as yet unknown functions (161).

D. Gene Regulation by the Glucocorticoid Receptor

1. Chromatin interactions of the glucocorticoid receptor

Within the nucleus, the ligand-activated GR has to find its target sequences in the genome. A major determinant of genomic GR binding and subsequent gene regulation is the accessibility of chromatin (33, 158). The DNA in all eukaryotic cells is tightly packed into nucleosomes, which are the basic elements of chromatin (45). Genome sequencing studies following DNase I treatment and formaldehyde-assisted isolation of regulatory elements (FAIRE) sequencing have revealed accessible DNA sites for DNA-binding proteins that are not wrapped into nucleosomes throughout the genome (377). Such analyses also revealed that patterns of accessible sites are cell type specific (372). So-called pioneering transcription factors (337) initiating chromatin remodeling define the chromatin landscape accessible for the GR (FIGURE 3B). Depending on the cell type, distinct pioneering transcription factors allow GR binding to open chromatin sites (158). Such pioneering factors facilitating GR binding to open chromatin are AP-1 in mammaeal cells (34) and myoblast cell line C2C12 (185); CCAAT/enhancer-binding protein (C/EBP)β in liver (122) and 3T3-L1 preadipocytes (355); HNF3 and NF1 in pituitary A20 cells (158); PU.1 in macrophages (380); and Gabpa, Prrx2, Zfp281, Gata1, and Zbtb3 in neuronal cells (281). For preadipocytic 3T3-L1 cells, it has been shown that multiple transcription factors including the GR occur clustered as “transcription factor hotspots” at distinct sites in the genome (337) (FIGURE 3B). Furthermore, these clusters are often organized into large genomic stretches (termed “super-enhancers”) which are decisive in determining cell fates (208, 413). These super-enhancers facilitate the binding of many transcription factors that interact with the mediator complex to ensure transcriptional activation (208, 413) (FIGURE 3B). At least in adipocytes, the GR participates in the occupancy of super-enhancers (338). Of note, in human osteoblasts, super-enhancers have been identified that also contain GR motifs (131). However, the functional relevance of these super-enhancers for osteoblast function needs to be proven.

Importantly, once the GR is bound to GR-binding sites in the genome, the chromatin accessibility is enhanced due to chromatin remodeling, thus allowing the recruitment of further transcription factors, including additional GR molecules, to the same sites. This has been described as “assisted loading” (397). Only for a minority of GR-binding sites, the ligand-activated GR creates de novo accessible chromatin sites and thus acts as a pioneering factor, at least in the cells and tissues analyzed to date (122, 158).

In summary, GR occupancy in the genome upon GC exposure is cell type specific and is determined to a large extent by cell type-specific accessible sites in the genome.

2. Glucocorticoid receptor-binding DNA sequence motifs

In addition to accessible chromatin, the GR requires particular DNA sequences in GR-binding sites to influence transcription. The classical GR-binding motif was initially identified as the glucocorticoid response element (GRE) in the

FIGURE 3. Glucocorticoid receptor activation, interaction with chromatin, and modes of transcriptional regulation. A: GCs enter cells and become activated by 11β-HSD1 or occasionally deactivated by 11β-HSD2. The activated GCs bind to a cytoplasmic protein complex containing heat shock proteins and the GR. Complexes with Hsp70 and Hsp40 render the GR as a low-affinity receptor; however, complexes with Hsp90 give rise to a high ligand affinity of the GR. Upon ligand binding, the chaperone FKBP51 is exchanged for FKBP52, thereby allowing shuttling of the complex into the nucleus and into contact with the chromatin. As well as nuclear actions, nongenomic effects of the liganded GR are also well-indicated to be crucial for the translocation of the GR (90, 390). B: interaction of the GR with the chromatin is in most cases facilitated by the prior activity of pioneering transcription factors that made the chromatin region accessible. Multiple binding sites in close proximity allow the binding of cell lineage-specific and hormone-inducible transcription factors. The GR and these locations are designated as transcription factor hot spots. The binding of and assembly of many transcription factors to large complexes interacting with the mediator complex at so-called super-enhancers facilitate potential transcription of lineage-specific and also hormone-inducible genes. C: ligand-bound GR regulates gene expression in multiple ways: direct binding of GR homodimers to positive and negative GR-binding sites (GBS); tethering as monomer proteins to other DNA-associated transcription factors such as NF-κB, AP-1, and IRF3 and thereby often reducing their activity; binding as homodimers in the vicinity of NF-κB- and AP-1-binding sites; and binding as monomers in the vicinity of lineage-specific transcription factors. D: GC effects that require either GR dimerization or rather GR monomer activities are shown.
promoter region of target genes (22). This classical GRE consists of an imperfect palindromic DNA sequence with two hexameric half-sites which are separated by three base pairs (GGAACAnnnTGTTCT). Parts of the consensus sequence can be flexible, which determines the outcome of the transcriptional activity of the GR (161, 228). However, genome-wide studies have revealed that the classical (positive) GRE GGAACAnnnTGTTCT is not a predictor of GR occupancy in the genome in vivo (158). DNA sequence motifs for GR binding are much more versatile.

An additional set of GR-binding motifs has been designated as “negative GREs” (nGREs) due to their association with genes downregulated by GC action. First identified in the proopiomelanocortin gene and for a long time defined by a conserved half-site (86), they have recently been extended to elements with two hexameric half-sites. These are arranged as an (imperfect) palindromic sequence (362) with a reduced spacer between the two half-sites which ranges from 0–2 nucleotides [CTCC(n)0–2GGAGA]. This specific site was functionally characterized in the Tslp (thymic stromal lymphopoietin gene) promoter. It leads to the recruitment of the co-repressors silencing mediator for retinoid and thyroid receptors (SMRT) and nuclear receptor corepressor (NCoR), which consequently recruit histone deacetylases (HDACs) and repress target genes (362). Although these sites were predicted in more than 1,000 genes by bioinformatics approaches (362), they were seldom found in genome-wide ChIP-Seq studies (380), indicating that only a minor portion of these sites is of functional importance.

Interestingly, half-sites of the classical GREs are occupied in the genome much more frequently than previously anticipated (203, 320). These sites are occupied by GR monomer proteins. The GR A477T mutation (GRdim), a mutant in which the dimerization interface in the DNA-binding domain is disrupted via an amino acid substitution in the D-loop of the GR DNA-binding domain (127, 295), occupies this half-site GRE motif as efficiently as the wild-type protein (203, 320). ChIP-exonuclease profiling in mice carrying the GRdim mutation confirmed the inability to form homodimers in vivo (203). In vitro studies, in contrast, showed homodimerization upon overexpression (153, 286). Some of the GRE half-sites have recently been found to be part of the recognition motif of the TEA domain protein/transcription enhancer factor (TEAD/TEF), allowing binding of a GR/TEAD heterodimer (354).

In summary, multiple GR-binding motifs are responsible for GR recruitment to DNA and positive and negative gene regulation. Genome-wide analyses have revealed that the classical GREs are represented to only a minor extent and that half-sites are more prominent in the genome. The occurrence of motifs of many other transcription factors in the vicinity of GR-binding sites indicates that crosstalk with other transcription factors is a major feature of gene regulation by GCs.

E. Crosstalk with Other Transcription Factors

The GR maintains intensive crosstalk with other transcription factors, thereby influencing their activity and vice versa (23). If the GR does not bind DNA, but rather participates in protein-protein interaction with other DNA-bound transcription factors, this process is designated as “tethering mode” (FIGURE 3D). Such protein-protein interactions occur between the GR monomer and factors involved in inflammation such as NF-κB, AP-1, IRF-3, STAT3, and STAT5 (75, 188, 358). These interactions are facilitated by co-regulatory proteins such as TRIP6 or glucocorticoid receptor-interacting protein 1 (GRIP1) (167, 298). Since tethering often leads to downregulation of genes encoding cytokines or metalloproteases, this mechanism was proposed to be causal for the anti-inflammatory activity of GCs, thus leading to the development of selective GR modulators and agonists that should favor the transpressive functions of the GR (75) but avoid transactivation of classical GRE-containing promoters. The promise of these compounds was a reduced side effect profile by maintaining anti-inflammatory efficacy. However, only a few compounds made it into clinical trials. A fact that could be explained by an insufficient suppression of inflammation by GR monomers. Preclinical studies were performed with GRdim mice (295) which express a dimerization-impaired GR that still acts as monomer protein. These mice demonstrated that efficient GR dimerization is required to suppress many inflammatory diseases, including septic shock, acute lung injury, contact allergy, and arthritis (20, 176, 339, 379, 396). Only in rare examples of inflammatory models, such as irritated skin inflammation (296) and experimental autoimmune encephalomyelitis (325), GCs reduce the inflammatory response.

Additionally, crosstalk of the GR with pro-inflammatory transcription factors also occurs when both the GR and NF-κB or AP-1 are directly bound to DNA in close proximity (380) (FIGURE 3C). ChIP-Seq experiments suggest that this situation is much more abundant than tethering (34, 380). This questions the overall relevance of tethering as a major mechanism for immune suppression. However, further evidence is needed to clarify whether the crosstalk based on such composite elements substantially exceeds tethering. Future experiments using ChIP-exonuclease sequencing will allow a higher resolution of mapping of GR-bound elements (203, 354).

The collective evidence suggests that compounds avoiding GR dimerization and just addressing the tethering mechanism will not exhibit full anti-inflammatory efficacies in inflammatory diseases (see sect. VII).
F. Co-regulators

Transcriptional regulation by the GR requires the interaction of co-regulatory proteins with the activation function domains. The activation function 1 domain (see above) interacts in a ligand-independent manner with ATPase BRG1, which recruits histone acetylases such as p/CAF and CBP/p300 (426). The activation function 2 domain acts in a ligand-dependent manner and recruits co-activators from the p160 family, such as SRC-1 or TIF2/GRIP1. These co-activators themselves possess histone acetylase activity, leading to further decondensation of chromatin and facilitating the recruitment of additional factors and finally activation of the basal transcription machinery (60, 217).

Transcriptional repression requires the recruitment of corepressors such as SMRT and NCoR (117). The interaction with HDACs, such as HDAC3, is pivotal to the removal of acetyl groups from histones, leading to tighter packing of the chromatin (117).

In some cases, co-regulators display co-activator as well as co-repressor activity. For example, GRIP1, which usually acts as a co-activator for the ligand-bound GR, can also inhibit the transcriptional activity of the GR, dependent on the cellular context (380). One attractive model is that the GR sequesters GRIP1 away from DNA-bound transcription factors and thereby reduces their activity (298).

G. Nongenomic Actions of the Glucocorticoid Receptor

Some of the GR actions are suggested to be carried out not only by acting on DNA and gene regulation, but also by nongenomic effects. These effects occur within seconds or minutes and are only observed following high-dose GC treatment (209). To date, there are four subcategories of nongenomic GC actions described: 1) membrane-bound GR (mGR) actions (19), 2) cytosolic GR actions (71, 204), 3) direct physiochemical interactions of GCs with cellular membranes (50), and 4) mitochondrial GR signaling (318).

The human mGR was first described in 1993 on malignant lymphocytes (108) and later confirmed in healthy peripheral blood mononuclear cells (19). However, the expression level of mGR revealed by immunostaining is much less than cytoplasmic or nuclear GR (395). Interestingly, the low expression can be increased upon lipopolysaccharide exposure (19). Treatment with membrane-impermeable dexamethasone linked to bovine serum albumin has been reported to induce mGR-dependent phosphorylation of p38 and prostaglandin synthase (361, 395).

Löwenberg and co-workers (210, 211) also revealed nongenomic immunosuppressive effects of the GR via a T-cell receptor (TCR)-associated protein complex. The association includes interactions with protein kinases, lymphocyte protein tyrosine kinase (Lck), and Fyn proto-oncogene (Fyn) (211). Intriguingly, knockdown of the GR or Hsp90 resulted in the disruption of proper TCR signaling by disruption of the enzymatic activity of Lck and Fyn (211). These studies suggest that upon ligand binding, the GR dissociates from the TCR multiprotein complex, resulting in the inhibition of Lck and Fyn kinases (210). However, an involvement of the unliganded GR in proper TCR signaling has been disproved in mice lacking the GR in T cells (233).

In addition to GR-dependent mechanisms, high-dose GCs leading to almost complete saturation of the cytosolic GR even exhibit direct effects on physical and chemical properties of the plasma membrane (50) such as fluidity (169) and ion uptake from membranes (49), thereby affecting the distribution of ion channels.

Finally, the GR could act in mitochondria in a genomic manner (77, 318). Intriguingly, a mitochondrion-specific GR construct (MLS Cox-GFP-GR) was sufficient to initiate rapid nongenomic translocation of the GR into the mitochondria, whereas the translocation into the nucleus was not affected (342).

To conclude, the nongenomic actions of GCs occur very rapidly, depend partly on high doses, and are associated with various signaling pathways. However, due to the low expression of the GR in mitochondria and the membrane, the physiological and pharmacological significance of these GR-mediated effects needs to be further investigated.

In summary, the GR is a versatile molecule that mediates GC actions mainly by alterations of gene expression via multiple cell type-specific interactions with chromatin, transcription factors, and co-regulators. Rapid nongenomic effects of the GR have been observed as well (353). However, it is likely that the GR actions on the genome are the basis of most observed GC actions in the stress response and therapy. The involvement of these multiple distinct mechanisms in bone physiology is only partially known and is the subject of intensive research that is discussed in the following sections.

III. INFLUENCE OF GLUCOCORTICOIDS ON THE GROWTH PLATE DURING LONGITUDINAL GROWTH

A major cause of morbidity in children under steroid therapy is severe growth retardation. The underlying mechanisms of direct and indirect actions of GCs on the growth plate are discussed in this chapter.
A. Growth Plate Homeostasis and Longitudinal Growth

During development and postnatal life, long bones grow in length by endochondral ossification and in width postnata- tally by appositional growth. For the endochondral ossification, cartilaginous growth plates develop on both diaphyseal ends of the bones. Chondrocytes, initially derived from limb mesenchyme during embryonic development, are located in three distinct zones of the growth plate: the resting zone, the proliferative zone, and the hypertrophic zone (46) (FIGURE 4). The resting zone contains self-renewing, slow-proliferating chondrocytes (1), generating highly proliferative chondrocytes that form columns along the bone axis and compose the proliferative zone. Chondrocytes further away from the end of the bone stop proliferating and become hypertrophic chondrocytes due to enlargement. The proliferating chondrocytes produce specific extracellular matrix (ECM) proteins like collagen II and aggregans (183), whereas the hypertrophic chondrocytes synthesize collagen X and start the mineralization of their surrounding matrix (29). The hypertrophic chondrocytes produce vascular endothelial growth factor (VEGF) to attract blood vessel migration. This, in turn, recruits monocyte-derived chondroclasts that degrade the chondrocyte-produced matrix. Subsequently, hypertrophic chondrocytes undergo apoptosis (113) and osteoblasts invade into the chondrocyte matrix lacuna, building a bone matrix, the primary spongiosa. These ongoing processes of proliferating chondrocytes, that mature during growth, mineralize their surrounding matrix and finally get replaced by osteoblasts, leading to longitudinal bone growth. With age, the growth plate depletes over time due to senescence of the chondrocytes and is completely replaced by bone at the time of sexual maturation.

GCs have profound effects on longitudinal bone growth. Intriguingly, while pharmacological exposure of children to GCs severely attenuates growth, withdrawal of GCs leads to a phenomenon of catch-up growth, i.e., an accelerated activity of the growth plate to rapidly achieve the size matching the age (215). Also, during malnutrition, enhanced endogenous GCs might impair growth in combination with the decline of anabolic hormones (110). Malnutrition-impaired growth in children leads to a rise of endogenous GCs, whereas the concentration of growth-promoting hormones such as thyroid hormones and insulin-like growth factor I (IGF-I) declines.

FIGURE 4. Glucocorticoids exert various effects on chondrocytes in the different zones of the growth plate and interfere with longitudinal bone growth. The growth plate consists of three distinct zones: 1) the resting zone containing self-renewing and slow-proliferating chondrocytes, providing highly proliferative chondrocytes for 2) the proliferation zone, and 3) the hypertrophic zone containing enlarged chondrocytes which mineralize their ECM, building a template for bone-forming osteoblasts. GCs inhibit proliferation of chondrocytes in the resting and proliferation zones of the growth plate via inhibition of pro-proliferative GH and IGF-I, as well as inhibition of ERK-dependent AP-1 activation. GCs induce apoptosis via the induction of caspases and interfere with the Bcl-2 family in proliferating and hypertrophic chondrocytes leading to compromised growth plate integrity. Pro-apoptotic Bcl-x and Bcl-2 are upregulated, and anti-apoptotic Bad-, Bax-, and Akt-phosphorylation is inhibited through GCs. Contradictory effects are reported regarding GC effects on the maturation of hypertrophic chondrocytes. Inhibition of GH and induction of lipocalin-2, Sox9, and ColXK point to the induction of chondrocyte maturation by GCs, but the controversial results on collagen I and II and on ALP activity prohibit a clear statement. Nevertheless, GCs affect hypertrophic chondrocytes by inhibiting their VEGF expression, resulting in a disturbed blood vessel migration and formation.
Despite the strong direct effects of exogenous GCs on chondrocytes and growth plates, endogenous GCs appear to play a minor role, at least in the adolescent and adult organism. Mice lacking the GR primarily in chondrocytes (GR\textsuperscript{Col2a1ERT2Cre}) exhibit normal long bone growth and trabecular architecture (378). Even though chondrocytes are essential for endochondral ossification and thus involved in long bone growth and trabecular bone formation, actions of endogenous GCs in chondrocytes appear to be unnecessary for maintaining bone mass as well as for early bone development (at the embryonic stage). This is in accordance with studies revealing normal skeletal development in GR-null mice (290). Although these mice die postnatally due to impaired lung development, skeletal analysis by X-ray imaging and alizarin red/alcian blue staining indicated no abnormalities (290). In line with this, mice with GR deletion in mesenchymal cells that give rise to chondrocytes, osteoblasts, myoblasts, and fibroblasts (GR\textsuperscript{Dermo1Cre}) die postnatally, but display a normal skeletal phenotype and normal growth plate development (194).

How exogenous GCs influence longitudinal growth remains completely unexplored, but presumably indirect and direct effects of GCs on the growth plate are required as described below.

**B. Endocrine Control of Growth Plate Homeostasis**

Growth plate homeostasis is centrally controlled, in particular by the growth hormone (GH) IGF-I axis and thyroid hormones which are targeted by exogenous GC therapy on different levels. This results in delay of endochondral ossification and growth retardation.

1. **GH and IGF-I**

GH and IGF-I stimulate longitudinal bone growth (146, 321) through the induction of chondrogenesis (321) and maturation of chondrocytes (403). Deficiency of GH leads to a reduction in postnatal growth (434), and lack of IGF-I results in pre- and postnatal growth retardation (416). GH induces liver IGF-I and thus some of the effects of GH are executed by liver-derived circulating IGF-I levels. This concept is termed “dual effector hypothesis.” Accordingly, the hepatic deletion of IGF-I and its complex compound acid labile subunit attenuates longitudinal growth (259, 421). However, GH also has direct effects on the growth plate resulting from enhancement of resting zone chondrocytes to generate proliferating chondrocytes (259) and stimulation of chondrocyte maturation. Thus both GH and IGF-I signaling are required for proper growth and can be affected by GCs on several levels.

The synthetic GC dexamethasone is able to inhibit GH receptor, IGF-I, and IGF-I receptor expression in primary growth plate chondrocytes (160). Contradictory studies report an increase of IGF-I receptor and GH receptor mRNA (129, 346) upon dexamethasone treatment. The discrepancies could be due to different culture systems, different species, and possibly due to loss of paracrine factors in isolated chondrocytes which are present in the growth plate (see below). Nonetheless, Chrysis et al. (65) showed that the dexamethasone-induced decrease of chondrocyte proliferation rate is significantly reversed by IGF-I treatment. Since inhibition of phosphatidylinositol 3-kinase (PI3K) signaling totally abolishes proliferation of chondrocytes in vitro (218) and prevents further inhibitory effects on proliferation after dexamethasone treatment (218), PI3K signaling might be involved in dexamethasone-induced growth retardation. In support of this notion, dexamethasone is able to inhibit the catalytic function of PI3K by an increase of the 85α subunit, resulting in reduced Akt phosphorylation (115). Thus inhibition of IGF-I signaling contributes at least partly to dexamethasone-induced growth retardation.

2. **Thyroid and parathyroid hormones**

The thyroid hormones triiodothyronine (T\textsubscript{3}) and its precursor thyroxine (T\textsubscript{4}) control the transition of chondrocytes to hypertrophic chondrocytes (357). Hypothyroidism inhibits longitudinal growth and endochondral ossification, whereas hyperthyroidism accelerates both processes (43, 259). Stevens et al. (357) have shown that thyroid hormones control the expression of parathyroid hormone-related protein (PThrP) in the growth plate. PThrP reduces the differentiation of prehypertrophic chondrocytes to hypertrophic chondrocytes, and PThrP expression is increased in hypothyroid growth plates (357). Accordingly, PThrP-knockout mice display skeletal deformities and decreased proliferation and accelerated maturation of chondrocytes (8). This effect of PThrP is partially regulated via runt-related transcription factor 2 (Runx2)-dependent pathways (123). GCs are able to inhibit PThrP expression in the growth plate, leading to growth retardation (64). Parathyroid hormone (PTH) application, addressing the same receptor as PThrP, partly normalizes the defects in growth plate architecture in dexamethasone-treated mice, but does not ameliorate the growth retardation (383). In contrast, T\textsubscript{3} treatment, which efficiently rescues dexamethasone-induced growth defects, does so at the expense of a disorganized growth plate (383). The precise mechanism how GCs affect signaling of PTH and PThrP as well as thyroid receptor actions and respective target genes remains elusive.

**C. Direct Effects of Glucocorticoids on the Growth Plate**

Profound effects of GCs on cartilage and chondrocyte cultures have been demonstrated. These refer to direct effects of GCs on the growth plate. The GC treatment of cultured...
metatarsal murine bones, primary growth plate chondrocytes (64), and ATDC5 or HCS-2/8 chondrocyte cell lines (336) has revealed cell autonomous effects of GCs on proliferation, differentiation, apoptosis, and senescence (213).

1. Proliferation and differentiation

Different in vitro and in vivo studies have revealed a strong inhibitory effect of dexamethasone on the proliferation of chondrocytes (61, 65, 241, 304, 429). It was hypothesized that inhibition of extracellular signal-regulated kinase (ERK)-dependent AP-1 activation may underlie the anti-proliferative effects of GCs on chondrocytes (234). However, in vivo, the impairment of apoptosis by deletion of the pro-apoptotic protein Bax (see below) appears to protect mice from GC-induced inhibition of proliferation, indicating a more complex mechanism of GC action in the regulation of chondrocyte growth (429).

Concomitant with the inhibition of proliferation, chondrogenic differentiation is severely affected by GCs. Chondrogenic matrix production and expression of genes that mark chondrocyte maturation are reduced in dexamethasone-treated rats (10) and chickens (61). Accordingly, GC-treated ATDC5 cells show a reduction of nodule formation and the absence of alkaline phosphatase (ALP) and alcian blue-positive ECM and matrix mineralization (336).

As with osteoblasts (see below), GCs can also stimulate chondrogenesis in certain tissue culture systems. The lineage-determining transcription factor Sox9 is induced by GCs, concomitant with Col2a1 and aggrecan (327, 328). Expression profiling of primary chondrocytes treated with dexamethasone displayed an enrichment of ECM genes involved in the maturation of chondrocytes, underscoring the promotion of chondrocyte differentiation by GCs (151). In line with this, GCs enhance chondrocyte hypertrophy as demonstrated by increased collagen X expression, at least in the chicken model (61). The induction of the Col10 gene has also been observed in GC-treated ATDC5 cells, ascribed to the dexamethasone-dependent induction of lipocalin, a secreted acute-phase protein that triggers collagen X expression (264).

Thus GCs inhibit chondrocyte proliferation, but may increase or decrease their differentiation. To what degree these different cellular effects contribute to a decrease in growth plate activity remains to be investigated.

2. Apoptosis

Chondrocyte apoptosis is strongly associated with systemic GC treatment. Dexamethasone administration in rats leads to apoptosis via activation of caspase-3 in all three zones of the growth plate compared with control animals (64). In vitro dexamethasone treatment of the HCS-2/8 chondrocyte cell line results in a significant increase of apoptosis due to an increased caspase-3 cleavage and activation of caspase-8 and -9 via cleavage of the pro-apoptotic factor Bid (65). Accordingly, siRNA knockdown of Bid results in a partial rescue of dexamethasone-induced apoptosis. Bid is activated by pro-apoptotic Bak, which is also directly regulated by dexamethasone in human chondrocytes (428).

Moreover, dexamethasone inhibits phosphorylation of Akt in chondrocytes (65, 103). Inhibition of Akt signaling results in the attenuation of anti-apoptotic Bad phosphorylation (428). Accordingly, induction of Akt signaling by IGF-I prevents the apoptotic effects of dexamethasone (63).

In addition, dexamethasone can also inhibit phosphorylation of the pro-apoptotic Bax, e.g., in human neutrophils and, strikingly, in human growth plate biopsies, leading to its translocation to the mitochondrial membrane, with a subsequent induction of apoptosis (109, 429). Bax-deficient mice are protected against dexamethasone-induced apoptosis of growth plate chondrocytes and growth retardation (429). However, this dramatic effect only occurs in male mice, implying a gender-specific role for Bax in GC-induced growth retardation. Concomitantly, the anti-apoptotic proteins Bcl-2 and Bcl-x are dramatically decreased in all layers of growth plate chondrocytes after GC treatment (64). Thus anti- and pro-apoptotic proteins of the Bcl-2 family appear to be key players in the dexamethasone-induced apoptosis of growth plate chondrocytes. However, the gender-specific protection of Bax against dexamethasone on longitudinal growth indicates the involvement of additional members of the Bcl-2 family. Functional evaluation of Bcl-2 family members using knockout mice will lead to a comprehensive picture of this process.

In summary, GC treatment disturbs the sensitive balance between apoptosis and proliferation in growth plate chondrocytes, thus disrupting longitudinal growth that eventually results in growth retardation. Endogenous GCs, in contrast, seem to play a minor role in growth plate chondrocytes, as indicated by the normal growth plate phenotype of mice lacking the GR in these cells (378).

3. Vascularization

Vascular invasion of the primarily avascular hypertrophic cartilage in the growth plate is necessary for proper endochondral ossification. Vascularization of the ossification center is mediated through sprouting capillaries from the bone collar and not via de novo angiogenesis involving endothelial precursor cells (399). The invasion of blood vessels from the metaphysis into the growth plate provides nutrition, pro-apoptotic signals, and translocation of chondrocytes and osteoblasts into the ossification center (112, 399). Invading blood vessels, together with signaling molecules, induce the degradation of the ECM as well as apoptosis of hypertrophic chondrocytes and bone formation.
The articular cartilage is composed of four zones: the superficial, the transitional, the radial, and the calcified zone (150). The calcified zone is missing in growing bone. In 1983, Glade et al. (116) analyzed the effect of daily intramuscular GC injection on articular cartilage in growing foals. Independent of dose and duration of dexamethasone treatment, all foals showed degenerative joint disease compared with control foals. Cartilage destruction included fissures and avulsions as well as lesions of the periarticular cartilage, which were comparable to equine osteoarthritis (OA) (116). Furthermore, just 3 months of dexamethasone treatment resulted in a decreased synthetic activity of cartilage matrix components (116). Likewise, prenatal treatment of piglets resulted in an overall reduction of articular cartilage. However, the extent of the decrease was found to depend on gender and bones affected (374, 375).

These few studies do not provide a comprehensive picture, but point towards adverse effects of GCs on articular cartilage. Taking the in vitro results of various chondrocyte cell lines into consideration (discussed above), it is obvious that the GC therapy of children during growth should be carefully considered. Further investigations are required to unravel the underlying molecular mechanisms.

IV. GLUCOCORTICOID ACTION ON BONE REMODELING

A. Role of Endogenous Glucocorticoids in Maintaining Bone Mass

Albeit detrimental in pharmacological doses, endogenous GCs are essential for normal bone development and play an anabolic role in maintaining bone mass. Accordingly, abolishing systemic corticosterone levels by adrenalectomy in rats causes a significant decrease of metaphyseal trabecular bone mass (88). Clinically, patients with Addison’s disease, which is characterized by inability of the adrenal glands to produce sufficient GCs, are at a higher risk of hip fractures, indicating impaired bone quality (36). However, muscle weakness might also contribute to this phenomenon.

Improving bone mass requires increased numbers and function of bone-building osteoblasts. These cells are derived from mesenchymal progenitor cells and differentiate into osteoblasts by activation of the key transcription factors Runx2 and subsequently osterix (Sp7) in response to a plethora of growth factors, bone morphogenetic protein (BMP)/transforming growth factor (TGF)-β, Wnt signals, PTH, IGFs, prostaglandins, and interleukin (IL)-11 (427). Additionally, physiological levels of GCs appear to promote osteoblast differentiation and maturation.

Osteoblast-specific deletion of the GR and cell-specific disruption of GC signaling in mouse models have revealed the
necessity of endogenous GC actions in osteoblasts for maintaining a healthy skeletal structure. Targeted inactivation of GC signaling by overexpression of the GC-inactivating enzyme 11β-HSD2 in osteoblasts and osteocytes (Col2.3–11β-HSD2 tg mice in which a 2.3-kb fragment of the Col1a1 promoter drives 11β-HSD2 expression) resulted in reduced femoral cortical and vertebral trabecular bone mass and decreased mechanical bone strength (162, 331, 332). Likewise, disruption of the GR specifically in the osteoblast lineage (GRRunx2Cre) did not affect overall bone growth of mice, but diminished bone mass in terms of decreased trabecular numbers in the vertebrae of adult mice (290).

During aging, endogenous GC production and sensitivity of osteoblasts and osteocytes increase in mice. Hence, in contrast to the bone-promoting effects of GCs in young and adult mice, endogenous GCs increase skeletal fragility in old mice as a result of cell autonomous effects on osteoblasts and osteocytes, leading to impaired bone angiogenesis and vasculature volume. This effect is abrogated in mice with impaired GC signaling in osteoblasts [OG2 11β-HSD2 transgenic (tg) mice] (409). To summarize, the studies of mice with cell-specific deletion of the GR or cell-specific disruption of GC signaling demonstrate important functions of endogenous GCs in the development and maintenance of bone mass.

A GC-promoting effect on osteoblast maturation and differentiation had been postulated much earlier (31, 147, 329). However, the underlying mechanism has proven elusive and still remains relatively doubtful. The above-mentioned mouse models of cell type-specific disrupted GC action have provided robust evidence that endogenous GCs drive osteoblast differentiation. Calvarial osteoblasts derived from GR-deficient mice or Col2.3–11β-HSD2 tg mice show an impaired differentiation potential indicated by reduced ALP activity and bone nodule formation (290, 331, 433). Accordingly, expression of osteogenic marker genes like Runx2, ColIa1, and Bglap2 is reduced in GR-deficient osteoblasts.

Mice with an impaired homodimerization interface of the GR (GRdim) display normal bone mass and trabecular numbers. Accordingly, ex vivo osteogenesis of GRdim cells is not attenuated, pointing to an essential role of monomeric GR actions in the anabolic GC effects on bone cells (290). However, recent studies have revealed that overexpression of the GR dimer-dependent anti-inflammatory-acting mediator glucocorticoid-induced leucine zipper (GILZ) under the control of the bone-specific 3.6-kb rat type I collagen promoter displays a high bone mass phenotype. This is correlated with increased bone formation rate, enhanced osteoblast numbers, as well as a shift of mesenchymal stromal cell differentiation towards the osteogenic lineage by potentially decreasing adipocyte function due to physical interaction with the adipogenesis-promoting C/EBPalpha (265; and see below).

Another intriguing mechanism that could explain the pro-osteogenic effects of GCs was proposed by Zhou and colleagues (432, 433). They proposed that Wnt signaling appears to be augmented by “physiological” but not by pharmacological GC concentrations. Low GC concentrations downregulate the Wnt antagonist secreted frizzled-related protein 1 (433). The highly conserved Wnt signal transduction pathway plays an important role in osteoblast differentiation and function. Wnt signaling is disrupted in primary calvarial cell cultures derived from mice with a blunted GR signaling in osteoblasts (Col2.3–11β-HSD2 tg mice). This redirects differentiation of the cells to the adipogenic lineage. In particular, Wnt7b and Wnt11b are implicated in this process (433). Thus basal doses of GCs appear to be important for the release of Wnt proteins, which then act in a paracrine manner to activate the canonical Wnt pathway. This leads to accumulation of β-catenin in the nucleus and expression of Runx2, a key transcription factor of osteoblast differentiation. In addition, GC-induced canonical Wnt signaling in osteoblasts also affects the surrounding chondrocytes by augmenting the expression of matrix metalloproteinase 14, an enzyme implemented in the breakdown of the ECM during tissue development and remodeling (432).

In summary, physiological levels of GCs exert anabolic effects on bone, especially on osteoblast proliferation and differentiation. These effects are mediated by the GR. However, osteoblastogenesis-promoting actions of GCs appear to be independent of receptor dimerization. The underlying mechanisms partially explaining the positive effects of GCs are stimulation of the Wnt signaling pathway and regulation of transcription factors involved in osteoblast differentiation. In the future, advanced molecular biological analyses such as gene expression profiles and genome-wide siRNA screens will help to identify the genes that mediate the physiological effects of GCs on bone cells and may contribute to unveiling the complexity of physiological GC signaling in bone.

**B. Glucocorticoid-Induced Osteoporosis**

Glucocorticoid-induced osteoporosis (GIO) is the most common form of secondary osteoporosis, contributing to ~25% of all osteoporosis cases with a predominant risk of vertebral fractures (89, 388). Application of 5 mg prednisolone per day for more than 3 months leads to a 50% increase in bone fracture risk (387). GC-induced bone loss occurs in two phases: an early rapid phase with bone loss due to excessive bone resorption (52) and a second, more progressive phase, in which bone formation is hampered. This is also reflected by longitudinal gene expression profiling studies that exhibit an early induction of osteoclast-
C. Minor Evidence for Systemic Effects of Glucocorticoids on Bone

Historically, systemic actions of GCs that indirectly affect bone metabolism were discussed. These systemic effects included a decreased absorption of calcium in the gastrointestinal system and a reduced renal reabsorption of calcium (52). Other systemic effects of GCs encompass reduction of the sex hormones testosterone and estrogens on the level of hypothalamic (310) and gonadal regulation (84, 142). Usually, in response to low blood calcium levels, PTH is produced and secreted in the parathyroid gland. PTH acts on kidney, bone, and intestine to maintain blood calcium levels. In bone, PTH acts directly on osteoblasts, stimulating them to secrete RANKL, a member of the tumor necrosis family (TNF), which in turn stimulates osteoclasts in the context of proliferation, differentiation, and survival. Hence, PTH promotes bone resorption and thus the release of calcium.

Consequently, the reduced calcium levels upon GC exposure were suggested to induce bone loss and may result in hyperparathyroidism. However, bone turnover in patients receiving GCs is low (73), in contrast to patients with high PTH levels that have an increased bone turnover. There are no indications that GCs alter the PTH level itself, but the number and affinity of PTH receptors on bone cells have been noted to be increased (381).

No real evidence exists that systemic GC effects on bone are crucial for GC-induced bone loss. Studies conducted by us and others using genetically modified mouse models have revealed that direct effects of GCs on osteoclasts, osteoblasts, and osteocytes form the main mechanisms underlying GIO (see below).

D. Effects of Glucocorticoids on Osteoclasts

1. Indirect glucocorticoid effects on bone resorption

The initial phase of GC-induced bone loss is characterized by a rapid but transient increase in bone resorption (85) due to an increase in osteoclast numbers and activity. Thereby, GCs exert direct as well as indirect actions on bone-resorbing cells. To drive monocyte differentiation into the osteoclastic lineage, M-CSF and RANKL are required. M-CSF triggers the early osteoclast differentiation, whereas RANKL, produced mainly by osteoblasts, osteocytes, but also activated T cells, is important in the later stages of differentiation and during osteoclast activation (368). Pharmacological levels of GCs increase levels of RANKL produced by cells of the osteoblast lineage. Concurrently, GCs downregulate the expression of osteoprotegerin (OPG), the decoy receptor of RANKL, by decreasing the amount of phospho-c-Jun protein which maintains steady-state transcription of the OPG-encoding Tnfrsf11b gene (133, 291, 371). The GC-triggered imbalance in the RANKL/OPG ratio has been suggested to enhance bone resorption. This hypothesis has also been emphasized by studies in mice expressing a humanized RANKL protein, which is neutralized by administration of denosumab, a recombinant antibody against human RANKL. These mice were protected from GC-induced bone loss and maintained bone mass and bone strength during 4 wk of GC treatment (135). Furthermore, a study in 2009 demonstrated that dexamethasone induces the production of RANKL in bone marrow adipocytes, thereby supporting osteoclast differentiation in vitro (141). However, whether this is also relevant in vivo has yet to be determined.

Recent work has shown that osteocytes and not osteoblasts are the major source of RANKL (244, 420). Osteocytes express higher amounts of RANKL and have a greater capacity to support osteoclastogenesis than osteoblasts and bone marrow stromal cells (244, 420). As discussed below, GC excess causes apoptosis in osteocytes. Thus it still needs to be elucidated whether GCs also induce osteoclastogenesis through upregulation of RANKL expression in osteocytes and whether this is linked to apoptosis, since apoptotic osteocytes are strongly linked to bone resorption (367). Notably, proinflammatory cytokines influence osteoclastogenesis. It is known that not only TNF-α (186) and IL-1, but also IL-6 and IL-17, can directly activate osteoclasts by recruiting CD11b+ cells from bone marrow (199, 302). Moreover, TNF-α strongly stimulates the production of RANKL (134) and M-CSF. IL-17, mainly released from T_{h17} cells, also enhances the expression of RANKL and M-CSF (382). In addition, T_{h17} cells themselves release RANKL. Thus T_{h17} cells are suggested to be a major trigger inducing osteoclastogenesis (315). GCs inhibit the expression of cytokines such as IL-1 and IL-6 in osteoblasts and also a plethora of pro-inflammatory factors in other mesenchymal cells and immune cells. Given that GC therapy is mostly used for treatment of immune-mediated diseases, it remains elusive how GCs influence the osteoclast lineage.

2. Direct glucocorticoid effects on osteoclasts

Apart from the GC-induced increase in pro-osteoclastogenic factors, GCs also exert direct actions on osteoclasts. However, studies of direct effects of GCs on osteoclasts in vitro and in vivo have revealed inconclusive results. Dexamethasone treatment of in vitro-generated osteoclasts, in the presence of M-CSF and RANKL, leads to a disruption...
of actin cytoskeleton reorganization due to decreased activation of rhoA, rac, and vav3. Thus formation of the basolateral ruffled border is disrupted, and consequently, osteolytic activity is impaired (171). These inhibitory effects on osteoclast function were recently supported by the discovery that the cytoskeletal effector protein calpain 6 is strongly suppressed by GCs in osteoblastogenic or osteoclastogenic cells, which is decisive for reduction of microtubule and resorptive activity (138). In contrast, enhanced release of COOH-terminal crosslinked telopeptide of type I collagen (indicating enhanced resorptive activity) has been observed in other studies of GC exposure of osteoclasts (343, 351). The conflicting results may be due to variations in GC concentrations, the duration of GC exposure, and the surface substrate of the osteoclast cultures (bone slices or plastic).

Osteoclast specific activity in vivo has been determined in GR<sup>−/−</sup>MCre mice with specific deletion of the GR in myeloid cells and osteoclasts or in mice overexpressing the GC-inactivating enzyme 11β-HSD2 under the tartrate-resistant acid phosphatase promoter in osteoclasts (154, 171). Thus Kim et al. (171) suggested a crosstalk from osteoclasts to osteoblasts, leading to an inhibition of osteoblast activity in the presence of GCs. However, another study using GR<sup>−/−</sup>MCre mice did not show any differences in suppression of bone formation within the 14-day treatment with prednisolone (290). Accordingly, GC administration for 7 days to TRAP-11β-HSD2 mice attenuated bone formation (154). Intriguingly, GCs induced a drop in osteoclast numbers in TRAP-11β-HSD2 mice but not in wild-type animals. This indicates a direct augmentative effect on osteoclast lifespan and an indirect non-cell autonomous suppressive effect on osteoclastogenesis. The increased lifespan of osteoclasts could explain the initial increased resorption upon GC treatment. In contrast, the suppressive effects of GCs on osteoclastogenesis could explain partly the long-term decrease of osteoclast numbers and activity observed after long-time GC exposure (290, 406).

Overall, GCs dynamically regulate bone resorption. An initial rise in bone resorption upon GC exposure is followed by hampering effects on osteoclast activity during long-term GC excess. The relevance of the different proposed mechanisms needs further clarification.

E. Effects of Glucocorticoids on Osteoblasts

A hallmark of GIO is the inhibition of bone formation, especially in the progressive phase of the disease. Histomorphometric and serum analyses of patients have revealed a reduced bone formation rate, indicated by reduced serum levels of type I collagen synthesis (NH<sub>2</sub>-terminal propeptide of type I procollagen) (78). Strong evidence in support of a central role for suppressed bone formation in GIO has been provided by studies in mice with a specific deletion of the GR in osteoblasts or with an abrogated GC signaling in osteoblasts. Such mice are protected from suppression of bone formation under GC excess and are thus resistant to GC-induced bone loss (255, 290). In contrast to endogenous GCs, pharmacological doses of GCs impair osteoblasts in terms of proliferation, differentiation, function, and survival.

1. The glucocorticoid receptor as a modifier of mesenchymal stem cell lineage decision

MSCs were first discovered in 1966 as a subpopulation of bone marrow cells capable of forming bone (100). These MSCs, capable of self-renewing, mainly reside in the bone marrow (but also in other tissues such as adipose tissue, skeletal muscle, and the synovium) and are responsible for the development, growth, and repair of long bones (12, 37, 159, 205). They can differentiate into multiple lineages and give rise to mature osteoblasts, chondrocytes, adipocytes, and stromal cells (278).

Shifting the balance from osteoblastogenesis to adipogenesis might be one of the mechanisms by which GCs diminish osteoblast differentiation (307) (Figure 5A). GCs induce the expression of adipocyte-specific transcription factors such as PPAR<sub>y</sub> and C/EBPalpha, beta, and gamma (44, 55, 196, 230, 231, 254, 273), resulting in an increased adipogenic differentiation at the expense of osteoblastogenesis. This shift in lineage cell fate decision may be recapitulated in patients receiving GCs, who concurrently accumulate fat in the bone marrow while losing bone mass (389; reviewed in Ref. 253). This is in line with observations from Cushing’s syndrome patients that display elevated endogenous GC levels. These patients are at increased risk of bone fractures from a concomitant increase of the adipose tissue (220). The same phenomenon can be observed in dexamethasone-treated mice; genome expression profiling revealed an induction of adipocyte-associated genes and a repression of osteoblastic genes (422). Strikingly, bone marrow-derived MSCs from dexamethasone-treated mice differentiate in osteoblastogenic culture conditions into an adipocytic phenotype (196), indicating a commitment switch towards adipogenesis at the expense of osteoblastogenesis (196, 230, 231, 254, 273), resulting in an increased adipogenic differentiation at the expense of osteoblastogenesis. This shift in lineage cell fate decision may be recapitulated in patients receiving GCs, who concurrently accumulate fat in the bone marrow (389; reviewed in Ref. 253). This is in line with observations from Cushing’s syndrome patients that display elevated endogenous GC levels. These patients are at increased risk of bone fractures from a concomitant increase of the adipose tissue (220). The same phenomenon can be observed in dexamethasone-treated mice; genome expression profiling revealed an induction of adipocyte-associated genes and a repression of osteoblastic genes (422). Strikingly, bone marrow-derived MSCs from dexamethasone-treated mice differentiate in osteoblastogenic culture conditions into an adipocytic phenotype (196), indicating a commitment switch towards adipogenesis at the expense of osteoblastogenesis. Changes in C/EBPalpha levels upon dexamethasone treatment were triggered by reduced methylation of the C/EBPalpha promoter, resulting in an upregulation of C/EBPalpha expression (196). Moreover, increased expression of PPARy2 and decreased expression of Runx2 upon dexamethasone treatment are accompanied by preferential adipogenesis of MSCs (201, 334). Recently, it was shown that prostaglandin E<sub>2</sub> attenuates osteogenic differentiation but promotes adipogenic differentiation of human bone marrow stromal cells (252). Dexamethasone treatment upregulated the expression of the prostaglandin receptors EP2 and EP4 in these cells, thus rendering them susceptible to prostaglandin E<sub>2</sub> and its ability to shift the balance from osteogenic to adipogenic differentiation (252).
An additional GR target gene that could be involved in cell lineage switch from osteoblastogenesis towards adipogenesis is GILZ. This target gene (Tsc22d3) can shift the balance between adipogenic and osteogenic MSC differentiation towards osteogenesis by inhibiting PPARγ expression (431). Overexpression of GILZ in murine MSCs increases ALP activity and mineralization, the hallmarks of osteoblast differentiation, whereas GILZ deficiency results in a reduced osteogenic capacity of MSCs. Moreover, transgenic overexpression of GILZ under the control of the 3.6-kb rat type I collagen promoter revealed increased bone formation and osteoblast numbers, resulting in a high bone mass phenotype in these mice (265). The authors of this study demonstrated an increased osteogenic differentiation of MSCs in
vitro accompanied by an increased Runx2 and Osterix expression, whereas adipogenic differentiation and PPARγ expression were decreased. The observed phenotype was suggested to be due to physical interaction of GILZ with C/EBPs resulting in an abrogated C/EBP-mediated PPARγ expression. Moreover, it has been shown in human fetal osteoblasts that dexamethasone-induced GILZ expression results in enhanced osteoblast maturation, whereas silencing of GILZ in these cells decreases the expression of typical osteoblast genes (192).

Interestingly, studies with mouse embryonic fibroblasts derived from GRdim mice revealed the requirement of an intact GR dimerization for adipocyte generation from mesenchymal progenitor cells in vitro by regulation of C/EBPdelta and KLF15 (13).

However, the timing of GC exposure might lead to different effects on MSC lineage decision. Dexamethasone can antagonize BMP2-triggered osteoblastogenesis in noncommitted preosteoblastic cells when applied early in cultures, thus triggering adipogenesis accompanied by suppression of the osteoblast-regulating factor osterix (230). However, treatment of cells with dexamethasone and BMP2 during the late phase of differentiation has synergistic effects on in vitro matrix mineralization. These results demonstrate that dexamethasone effects depend on the stage of differentiation.

It remains to be determined whether the GC-induced switch from the osteogenic to the adipogenic phenotype reflects a mechanism contributing to GIO or whether it is merely an artifact of MSC cultures. Most studies have tested the effects on the respective lineages using either osteoblast progenitor cells and chondrocytes, but not adipocytes, are derived from these skeletal stem cells. Worthley et al. (417) suggest that during development only osteochondroreticular stem cells are responsible for the formation of articular and growth plate cartilage, bone, and stromal cells. However, in postnatal bone at least two skeletogenic stem cells exist, osteochondroreticular stem cells and perisinusoidal MSCs, the latter ones being capable of additionally giving rise to adipocytes (417).

In the future, novel lineage tracing mouse models should be able to address how GCs tip the balance of skeletal stem cells into different lineages in vivo and whether a lineage switch of progenitor cells occurs in vivo at all.

2. Glucocorticoids and osteoblast proliferation

GC-mediated inhibition of proliferation of cells of osteoblastic lineage contributes significantly to GIO. GCs thereby have an impact on different mitogenic signaling pathways and have been shown to disrupt physiological cell cycle progression (FIGURE 5B). Several in vitro studies of osteoblasts and osteoblast progenitors have demonstrated a strong interference of GCs with the canonical Wnt/β-catenin and BMP signaling pathways, essential for maintaining osteoblast proliferation and differentiation. Wnt signaling prevents osteoblast apoptosis and increases osteoblast cell cycle progression and thus increases proliferation (reviewed in Ref. 237).

GCs abrogate further mitogenic signals at the level of phosphorylated ERK via the induction of dual specificity phosphatase 1/MAPK phosphatase 1 (DUSP-1/MKP-1) in cultured osteoblasts (139). Dephosphorylation of ERK hampers the response to mitogenic signaling, thereby suppressing cell proliferation. However, studies in DUSP-1−/− mice could not confirm a pivotal role for DUSP-1/MKP-1 in vivo, since ablation of DUSP-1 did not prevent GC-induced bone loss (68). Hence, regulation of DUSP-1 by GCs is critical for GC-induced inhibition of osteoblast proliferation, but it is not sufficient to reduce bone mass in GIO.

Attenuation of cell cycle progression is another mechanism by which GCs impair proliferation of human osteoblasts and their progenitors. Chang et al. (58) reported in 2009 that pharmacological doses of dexamethasone arrest cells in the G0/G1 phase of the cell cycle, together with gene expression changes of the cell cycle regulators p27kip1 and cyclin D2 (58). Similarly, dexamethasone strongly inhibits the G1/S cell cycle transition in primary mouse osteoblast cultures during and after the commitment stage to the osteoblast lineage by reducing the expression of cyclin A, an important regulator of G1/S cell cycle transition (105, 350). In MC3T3-E1 cells, an immortalized mouse osteoblast cell line, dexamethasone also inhibits cell cycle progression (350), partly by aberrant GR activation and subsequent p53 activation (195). In particular, in mouse calvarial primary cells and cell lines of the osteoblastic lineage, inhibition of proliferation is a prerequisite for osteoblast differentiation commitment (350). In primary human osteoblasts and osteosarcoma cell lines, the antimitogenic effect of GCs is associated with downregulation of the cell cycle regulators CDK2, 4, 6, cyclin D, c-Myc, and E2F and upregulation of cyclin-dependent inhibitors p21 and p27, as well as the tumor suppressor gene p53 (58, 195, 306).

3. Glucocorticoids and osteoblast differentiation

Among the various skeletal effects of GCs, the suppression of osteoblast differentiation appears to be predominant. Studies in cultured osteoblasts, as well as genetic mouse models, have established that GC treatment has profound
effects on osteoblast differentiation. While a direct interference with Runx2 has recently been described as a potential mechanism for the inhibition of osteoblast differentiation (181), numerous lines of evidence exist for the involvement of multiple transduction pathways. Indeed, high doses of GCs interfere with pathways that are crucial for osteoligand differentiation, e.g., Wnt and BMP2 signaling pathways. Moreover, GCs can influence the expression pattern of microRNAs (miRNAs) or stimulate inhibitory pathways such as Notch (see below) to impair osteoblastogenesis (Figure 5B).

**F. Wnt Signaling**

Wnt/β-catenin signaling is crucial for the maintenance of osteoblast function. Its impairment by genetic mutations leads to severe abnormalities in bone (reviewed in Ref. 17). The canonical Wnt pathway is triggered by binding of Wnt family members to the receptor complex composed of frizzled or either low-density lipoprotein receptor-related protein 5 (LRP5) or 6 (LRP6). Consequently, the constitutively active β-catenin degradation complex composed of Axin, APC, and GSK3β is inactivated due to phosphorylation of the adapter protein dishevelled and GSK3β. Subsequently, β-catenin is not phosphorylated and therefore stabilized. It can enter the nucleus and interacts with lymphoid enhancer-binding factor 1 to activate transcription of osteoblast-essential genes such as Runx2 (17). In the absence of Wnt proteins or by expression of inhibitors of the Wnt signaling pathway β-catenin is constantly degraded. Wnt-inhibitors bind either directly to Wnt ligands (such as secreted frizzled-related proteins) or to proteins interacting with LRP5/6 co-receptors [such as sclerostin and dickkopf-1 (DKK1)].

GCs oppose Wnt signaling by different mechanisms. High (but not low) GC doses reduce the expression of Wnt7b and Wnt10, thus attenuating osteoblast differentiation. Different effects of low and high GC concentrations on Wnt signaling might explain, at least in part, the biphasic effects of GCs on osteoblast differentiation (219). High doses of GCs further enhance the expression of DKK1 (48, 260), secreted frizzled (219, 402), Wif1, and sclerostin (422). Accordingly, decrease of DKK1 expression by antisense constructs alleviates dexamethasone-induced bone loss in rats (401). Furthermore, children chronically treated with high doses of GCs display elevated DKK1 levels in their serum (41). Additionally, GCs activate glycogen-synthase kinase 3β and thereby destabilize β-catenin (260, 348). Finally, GCs also reduce the transcriptional activity of the final Wnt transducer LEF/TCF by activation of HDAC1 (349).

**G. Interference with BMP Signaling, Notch, and AP-1**

Regarding BMP signaling, GCs repress BMP2, a key regulator of osteoblast differentiation which can antagonize the GC-induced suppression of differentiation but not the inhibition of proliferation (216). In addition, GCs increase the expression of BMP2 antagonists, namely, follistatin and Dan (126).

The Notch signaling pathway participates in many developmental processes and is activated by interaction of membranous Notch receptors with membrane-bound ligands of neighboring cells. Upon stimulation, juxta- and intramembranous cleavage of the Notch proteins is induced; the Notch intracellular domain is released and transmigrates into the nucleus to regulate gene expression. Notch signaling interferes with Runx2 activity on several levels. Through the induction of Notch1 and Notch2 mRNA in osteoblasts, GCs could attenuate osteoblast differentiation (274).

Studies in mice with an impaired GR dimerization function (GRdim mice) have revealed a decreased osteoblast differentiation by GCs via a mechanism that involves interference of the GR monomer with AP-1. Interaction of the monomeric GR with the proinflammatory transcription factor AP-1 results in suppression of anabolic cytokines like IL-11 and leukemia inhibitory factor which activate JAK2 and STAT3 signaling. Addition of recombinant IL-11 and leukemia inhibitory factor to osteoblast cultures prevents the GC-induced suppression of differentiation (290).

In summary, GCs significantly impair osteoblast differentiation by interfering with multiple bone anabolic pathways.

**H. Glucocorticoid Regulation of MicroRNAs During Osteoblast Differentiation**

In recent years, miRNAs have been discovered and subsequently shown to play an important role in MSC differentiation. miRNAs are short noncoding RNAs, 18–25 nucleotides long, transcribed from intergenic as well as from genomic regions of the genome (238, 305). They usually bind to the 3'-untranslated region of specific target mRNAs and inhibit gene expression by either targeting those miRNAs for degradation or simply inhibiting their translation (92). One important step in the processing of miRNAs in the cytoplasm is the cleavage of pre-miRNAs into miRNAs mediated by an enzyme called Dicer. Deletion of this miRNA-processing enzyme in chondrocytes, osteoblasts, and osteoclasts has uncovered the emerging role of miRNAs in bone formation, cell differentiation, and cell function (111, 179, 235). Moreover, downregulation of Dicer in human MSCs suppresses osteogenic differentiation (263). To date, several miRNAs have been recognized to play a significant role in osteogenesis by acting either as inhibitors or as inducers of osteoblastogenesis (14, 263). For example, miRNA-29 has been identified as an osteoblastogenic inducer increasing ALP activity and Runx2 protein levels and at the same time targeting negative regulators of osteoblastogenesis, such as HDAC4 and TGF-β (202). Wnt signaling increases expres-
methasone induces apoptosis in human chondrocytes by miRNA expression during chondrogenesis are only poorly reviewed, see Ref. 330). Unfortunately, GC-induced effects on postnatal) and in cartilage-related diseases (for a detailed review, see Ref. 330). GCs reduce the expression of miRNA-29a via HDAC4 activity, reducing acetylation of histone 3 at lysine 9 in the miRNA-29a promoter (178). Moreover, GC-induced loss of miRNA-29 signaling increases deacetylation and ubiquitylation of β-catenin, thus attenuating the promoting effect of the canonical Wnt signaling on osteoblast differentiation. Therefore, enhancement of miRNA-29 signaling in osteoblasts represents an alternative strategy to minimize the negative effects of GCs on osteogenic cells.

In 2014, two other GC-dependent miRNAs were identified, namely, miRNA-17/20a (333). Both can counteract GC actions on osteoblasts/osteoclasts by targeting the 3′-untranslated region of RANKL (Tnfsf11) mRNA. Expression of miRNA-17/20a in murine calvarias is significantly decreased after 3 days of dexamethasone treatment, subsequently leading to an increase in RANKL expression in osteoblasts. Accordingly, depletion of miRNA-17/20a results in increased osteoclast differentiation in an osteoclast/osteoblast coculture system. Collectively, these data indicate that GCs increase RANKL expression through down-regulation of miRNA-17/20a in osteoblasts, thereby indirectly enhancing osteoclastogenesis and bone resorption. Also in 2014, Li et al. (200) identified 16 deregulated miRNAs in dexamethasone-induced human bone marrow-derived MSCs during osteogenic differentiation. Target genes of the upregulated miRNAs following dexamethasone treatment included Runx2, Stat5b, Smad1, and Smad5, all of them associated with bone formation. Thus dexamethasone-induced upregulation of these miRNAs leads to an inhibition of osteoblast differentiation of MSCs. Moreover, target genes of the downregulated miRNAs following dexamethasone administration have been identified as ones that normally function as suppressors of osteoblastogenesis. For example, miRNAs against BMP3, HDAC4, and TGF-β1 were downregulated after dexamethasone treatment, thus counteracting osteogenic differentiation.

In parallel to their effects on osteoblastogenesis, miRNAs play an important role during chondrogenesis (pre- and postnatal) and in cartilage-related diseases (for a detailed review, see Ref. 330). Unfortunately, GC-induced effects on miRNA expression during chondrogenesis are only poorly understood. Xing et al. (419) showed in 2014 that dexamethasone induces apoptosis in human chondrocytes by inhibiting the expression of miRNA cluster 17–92 (419). miRNA expression profiles in MSCs during chondrogenesis have yet to be investigated.

In summary, GC-regulated miRNAs likely impairs osteoblast differentiation by interfering with multiple bone anabolic pathways.

1. Glucocorticoids and osteoblast apoptosis

Many studies suggest that GC-induced apoptosis in osteoblasts and osteocytes contributes to GC-induced bone loss and impairment of bone quality. In a fundamental study, GC-mediated promotion of osteoblast apoptosis was demonstrated in GC-treated mice and humans by TUNEL staining (408). The GC-induced cell death in osteoblasts appears to be a cell autonomous effect, since apoptosis is not observed in prednisolone-treated mice with an abrogated GC signaling specifically in osteoblasts (255).

As discussed in section II, particular GR isoforms may exist that render osteoblast-like U2OS cells apoptotic, since overexpression of GR C isoforms (NH2-terminal truncated version) enhances dexamethasone-induced apoptosis in these cells (213). Whether the repertoire of GR isoforms determines the rate of GC-induced apoptosis in primary cells currently remains elusive.

Several in vitro studies suggest potential mechanisms of GC-induced osteoblast apoptosis. A study from 2008 in murine osteoblasts reported a GC-mediated increase in pro-apoptotic factors of the Bcl-2 family, like Bim, and a corresponding activation of the downstream effectors caspase-3, -7, and -8 (93). Accordingly, in MC3T3 cells, dexamethasone was shown to elevate levels of the pro-apoptotic protein Bax (138). Bax itself promotes and contributes to the formation of pores in outer mitochondrial membranes, and thereby leads to apoptosis. Other proapoptotic factors that appear to be increased and activated by GCs are Bak (58) and the tumor suppressor and apoptosis regulator p53 (195). Consistently, expression of Bcl-XL, a pro-survival Bcl-2 protein, is downregulated by dexamethasone treatment (58). Other mechanisms of GC-induced apoptosis in osteoblasts include an increase of reactive oxygen species with a subsequent constitutive activation of Jun NH2-terminal kinase (5). Furthermore, in human osteoblasts, dexamethasone decreases β1 integrin expression and thereby prevents cell-matrix adhesion. This finally results in cell death (247). Thus GCs induce apoptosis in the osteogenic lineage by activation of different pro-apoptotic and inhibition of anti-apoptotic signaling pathways. However, functional evidence of the importance of GC-induced apoptosis in bone cells in GC-induced bone loss is still lacking. Of note, in mice the induction of apoptosis in osteoblasts by GCs has been reported to affect no more than 10–20% of all osteoblasts (280, 290). Despite this minor apoptosis rate, the mice still developed an overall bone loss, indicating
that apoptosis alone is not sufficient to cause GC-induced bone loss. In contrast, osteocytes are greatly affected by GCs towards either apoptosis or autophagy, which contributes to impaired bone quality (discussed below).

2. Effects of glucocorticoids on osteocytes

An advanced microscopy analysis of bones derived from GC-treated mice has revealed a larger number of empty osteocyte lacunae (187). Osteocytes are terminally differentiated osteoblasts with characteristic dendrites that form a lacunar-canalicular network throughout the bone tissue. Osteocytes are the most abundant cell type in bone (90−95% of all cells). Their tight network allows the osteocytes to communicate, sense mechanical alterations, direct repair, and maintain bone integrity by orchestrating osteoblasts and osteoclasts (70, 370). A reduction in osteocyte number or function and the concomitant disorganization of the canalicular network contributes to an impaired bone quality and strength, as is observed in GIO (156) and underlines that GCs influence osteocyte function and number.

GC excess may modify the perilacunar mineralization around osteocytes through upregulation of FGF23 (423). FGF23 is a hormonal factor mainly produced by osteocytes. Increased FGF23 reduces serum phosphorus and 1,25-(OH)2-vitamin D3 levels by acting on the kidney via FGF receptors and Klotho. Interestingly, in GC-treated mice FGF23 serum level increases in a dose-dependent manner (224, 423).

In addition, osteocytes are a major source of RANKL, the main factor of osteoclast differentiation and activation (244). GCs have been shown to enhance the expression of RANKL (371) and concomitantly the expression of inhibitors of pro-osteogenic Wnt signaling, such as DKK1 and SOST. However, it still has to be elucidated how decreased osteocyte numbers lead to enhanced expression of these osteocyte-specific genes in vivo.

Recent studies have focused on the underlying mechanisms of GC-induced bone fragility and reduced osteocyte numbers. Intriguingly, apoptosis of osteocytes was observed only under high and prolonged GC exposure (2.8−5.6 mg/kg per day), whereas low doses of GCs (<2.8 mg/kg per day) induce autophagy (155). Autophagy is one of the most important mechanisms enabling cells to survive stress through breakdown of cellular components, thereby maintaining cellular energy levels. Xia et al. (418) showed that dexamethasone treatment of MLO-Y4 cells, an osteocytic cell line, increases autophagy markers and the accumulation of autophagosome vacuoles in vitro. According to these data, at the initial period of GC exposure osteocytes respond to “GC stress” with autophagy to “save themselves.” But with prolonged high GC exposure, the osteocytes may undergo apoptosis, resulting in an overall reduced osteocyte number and a loss of the lacunar-canalicular network, which substantially impairs bone quality. This hypothesis is challenged by a recent study of the O’Brien group (277). They demonstrated that although GCs stimulate autophagy in osteocytes, suppression of autophagy in this cell type does not affect the negative impact of GCs on the skeleton (277).

GC-induced apoptotic mechanisms in osteocytes are partly similar to the ones osteoblasts. In addition, GCs promote osteoclasts. Osteocyte apoptosis via a GR-dependent mechanism by rapid activation of pro-apoptotic kinases Pyk2 and JNK (279). Activation of JNK increases reactive oxygen species, thereby inducing final cell death. Activation of Pyk2 results in reorganization of the cytoskeleton and loss of cell detachment, eventually resulting in cell death.

A higher sensitivity of osteocytes in response to endogenous GCs might occur with aging. Weinstein and colleagues observed an increased 11β-HSD1 expression in aged mice, indicating a higher local exposure to active GCs. Intriguingly, age-associated decreased vascularization is diminished in 11β-HSD2-overexpressing mice (407). This study links bone aging to an increased activity of the GC-activating enzyme 11β-HSD1.

In summary, recent efforts in osteocyte bone biology have emphasized osteocytes as important targets of GC excess, contributing substantially to GC-induced bone fragility and bone loss. The GC-induced reduction in perilacunar mineralization and reduction in osteocyte viability and numbers may represent a mechanism by which GCs impair bone strength.

V. MECHANISM OF GLUCOCORTICOID ACTIONS IN RHEUMATOID ARTHRITIS: LESSONS FROM ANIMAL MODELS

Rheumatoid arthritis (RA) was one of the first diseases to be treated with corticosteroids. Since the 1950s, GCs have been among the most frequently used drugs in RA therapy, although their use is compromised by severe side effects such as osteoporosis (53). RA is a chronic inflammatory disease affecting 1% of the world’s population (132). Whereas the etiology is still unknown, disease development is associated with genetic (412) and environmental factors, e.g., smoking and obesity (3, 363), and with different prevalence in women and men (145, 412). RA is characterized by production of autoantibodies, “rheumatoid factors” and anti-citrullinated protein antibodies. Furthermore, genetic factors known for their association with autoimmunity (206, 366) are associated with RA development. Human leukocyte antigen-DRB1 and -DR4, as well as single nucleotide polymorphisms in the protein tyrosine phosphatase, non-receptor type 22 gene have been identified as RA-associated genes (27, 121, 366). Moreover, infiltration of leukocytes into joints leads to inflammation and hyperplasia of
the synovium, resulting in cartilage and bone destruction, the key factors of disease progression. The participation and interplay of various immune and non-immune cells define RA as a complex disease with many possible therapeutic targets.

Despite their comorbidities, GCs are still used in a broad range in clinical practice, either in monotherapy (384) or in combination with disease-modifying antirheumatic drugs (15). However, GC resistance (175) and reduced bone mineral density (261, 384) compromise the beneficial effects of GC therapy. Potential ways to avoid the deleterious effects of corticosteroid therapy in RA are still a matter of continuous debate, in particular concerning application and dosage (152, 404, 411).

Animal models are widely used to study RA mechanistically. Although animal models of arthritis do not display the full complexity of human RA, their use has increased our knowledge about pathology and therapeutic approaches. It is well established that GC treatment of arthritic animal models leads to reduced swelling and infiltration of immune cells into affected joints (20, 364). However, the underlying mechanism, as well as target genes, may differ between the different animal models. Collagen-induced arthritis (CIA) is one of the frequently used arthritic models in the field and is induced by native type II collagen extract in combination with incomplete Freund’s adjuvant (376). Mice subjected to CIA display a broad range of similarities to the human situation, since they produce autoantibodies and develop synovial infiltration and hyperplasia, as well as cartilage and bone erosion in various joints. CIA has been shown to be critically dependent on the activity of mast cells (324), macrophages (335), and T cells (62). Susceptibility towards CIA is limited to a few mouse strains, such as DBA/1 mice, thus excluding various transgenic mouse lines as tools to reveal underlying mechanisms. In contrast, antigen-induced arthritis (AIA) is suitable for various mouse lines and displays a single arthritic joint. It is induced by immunization with methylated bovine serum albumin in complete Freund’s adjuvant with *Bordetella pertussis* vaccine, followed by an intra-articular methylated bovine serum albumin injection into a single knee joint (39). AIA is characterized as a chronic T-cell-dependent and antigen-specific model of arthritis that does not display the polyarthritic inflammation of small joints of RA patients. The K/BxN serum transfer model of arthritis displays a chronic polyarthritis inflammation, especially of small joints, triggered by autoantibodies against glucose-6-phosphate isomerase (236), which have been observed to be associated with the extra-articular manifestation of disease in RA patients (385). Upon transfer of serum from K/BxN mice into recipient mice, neutrophils (414) and mast cells have been proposed to be responsible for the joint inflammation (236). However, elimination of mast cells using the Cre-loxP system does not reduce inflammation in this model (324). In fact, nonclassical Ly6C-negative monocytes have been found to be essential for the induction of inflammation in this arthritis model (232). A similar polyarthritis to that observed in the K/BxN serum transfer model exists in mice overexpressing human TNF-α (hTNF-tg mice), where multiple cell types, including inflammatory synovium, are involved (198).

A. Mechanisms of the Glucocorticoid Receptor for Suppression of Inflammation in Arthritis

In 1986, Paska et al. (266) demonstrated a reduction of inflammation in CIA mice in response to orally administered prednisolone treatment (266). This was the first time the clinical situation had been confirmed in an animal model. Subsequent studies have corroborated this finding in various mouse models of arthritis (e.g., Refs. 20, 267). Since the GR (as described in sect. I) can act either as monomer or as homodimer, thus involving various mechanisms to induce or suppress gene expression, elucidating the underlying GR action modes could provide new therapeutic possibilities for the treatment of arthritis. To unravel the molecular mechanisms of action of the GR responsible for suppression of inflammation in arthritis, De Bosscher (75) utilized the nonsteroidal compound A (CpdA) that specifically induces tethering of the GR monomer to proinflammatory transcription factors. When CpdA was administered to mice subjected to CIA, their inflammatory symptoms were relieved (80, 292). Furthermore, CpdA treatment of fibroblast-like synoviocytes (FLS) derived from RA patients prevented the induction of proinflammatory mediators under the control of AP-1 and NF-κB to a similar extent as dexamethasone treatment (119). From these studies, it was concluded that GR monomer activity might be sufficient to reduce inflammation. However, this interpretation has been challenged by the finding that CpdA can induce GR dimerization-dependent anti-inflammatory genes, e.g., DUSP-1/MKP-1, in some cell types (157, 390).

The requirement of an intact GR dimerization interface for the treatment of arthritis has been further demonstrated from GC-treated GRdim mice that fail to reduce inflammation in several arthritis models [AIA (20), glucose-6-phosphate isomerase-induced arthritis, and K/BxN serum transfer arthritis (unpublished data)]. In AIA, the requirement of an intact GR dimerization was found to be critical in the inhibition of IL-17-producing T-cell activation (20). Thus GR dimer-dependent induction of anti-inflammatory-acting genes appears to be required to efficiently suppress inflammation in arthritis.

Presently, there are a few known genes that could serve as candidates to mediate the immunosuppressive effects of GCs. DUSP-1/MKP-1, which is induced by the GR, is critical for the suppression of inflammation in various inflammatory mouse models (2, 390). DUSP-1 is a classical GR
homodimer target gene (166) that inhibits MAP kinase signaling, such as JNK1/2, which is sufficient to ameliorate TNF-induced septic shock (390). DUSP-1 knockout mice develop an early onset and higher score of arthritis, demonstrating the immune-modulatory role of DUSP-1 (392). Whether these mice are impaired in their response towards exogenous GCs is currently unknown.

GILZ is another potent anti-inflammatory GC-induced target protein (72). Indeed, single injections of adenoviral GILZ into both knee joints of CIA mice result in a significant reduction of arthritis severity (248). In line with this, depletion of endogenous GILZ by siRNA augments the severity of CIA in terms of clinical and histological score and increases TNF-α and IL-1β expression in the joints (24). Nevertheless, the role of GILZ as an anti-inflammatory target of GC therapy in arthritis is more complex. It has been reported that GILZ deletion or knockdown does not alter disease development in K/BxN serum transfer arthritis nor in CIA (248). The response of GILZ-deficient animals towards dexamethasone treatment has not been reported.

The most promising mediator of the anti-inflammatory actions of GCs in RA is annexin-1, a Ca^{2+}-dependent phospholipid-binding protein. Annexin-1 (encoded by the Anxa1 gene) activates a G protein-coupled receptor (ALXR), leading to inhibition of leukocyte migration. Intriguingly, GC effects are abrogated in Anxa1 knockout mice with K/BxN serum transfer arthritis (267).

B. Role of Endogenous Glucocorticoids

In addition to exogenously applied GCs, the inflammatory response in arthritis is influenced by endogenous GCs released upon hypothalamus pituitary adrenal axis activation triggered by severe inflammation. Pharmacological inhibition of the GC-activating enzyme 11β-HSD1 in RA patients through metyrapone results in exacerbation of the clinical signs of RA (311). In contrast to this finding, systemic elevation of 11β-HSD1 is accompanied by persistent arthritis in humans (245). The role of endogenous GCs in vivo also remains controversial. However, prevailing evidence points to an anti-inflammatory function. Inhibition of GR activity by RU486 (GR antagonist) leads to a higher susceptibility of Lewis rats to streptococcal cell wall-induced arthritis (356). Accordingly, adrenalectomy leads to lethality of rodents with AIA, an outcome that can be prevented with low-dose dexamethasone treatment (275). In rats subjected to adjuvant arthritis, depletion of endogenous GC signaling by adrenalectomy is accompanied by increased serum levels of IL-1 (63) and the pro-inflammatory anti-GC-acting macrophage migration inhibitory factor (191). Accordingly, global 11β-HSD1 knockout mice display an earlier onset of K/BxN serum transfer arthritis and an increased recruitment of inflammatory cells in a pleural inflammation model (69). However, the peak of inflammation is the same in control and 11β-HSD1-deficient mice (69). This view of an anti-inflammatory role has been challenged following analysis of Col2.3–11βHSD2 tg mice which display impaired GC signaling in osteoblasts (51). Upon induction of K/BxN serum transfer arthritis in these mice, a slightly attenuated score of inflammation is detected, suggesting a pro-inflammatory role of GCs in arthritis. However, the same group could not observe this effect in AIA, leaving open the relevance of osteoblast-specific signaling in the inflammatory response of arthritis (352).

In summary, the majority of studies point to an important role for endogenous GCs in the resolution of systemic inflammation in RA; however, the effects of endogenous GCs on local joint inflammation require further analysis.

C. Glucocorticoid Effects on Bone in Mouse Models of Rheumatoid Arthritis

Very little is known about the mechanisms of GC therapy in arthritis and the subsequent GC actions on bone stability and integrity in the context of inflammation. Recently, Takahata et al. (364) have shown an additive adverse effect of GC therapy and human TNF expression on the mechanical stress and stiffness of tibia and vertebra bodies in a TNF-tg mouse model of arthritis. GC treatment of TNF-tg mice leads to a significant increase in osteoclast activity and a slight increase in osteocyte apoptosis, explaining the worsened bone quality. Furthermore, an in vitro study suggests that GC-induced Dkk1 expression in FLS could contribute to deleterious GC actions on bone by inhibiting Wnt activity on osteoblasts (124). This is supported by an increased Dkk1 expression in FLS, but also in microvessels and chondrocytes of RA patients, resulting in reduced bone formation (81). Furthermore, inhibition of DKK1 leads to a strong reduction of bone-resorbing osteoclasts (81). In contrast, prednisolone suppresses osteoclast activity in vivo in AIA (136) and K/BxN serum transfer arthritis (148) models. Thus, in these mouse models, GCs appear rather to have beneficial effects by reducing arthritis-induced bone damage by inhibiting bone resorption. Perhaps animal models have not yet been sufficiently exploited to demonstrate the well-known effects of GC treatment of patients and their impaired bone quality. In conclusion, the mechanisms of GC action on inflammation and bone resorption in arthritis appear to be rather complex. Exogenous and endogenous GCs might have different and overlapping functions in the stimulation and attenuation of inflammation. Only a few GR target genes inhibiting inflammation are known (e.g., Anxa1 and Dusp1).

Studies in transgenic animals have revealed that T cells appear to be critical cell types in RA (20), although other cell types may conduce the observed GC-induced effects equally. In particular, FLS are key players in the progression of RA (38). Unfortunately, it is difficult to target FLS spe-
specifically with Cre-loxP technology since mouse lines exclusively addressing FLS are not available. However, liposomal encapsulated prednisolone linked to a fibroblast-specific peptide has revealed a suppression of AIA due to FLS-specific actions of GCs (391). Further research is required to define precisely the relevant cells and their GC-responsive effector genes in the different arthritis models.

VI. OSTEOARTHRITIS

With more than 10% of the adult population in the United States being affected in 2005, osteoarthritis (OA) is the most common form of arthritis (189, 240). A hallmark of OA is the degeneration of joints, especially the damaged areas of articular cartilage and subchondral bone. The damaged areas are accompanied by spurs of newly ectopically formed bone in the joints called osteophytes. These abnormalities in the synovial joint tissues result in symptoms of pain, stiffness, and functional disability (83). As with RA, the etiology of OA is not completely understood. Biomechanical stimuli, mechanisms controlling the anabolic and catabolic activities of chondrocytes, as well as inflammatory processes in osteoarthritic tissues appear to regulate the initiation and progression of the disease (207). In normal adult cartilage, chondrocytes maintain the ECM by low-turnover replacement of certain matrix proteins. In OA, this balance between synthesis and degradation of ECM proteins is disturbed (118). Soluble mediators released by cartilage, subchondral bone, and synovium, such as inflammatory cytokines or prostaglandins, can lead to an enhanced production of degradative proteinases, associated with a gradual degradation of proteoglycans and type II collagen.

Since the 1950s, GCs have been used as a therapeutic drug for OA. GC-associated pain relief and functional improvement are mediated by their anti-inflammatory effect (137). To the best of our knowledge, to date, neither the United States Food and Drug Administration nor any other agency has approved a drug for osteoarthritic disease modification. Available drugs only provide symptomatic relief, but no curable effect. This lack of availability of a curative drug is partly attributed to the poor understanding of the mechanisms initiating and driving the disorder (95). Therefore, GCs are still widely used for the management of OA in clinical practice. The short-term benefit of intra-articular corticosteroid administration in the treatment of knee OA with few side effects appears to be well proven (11, 223, 227, 430). In contrast, the value of long-term therapy remains controversial (11, 30, 294, 424).

The beneficial effects of GCs are caused partly by stimulatory effects on the synthesis of ECM components, like glycosaminoglycan and derived proteoglycan in chondrocyte cultures (168, 365). In addition, a dose-dependent inhibition of glycosaminoglycan loss by GCs could be detected in human and bovine cartilage explants which had been subjected to combined catabolic effects of mechanical injury, TNF-α and IL-6/soluble IL-6 receptor (214). A second mechanism of cartilage protection by GCs is the inhibition of the expression of stromelysin, a key enzyme of ECM catabolism, as revealed in an experimental dog model of OA in which the anterior cruciate ligament of the knee had been sectioned (269, 272). The same effect on catabolic ECM proteins could be shown in vitro in healthy human chondrocytes and chondrocytes isolated from cartilage explants of OA patients (82, 359). In rabbits exposed to drill hole bone-injury surgery to induce a posttraumatic OA model, as well as in the canine OA model mentioned above, a GC-mediated inhibition of inflammation and cartilage damage has been associated with reduced levels of IL-1β (143, 269). Hence, the cartilage-protective effect of GCs appears to be mediated through a direct reduction in the expression and synthesis of proteolytic enzymes, an inhibited stimulation of protease synthesis by cytokines, as well as an increased synthesis of proteoglycans. These beneficial effects have been shown to result in macroscopic changes, like reduced numbers and size of osteophytes and a reduced histological grading of cartilage lesions in animal models as well as in patients after GC administration (270–272).

Apart from these positive effects of short-term applied GCs, it is widely recognized that repeated intra-articular injections of GCs potentially result in the degeneration of the affected osteoarthritic tissue, so-called steroid-induced arthropathy (102, 234, 243). In part, this serious side effect is possibly attributed to a decreased expression of major cartilage matrix components. In this regard, GC administration has been shown to cause a decrease in type II collagen expression in cultured rat articular chondrocytes, as well as in rat articular cartilage. These in vitro examinations indicate a suppression of the collagen type II promoter by a GC-dependent inhibition of AP-1 activity (234). In rabbit articular cartilage, a reduced synthesis of proteoglycans accompanied by a degeneration of the tissue has been demonstrated in response to GC administration (28). In chondrocyte cultures from human osteoarthritic cartilage samples, the expression of aggrecan, the major proteoglycan in cartilage, is inhibited by dexamethasone by 30–46% (359). In addition, in a murine chondrocyte cell line, GC exposure results in a significant reduction of cell number and proliferation (241).

These controversial data on GC effects on the anabolic and catabolic chondrocyte metabolism prompted studies trying to correlate the effects to the concentrations used. The GC concentrations that inhibit matrix synthesis followed by a deterioration of cartilage tissue have to be higher than those needed to suppress metalloproteinase synthesis and effectively reduce negative changes in osteoarthritic joints (96, 102, 300, 373). To our knowledge, studies on transgenic mice with cell type-specific altered GC signaling are scarce.
in OA research. More such studies would be invaluable at shedding light on the complex actions of GCs in OA in the future.

VII. ROLE OF BONE IN GLUCOCORTICOID-INDUCED INSULIN RESISTANCE

Apart from skeletal side effects, patients receiving long-term GC treatment also develop clinical features of type 2 diabetes, such as insulin resistance and obesity (317). Currently, the precise mechanisms by which GCs affect glucose metabolism at the molecular level are not well understood. In the last 20 years, great efforts have been made to identify targets and signaling pathways that are responsible or contribute to GC-induced insulin resistance to improve the clinical outcome of long-term GC therapy. In particular, highly metabolic organs like liver, muscle, adipose tissue, and pancreas have been the main targets of extensive investigations (discussed in detail elsewhere) (268, 386). In brief, GCs may induce insulin resistance through downregulation of the insulin receptor (IR) adaptor protein insulin receptor substrat-1 IRS1 (in fat and muscle) and of insulin receptor substrat-2 IRS2 (in fat). GCs upregulate phosphatases like PTP1B and p38MAPK in liver and muscle and counteract by this insulin signaling in these tissues (6, 7, 47, 309). As a result, phosphorylation of IR and IRS1 is decreased in response to GCs (21, 309). Moreover, in rat skeletal muscle, a reduced glucose transporter (GLUT4) migration to the cell surface was observed after GC treatment (410). Furthermore, short-term GC treatment in mice leads to a transcriptional change of 518 genes in the liver, 104 of them linked to metabolic signaling pathways like glucose metabolism/transport, gluconeogenesis, glycolysis, lipid metabolism, and many other metabolic pathways (101). Among the downregulated enzymes are IRS1 and pyruvate kinase, a glycolysis-associated enzyme that catalyzes the production of pyruvate from phosphoenolpyruvate. The insulin-controlled transcription factor Foxo1 belongs to the upregulated genes after GC treatment in wild-type mice. Foxo transcription factors regulate, in addition to a subset of other genes, the expression of enzymes that are important for the regulation of gluconeogenesis in the liver [e.g., Pepck (phosphoenolpyruvate carboxykinase) and glucose-6-phosphatase] (18, 242). As a consequence, GC-induced insulin resistance promotes hepatic gluconeogenesis and therefore increases hyperglycemia. Other effects of GCs on metabolic organs are increased proteolysis, lipolysis, and free fatty acid production (268, 386).

Similar to the classical metabolic organs, the skeleton can influence systemic glucose metabolism through the bone-pancreas-endocrine loop (for detailed information, see Refs. 104, 165). Osteocalcin (OCN), a noncollagenous protein mainly produced by osteoblasts and osteocytes, is a bone-specific metabolically active hormone that controls insulin production and sensitivity. Mice lacking OCN show, in addition to a rise in bone formation, increased blood glucose levels, lower insulin serum levels, and enhanced insulin resistance, a phenotype resembling type 2 diabetes (87, 190). Prior to secretion, OCN undergoes a vitamin K-dependent carboxylation at three defined glutamic acid residues (125). However, only undercarboxylated OCN functions as a hormone to promote β-cell proliferation and insulin expression (190, 405). Therefore, changes in the bioactivity of circulating OCN modulate whole body glucose metabolism.

As discovered by Brennan-Speranza et al. in 2012 (40), OCN appears to play an important role in the context of GC-induced insulin resistance. Overexpression of 11β-HSD2 under the control of the rat 2.3-kb collagen type I promoter results in selective oxidation of GCs into the inactive metabolite cortisol. This restricted GC inactivation, especially in osteoblasts and osteocytes, results in a diminished GC-mediated reduction of OCN serum levels (40). Furthermore, disruption of GC signaling in osteoblasts and osteocytes attenuates the development of obesity, improves glucose tolerance, and partially protects from insulin resistance in response to GCs. Thus targeted disruption of GC signaling in osteoblasts improves whole body glucose homeostasis mediated by elevated undercarboxylated OCN levels. Restoration of OCN via gene transfer in the liver of GC-treated mice restores insulin and glucose sensitivity. Moreover, heterotrophic expression of OCN reduces hepatic steatosis and rescues insulin signaling to levels detected in wild type mice (FIGURE 6).

The precise mechanism by which GCs change the bioactivity of circulating OCN levels is still unclear. Presently, several signaling pathways are known to regulate and influence undercarboxylated OCN levels. For example, OCN can be regulated by Esp (embryonic stem cell phosphatase), a gene specifically expressed in osteoblasts that encodes the tyrosine phosphatase OST-PTP (osteotesticular protein tyrosine phosphatase) (190). Mice selectively overexpressing Esp in osteoblasts develop insulin resistance and display lower levels of undercarboxylated OCN. Rached et al. (288) showed that particularly Foxo1 in osteoblasts diminishes OCN levels by stimulating Esp expression (288). Thus Foxo1 knockout mice are hypoglycemic and show an improved glucose tolerance due to increased β-cell proliferation and insulin sensitivity. In addition, Foxo1 not only decreases OCN bioactivity indirectly by upregulation of Esp expression, but also through binding to the OCN promoter region to decrease the promoter activity. Collectively, these results indicate a model system in which phosphorylation of Foxo1 via insulin would render it unable to stimulate Esp transcription and suppress OCN expression, resulting in rising levels of active OCN in the serum. Since short-term GC treatment leads to an upregulation of Foxo1 expression, Foxo1 appears to be a potential target of GC-induced changes in systemic glucose metabolism.
The role of bone in glucocorticoid-induced changes in whole body fuel metabolism. GCs impair osteoblast function and hence reduce circulating levels of bioactive (undercarboxylated) OCN. The precise mechanism by which GCs influence the secretion of OCN (either directly by gene expression/signaling pathway modifications or indirectly via increased bone resorption in the resorption lacuna) is still unknown. Elevated serum OCN levels can stimulate the insulin secretion of pancreatic β-cells and impact the glucose metabolism of peripheral organs (liver, muscle, adipose tissue), thus changing whole body glucose homeostasis. Direct effects of GCs on peripheral metabolic organs have not been taken into consideration in this hypothetical mechanism.

Moreover, insulin signaling in osteoblasts can favor bone resorption, thereby influencing undercarboxylated levels of OCN in the serum. Osteoblast-specific IR \(^{-/-}\) mice display an increased level of OPG (99). OPG functions as a decoy receptor for the RANK ligand (RANKL), which normally induces osteoclast differentiation (369). Therefore, insulin signaling leads to a reduced expression of Opg that allows RANKL to bind to RANK (receptor activator of nuclear factor-κB) on preosteoclasts to stimulate osteoclast differentiation. Due to increased osteoclastogenesis, higher bone resorption occurs and an acid pH is created in the resorptive environment. It has long been recognized that simply lowering the pH enables proteins to be decarboxylated (91). Therefore, previously hydroxyapatite-bound OCN is decarboxylated in the resorption area and released in its active undercarboxylated form into the bloodstream (99). Further studies need to be performed to precisely identify the molecular basis of GC actions in osteoblasts that diminish undercarboxylated OCN levels in the serum and thus have an impact on whole body fuel metabolism.

Similar to the results in rodents, evidence appeared that serum OCN levels and whole body glucose metabolism are relevant for humans. Indeed, obese diabetic patients show lower levels of serum OCN than nondiabetic controls and undercarboxylated OCN levels are inversely associated with glucose levels and fat mass in type 2 diabetes (144, 173, 360, 425). At least in mouse models, OCN infusions can improve various aspects of glucose metabolism and fat mass in vivo and therefore weaken the development of obesity and diabetes (97, 98). For example, low doses of OCN can increase β-cell-mediated insulin secretion, improve glucose tolerance, and favor insulin sensitivity. Mice under a high-fat diet develop fewer fat pads, gain less weight, and show decreased insulin resistance. A possible concern is that the Esr gene, which encodes OST-PTP, one of the major regulators of the osteoblast-pancreatic-endocrine loop in rodents, is a pseudogene in humans. However, a substitute of OST-PTP (called PTP1B) exists in human osteoblasts. This phosphatase can replace OST-PTP due to the fact that it also dephosphorylates IR in many cell types. Nevertheless, another important concern has arisen from a recent publication by the Karsenty group showing that, at least in a mouse model, chronic exposure to high levels of OCN can lead to a resistance of the pancreatic β-cells towards OCN, thereby possibly worsening the metabolic effect rather than improving it (97).

But what about using OCN therapy in the context of improving GC-induced metabolic side effects? It has been shown that OCN levels decrease in patients receiving long-term GC therapy (287, 297). A similar reduction of OCN levels has been observed in patients with metabolic syndromes, underlining the connection between bone and glucose metabolism in humans. Moreover, levels of undercarboxylated OCN decline in humans treated with GCs, an effect that could potentially be mediated by a GC-induced activation of the vitamin K-dependent carboxylase (114, 140, 398). Therefore, combining GC administration with OCN treatment might be beneficial to avoid metabolic side effects of GC treatment in humans. However, is an OCN replacement strategy able to compensate for other GC-induced side effects, like GC-mediated bone loss? The discovery of multiple GR binding sites in the promoter region of the rat OCN gene has led to the proposal that GC-mediated transrepression of OCN might be one of the reasons for bone loss seen after long-term GC treatment (128). However, in contrast to the expectation that loss of OCN decreases bone formation, a transgenic mouse model revealed an increase in bone formation and rather improved bone functionality (87). Thus GR actions on bone metabolism are, at least partly, mediated by the monomeric GR, whereas the contribution of monomeric or dimeric GR action to insulin resistance is currently poorly understood (290). Therefore, different modes of GR actions can contribute to the side effects of long-term GC treatment.

In summary, there is clear evidence that OCN levels are linked to fuel metabolism in animals, and these findings are supported by clinical studies in humans. In the context of GC-induced insulin resistance, further studies are needed to uncover the precise effect of GCs on bone and glucose metabolism to assess OCN as a promising candidate for therapeutic use. Since GCs cause diverse effects on metabolic...
organs, it seems unlikely that GC actions on bone metabolism and OCN activity are solely responsible for GC-induced metabolic side effects. Moreover, OCN is described as a negative regulator of bone formation in rodents, thus maybe counteracting therapeutic effects in the context of osteoporosis rather than improving them.

VIII. STRATEGIES TO IMPROVE GLUCOCORTICOID TREATMENT

Despite severe metabolic and musculoskeletal side effects, corticosteroid therapy is frequently used to combat inflammatory diseases. These side effects are especially dramatic in arthritic disease, in which bone integrity is already compromised and long bone growth, particularly in children, can be severely attenuated.

Based on the mechanistic knowledge of GR function, one could envisage the following future strategies: 1) developing selective GR ligands, 2) establishing tissue-specific delivery of GCs, or 3) targeting GR-regulated pathways or genes to specifically counteract side effects.

A. Selective Glucocorticoid Receptor Modulators

The search for GR ligands favoring transrepression of cytokine genes under the control of AP-1, NF-κB, IRF-3 instead of GR-mediated transactivation began in the late 1990s (316). Due to a long-standing dogma that inhibition of these proinflammatory transcription factors would be sufficient to suppress inflammation, these selective GR modulators promised to be successful in inhibiting inflammation while producing fewer side effects (127, 393). Unfortunately, very few of these compounds have found their way into clinics. To date, only a few GR modulators applicable for skin diseases requiring topical application are in clinical trials. This overall failure has multiple reasons. The first generation of these steroidal selective GR modulators were selected on the basis of a few reporter gene assays in vitro, but subsequently failed in their pharmacology in vivo. Another issue is the fact that complete anti-inflammatory efficacy requires transactivation of anti-inflammatory genes for the suppression of inflammation in many disease models (Ref. 66 and see sect. V). On the other hand, for GIO, GR-mediated transrepression of certain genes appears to attenuate osteoblast differentiation (290), indicating that selective GR modulators might still cause bone loss. Finally, it has only been in recent years that ChIP sequencing approaches have demonstrated the complexity of the mechanisms of GR-dependent gene regulation. The recently uncovered cell type-specific GR genome interactions were not considered when the first concepts for selective GR ligands were developed. Furthermore, binding of GR monomers to DNA appears to be more common than previously anticipated, independent of gene upregulation or downregulation (320). This questions the concept of whether interference of GR dimerization is sufficient to abrogate transrepression in general. Therefore, cell type-specific readouts for cellular function in response to GCs need to be developed. These would serve as new tools to identify ligands of the GR that mediate an anti-inflammatory response, e.g., in a T cell or macrophage, but spare the differentiation potential of an osteoblast or the proliferative potential of a chondrocyte. High-content screening platforms, a mainstay of the pharmaceutical industry and currently being introduced into academia, will allow the selection of these specific compounds. One proof-of-principle compound is the nonsteroidal CpdA that does not antagonize AP-1-dependent IL-11 expression and thus does not interfere with osteoblast differentiation (289). Moreover, this substance is bone-sparing in mice (371). Future cell-based screens may result in the discovery of additional GR ligands that spare osteoblasts. Some of these ligands may serve as lead compounds for the development of novel bone-sparing anti-inflammatory GC drugs.

B. Activating Bone Cell-Protective Pathways

As well as the search for novel GR regulators, GR-modulated pathways could be directly addressed. One option is the inhibition of sclerostin to increase Wnt signaling and thus improve osteoblast function. Using neutralizing antibodies against sclerostin, which is mainly expressed in osteocytes, would ensure a rather cell-specific effect and presumably avoid additional side effects. Although antibody treatment of prednisolone-exposed mice has been found to have only minor effects (222), optimized conditions may protect bone from GIO. Alternatively, application of miRNA-29a could restore Wnt signaling. miRNA-29a has been identified as an osteoblastogenic inducer increasing ALP activity and Runx2 protein levels and at the same time targeting negative regulators of osteoblastogenesis, such as HDAC4 and TGF-β (178).

In a novel approach, unidentified pro-osteoblastic and pro-chondrogenic factors and pathways could be obtained by unbiased siRNA screens using marker genes and enzymes as screening readouts. Such efforts have been performed by the Glimcher laboratory (435). Here, a lentiviral-based shRNA library that targeted 1,500 genes was transduced into primary MSCs. This led to the identification of the microtubule-associated protein DCAMKL1. As DCAMKL1 inhibition improves osteoblast differentiation in vitro and in vivo, it presents an attractive new target for the improvement of bone mass in general. The enlargement of such screens to a genome-wide scale will provide novel possibilities to improve the integrity of the skeletal system, even under conditions of long-term corticosteroid therapy.
Our knowledge of the molecular mechanisms of the GR underlying the multiple effects of GCs on the skeleton is still far from complete. However, in the last decade much has been learned from mechanistic studies of genome-wide analyses, including DNA binding of the receptor and transcriptomics in response to GCs. Although some primary cell types and tissues such as macrophages, adipocytes, and liver have been studied, the findings gained by these novel techniques have mainly come from cell lines. Therefore, the situation in the individual cell types of the skeleton remains to be determined. ChIP-Seq studies, e.g., in liver, macrophages, and adipocytes, revealed GR-binding sites in close proximity to tissue-specific transcription factors of the respective cell types, suggesting there might be cell type-specific effects of GCs. Thus it is tempting to speculate that specific transcription factors of the chondrocyte-, osteoblast-, osteocyte-, and osteoclast-lineage cooperate with the GR to mediate GC effects on bone growth and homeostasis. Exploitation of these cooperative GR transcription factor networks and their control over bone cell functions should be addressed in the near future. Defining the functionality of GR-containing transcription factor complexes in terms of certain genomic loci for execution of hormonal effects on the function of skeletal cells remains a challenge. The advantages of genome editing such as CRISPR/Cas9 will allow this demanding task to be tackled in a genome-wide manner.

Another exciting area that still needs to be unraveled is the impact of GCs on cell lineage decisions. Most of our knowledge of the differentiation of bone cell types from progenitor cells derives from tissue culture models. The concept that MSCs become more prone towards adipocyte differentiation and less towards osteoblast differentiation upon exposure to GCs has been a general hypothesis throughout many decades. However, recent cell lineage tracing studies in mice suggest that adipocytes may not derive from the same progenitors as osteoblasts. Thus, whether the plasticity of mesenchymal cells present in vitro also occurs in vivo is not clear. In vivo studies deciphering the lineage decisions of bone progenitor cells are necessary to understand bone homeostasis under corticosteroid therapy.

When studying the pharmacological consequences of GCs, one has to be aware that GCs are usually applied in inflammatory conditions that have themselves an impact on the bone, such as RA. The complex action of GCs on modulating the immune response and thereby impacting on bone growth, formation, and resorption is still poorly understood. The unraveling of these mechanisms will be decisive for the achievement of therapeutic applications for arthritis that protect the bone even beyond the use of GCs. The underlying mechanisms of GCs in OA therapy are even less well understood, despite them also being clinically used for OA. Studies in transgenic mouse models addressing GC effects in such bone degenerative diseases are needed.

Due to the emerging role of bone in energy and glucose metabolism, it is conceivable that GC-induced diabetes may rely in part on bone cells. Whether the GR in osteoblasts is essential for GC-induced insulin resistance still needs to be proven to develop rationales for bone-protecting therapies that might also avoid diabetes.

To establish alternatives for corticosteroid therapy based on our current knowledge, the classical concept of selective GR modulators only addressing the GR monomer has to be reconsidered. Novel bone cell-dependent screenings are one possibility to search for ligands with fewer effects on bone. To find substances that retain their anti-inflammatory efficacy similar to classical GCs is a challenge, but not impossible. In parallel, improving cell type-specific delivery of GCs is another promising possibility to lower systemic concentrations of pharmacological GCs and thereby protect skeletal tissue, cartilage, and bone.

The quest for the molecular mechanisms of this ligand-induced transcription factor in skeletal disease is ongoing. Upcoming results will not only provide insight into the effects of GCs as a drug, but also the endogenous hormone during skeletal maturation and aging.

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438 Physiol Rev • VOL 96 • APRIL 2016 • www.prv.org


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GLUCOCORTICOIDS IN CARTILAGE AND BONE


