Chapter 1

General introduction

Historical aspects of glucocorticoids

Glucocorticoids (GCs) constitute a well-characterized class of steroid hormones that are produced by the adrenal glands. The name glucocorticoid derives from their role in the regulation of glucose metabolism (‘gluco-‘), their synthesis in the adrenal cortex (‘-cortic-‘) and their steroidal structure (‘-oid’). They were first isolated in 1936 and in 1948 they were first used clinically as anti-inflammatory drugs in the treatment of rheumatoid arthritis patients [1]. The importance of the discovery of GCs culminated in a Nobel Prize in Physiology or Medicine for Edward Kendall, Tadeus Reichstein and Philip Hench in 1950 for their work related to the structure and physiological effects of these steroid hormones [2, 3]. Since then, many synthetic GCs such as prednisolone and dexamethasone have been produced and they have widely been prescribed as forefront immunosuppressive drugs, in cases of many inflammatory, autoimmune (e.g. multiple sclerosis), allergic (e.g. asthma) and infectious (e.g. septic shock) disorders, graft rejections and malignancies of the immune system (e.g. lymphoblastic leukemia) [4-6].

Glucocorticoids: biosynthesis & secretion

GCs are synthesized from cholesterol in the mitochondria via a cascade of enzymatic reactions that include cytochrome P450c21 and P450c17, and result in the production of cortisol which is the main GC in primates and fish, and corticosterone, the main GC in rodents [7, 8]. Availability of active GCs is regulated by two isoenzymes: 11β-hydroxysteroid dehydrogenase type 2 inactivates GCs by converting cortisol to cortisone (an inert GC) whereas 11β-hydroxysteroid dehydrogenase type 1 mainly regenerates the active forms of GCs [9].

Biosynthesis and secretion of adrenal GCs are controlled by upstream signaling cascades that constitute the Hypothalamic-Pituitary-Adrenal (HPA) neuroendocrine axis. Hypothalamic neurons release Corticotrophin-Releasing Factor (CRF) and Arginine vasopressin (AVP) that reach the pituitary and synergistically trigger the release of the Adrenocorticotropic Hormone (ACTH). The latter arrives, via the blood circulation, at the adrenals where upon binding to the melanocortin 2 receptor (MC2R), it orchestrates signal transduction (e.g. upregulation of steroidogenic gene transcription) that ultimately leads to the production and subsequent secretion of active GCs into the blood stream. Circulating GCs feed back to the level of the pituitary and the hypothalamus in order to suppress the production of ACTH and CRF, thereby negatively regulating their own secretion [10].

Glucocorticoids: modulation of secretion & physiological effects

During a 24 hour period, circulating levels of GC are not stable but fluctuate according to a circadian rhythm, imposed by the suprachiasmatic nucleus of the hypothalamus that interacts with CRF positive neurons and thereby directs the increase in circulating levels of GCs during active periods (daytime for diurnal animals) and lowers them significantly during resting periods (nighttime for diurnal animals) [11]. Importantly, circulating levels of GCs also peak upon stressful challenges. Stress can be defined as a physiological and complex reaction of the body towards (perceived) physical and emotional events that threaten or disrupt the homeostatic status of an organism. The hypothalamus is the brain region that perceives and integrates neuronal and humoral information concerning changes in body homeostasis and together with the sympathetic nervous system initiates a biological response in order to cope, recover and adapt to stressors [12-15]. Sympathetic

innervation mediates the rapid release of catecholamines from the adrenal medulla, whereas hypothalamic activation, via the HPA axis, leads to a relatively slow increase in the secretion of GCs from the adrenal cortex [16]. GCs are involved in an array of biological processes. Briefly, GCs catalyze carbohydrates, proteins and lipids and divert energy to the brain and the muscles, they promote liver gluconeogenesis, enhance cardiovascular tone, but suppress growth and reproduction as well as immune reactions [12, 13]. Most likely, the physiological significance of this latter effect is to prevent the initial immune response from overshooting, which would be damaging to the organism [13]. For that purpose GCs suppress the production of cytokines, hinder leukocyte trafficking and interfere with the maturation and lifespan of a number of immune cells (e.g. they inhibit dendritic cell maturation, and induce apoptosis of T cells, eosinophils and basophils) [4, 17]. Among others, these effects have constituted the basis of the clinical use of GCs as very potent anti-inflammatory drugs. Nevertheless, the interplay between GCs and the immune system is very complex since GCs have also been demonstrated to enhance or prime certain inflammatory processes such as regulatory T cell and Th2 differentiation, as well as antibody production [4, 17]. Furthermore, not all patients respond to GC treatment [18] and in addition to that, chronic exogenous administration of GCs can lead to deleterious side effects, such as skin atrophy, decreased wound healing, osteoporosis, muscle atrophy, glaucoma, psychosis, diabetes mellitus and hypertension [19]. Taken into consideration these issues, since the discovery of the effects of GCs on inflammation more than 70 years ago, intense scientific research is aiming to unravel the exact regulation and mechanisms of GC-mediated signal transduction towards specific cellular and molecular targets, the physiological role of GCs in various biological processes (at basal as well as elevated hormone levels) and their interaction with other molecular factors, especially within immune-related signaling pathways.

The Glucocorticoid Receptor: structure & activation

The physiological and pharmacological effects of GCs are mediated by the Glucocorticoid Receptor (GR), which is a member of the steroid receptor family of ligand-dependent factors, such as the estrogen and androgen receptor. In turn, these steroid receptors belong to the superfamily of nuclear receptors to which the Vitamin D Receptor and Thyroid Hormone Receptor belong as well. In mammals, the GR is expressed in nearly all nucleated cells of the body and is necessary for life, since its deletion hinders the maturation of the embryonic lungs [20] and the production of insulin-like growth factor-1 (IGF-1) from the liver that promoters postnatal growth [21].

The human GR gene was first cloned in 1985 and is encoded by a single gene, located in chromosome 5 [22]. It consists of 9 exons of which exon 1 is non-coding and represents the 5' untranslated region [23]. The GR is a modular protein that is composed of distinct functional domains. Exon 2 encodes for the N-Terminal Domain (NTD) that is the immunogenic domain of the GR and harbors the activation fanction-1 (AF-1) region, which is required for optimal trasncriptional activity. Exon 2 and 3 encode for the DNA Binding Domain (DBD) that contains two zinc finger motifs through which the GR can bind to specific DNA sequences. The DBD also contains sequences corresponding to a weak dimerization interface as well as part of the first (ligand-independent) nuclear localization signal (NLS1). The rest of the NLS1 expands within the region adjacent to the DBD called the hinge region (encoded in exon 5) that confers structural flexibility in the receptor dimers and separates the DBD from the C-terminal part of the GR protein, the Ligand Binding Domain (LBD, encoded within exons 5-9). The latter is characterized by

12 α-helices and 4 β-sheets that form a hydrophobic pocket for GC binding. The LBD contains the activation function-2 (AF-2) domain that upon hormone binding, interacts with transcriptional co-activators (which promote induction of transcription) or co-repressors (which hinder transcription). Moreover, the LBD harbors a strong dimerization interface, an interaction interface with heat shock protein 90 (hsp90) and the second nuclear localization signal (NLS2). Upon ligand binding, the LBD undergoes a conformational change and hsp90 is released, thereby unmasking the NLS2 signal that subsequently allows translocation of the activated GR to the nucleus [3, 24, 25].

In the absence of hormone, the GR resides predominantly in the cytoplasm where it is embedded in a multiprotein complex containing heat shock proteins (e.g. hsp90, 70, 50) and immunophilins (e.g. FKBP51). Upon hormone binding, a conformational change occurs, the ligand-bound GR is released from its protein complex, phosphorylated at Ser 211 and is actively imported into the nucleus where it acts as a transcription factor [3, 24, 26].

The Glucocorticoid Receptor: modes of transcriptional activity

Activated GR modulates that transcription rate via DNA binding-dependent and -independent mechanisms that confer up- or downregulation of gene expression. For its DNA binding-dependent transcriptional activity, the GR forms a dimer and recognizes glucocorticoid response elements (GREs) within promoters of target genes. GREs are palindromic motifs, similar to the 15bp consensus sequence 5'-AGAACAnnnTGTTCT-3'[26]. Genes can have one or many GREs in order to be used for GR-mediated regulation of transcription. GREs can also be combined with other response elements specific for different transcription factors (e.g. C/EBP and FoxA) that synergistically with the GR promote gene expression (e.g. in the case of the gluconeogenic phosphoenolpyruvate carboxykinase (PEPCK) gene) [26]. This mode of upregulating transcription is termed as *transactivation* and can be observed, for instance, in the case of many metabolic enzymes (e.g. the tyrosine aminotransferase and tyrosine oxygenase gene involved in amino acid degradation, and the 6-phosphofructo-2-kinase gene involved in gluconeogenesis) as well as anti-inflammatory mediators (e.g. glucocorticoid-induced leucine zipper (GILZ), lipocortin, Map kinase phosphatase-1 (MKP-1), and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκB-α)) [26, 27]. Moreover, so-called negative GREs (nGREs) are present in genes of which the transcription rate is downregulated by GR [28]. For instance, the CRF and pro-opiomelanocortin (POMC) genes (the latter encodes an ACTH precursor protein) contain nGREs via which GC-mediated negative feedback on the HPA occurs, as well as the osteocalcin gene, whose product is important for bone formation and mediates GC-induced osteoporosis, a well-known side effects of GC treatment [26]. Once bound to GREs, the GR dimer via its AF-1 and AF-2 surfaces acts as a scaffold for coactivators (e.g. the SRC-1, -2, and -3 proteins) that that possess enzymatic activity in order to modify (e.g. acetylate) the core histones, thereby relaxing the chromatin structure for induction of gene transcription [2, 3, 24, 26], or recruit basal elements of the transcriptional machinery and in this way bridge the DNA-bound receptor to the RNA-polymerase II complex for initiation of transcription. GR-bound in nGREs may favor recruitment of chromatin remodeling factors with enzymatic activities (e.g. deacetylation) that would compact chromatin, thus hindering transcription. Alternatively, nGREs can overlap with other response elements, thus preventing binding of other transcription factors responsible for induction of genes [2, 26].

DNA binding-independent mechanisms of ligand-bound GR involve physical interaction of the activated receptor (as a monomer) with other transcription factors, thereby enabling either (synergistic) induction of genes (e.g. in the case the induction of IGF-1 by STAT5 [23]) or suppression of genes (e.g. in the case of NF-κB or AP-1 activity on the promoter of a large number of cytokine genes [2, 3, 10, 23, 26, 29]). The latter mode of action is termed *transrepression* and comprises one of the classical mechanisms by which GCs exert their anti-inflammatory effects [2, 3, 10, 24, 26, 29, 30].

Receptor variants deriving from a single Glucocorticoid Receptor gene

GCs are one of the most pervasive hormones in mammals and the GR is ubiquitously expressed. The receptor orchestrates the expression of a plethora of genes involved in various physiological processes such as intermediate metabolism, stress response, inflammation, growth and brain function [31]. These diverse effects in different tissues that ensure homeostasis can be exerted by the specific availability of several GR subtypes with different molecular profiles [32].

Its exon 1 (that is non-coding and represents that 5' untranslated region) harbors different transcriptional start sites, which are associated with unique promoters (that do not contain classical TATA or CCAAT boxes) and they are thought to account for differential gene expression regulation of the GR gene throughout the body [23]. Exon 2 encodes for alternative translational start sites that give rise to 8 GR isoforms with progressively shorter N-termini, from which the classical GR protein represents the longest variant. These isoforms differ in their cellular distribution and transcriptional properties [25].

Moreover, several subtypes derive from alternative splicing of the human GR pre-mRNA. The canonical receptor isoform (hGRα) is translated from an mRNA that contains 9 exons and is the predominant splice variant expressed in most cells. A much less expressed GR subtype, the hGRβ isoform, derives via alternative usage of an acceptor splice site within exon 9 [22, 33]. hGRα and hGRβ are identical between amino acids 1 and 727, thus sharing the same NTD and DBD, and only differ in the C-terminal part of their LBD. The hGR α-isoform is 777 amino acids long and is able to interact with GCs and subsequently modulate gene transcription. The hGR β-isoform contains 742 amino acids, and has a shorter LBD with a unique C-terminal 15 amino acid sequence, which renders it unable to bind GCs [22, 33, 34].

Using *in vitro* reporter assays, hGRβ was shown to have a pronounced dominant-negative effect on hGRα's transactivational properties [35-37], and hGRα-mediated transrepression of *in vitro* reporter assays was also reported to be inhibited by hGRβ [37]. Further cell culture-based research supports an inhibitory role for hGRβ on both hGRα-induced transactivation and transrepression of endogenous genes (MKP-1, myocilin, fibronectin, tumor necrosis factor α (TNFα) and interleukin 6 (IL6) [38-40], as well as on hGRα-mediated regulation of cell death, proliferation and phagocytosis [41-43]. These findings are in line with clinical data demonstrating a positive correlation between high expression levels of the hGR β-isoform and GC resistance of patients suffering from immune-related disorders, like asthma [40, 44-48], ulcerative colitis [49-51], leukemia [52-54] and rheumatoid arthritis [55, 56]. Interestingly, hGRβ expression has been shown to be increased in several types of human cells (e.g. HeLa, neutrophils, PBMC, ASM cells) upon stimulation with various cytokines (e.g. TNFα, IL1, IL2, IL4, IL7, IL8, IL17A and F, IL18, interferons) [41, 44, 57-60]. Furthermore, a single nucleotide polymorphism within the 3' UTR of the hGRβ mRNA, leads to a more stable mRNA of this receptor [55]. This polymorphism has been associated with increased insensitivity for GC-mediated

transrepression of IL2 in positive carriers [61] as well as occurrence of rheumatoid arthritis [55].

Recent data support the notion that hGRβ can have its own intrinsic transcriptional activity. Overexpression of this receptor isoform has been demonstrated to attenuate induction of luciferase reporter constructs by NF-κB and AP-1[62], as well as GATA3-mediated activation of the IL5 and IL13 promoters driving a luciferase genes [63]. Additionally, transcriptome analyses of cultured cells showed that hGRβ can direct gene transcription independently of hGRα activity [64, 65].

However, the GR β-isoform's physiological relevance has often been questioned [66]. First, in many studies the dominant-negative role of hGRβ on hGRα's transcriptional properties could not be demonstrated in *in vitro* reporter assays [62, 63, 66-71]. Second, despite the fact that hGRβ is expressed in almost all human tissues, its expression levels are significantly lower compared to hGRα [35, 36, 41, 72-74]. This raises doubts about its *in vitro* dominant negative effect, since in most studies this requires transfection of a 10-molar excess of GRβ expression vector compared to hGRα [35-37]. Until recently, a third point of controversy has been the fact that expression of a GR β-isoform was only found in humans, and that rodents contained a mutation in the splice site required for GRβ expression [75]. Recently however, a β-isoform was discovered in mice [76] as well as zebrafish [77] and its molecular characterization showed that it has a defective LBD unable to bind GCs as well as a significant inhibitory activity on GRα's transactivational properties in reporter assays [76, 77]. This has opened up a new chapter in GRβ research providing new tools and, most importantly, *in vivo* systems to investigate more thoroughly the exact role of this receptor and its mechanisms of action.

Apart from hGRβ, three more alternative splice variants have been identified (reviewed by Oakley et al. [25]). The hGR γ-isoform has an insertion of an arginine residue between the two zinc fingers of the DBD and is a result of the utilization of an alternative splice donor site in the intron separating exons 3 and 4. This isoform can bind DNA but has a different transcriptional profile from hGRα on a subset of genes, and its presence has been associated with various cancer tissues. hGR-A is derived from alternative splicing that links the end of exon 4 to the beginning of exon 8, and thus misses the N-terminal part of its LBD (encoded by exons 5-7). hGR-P is derived from a failure to splice at the exon7/8 boundary, which causes a lack of exons 8 and 9 that encode the C-terminal half of the LBD. Both hGR-A and hGR-P were discovered in GC-resistant multiple myeloma cells, and the latter receptor appears to be the predominant receptor variant in GC-insensitive cancer cells. hGR-P has been shown to either repress or stimulate GRα's transcriptional activity on reporter genes in a cell-specific manner [25].

Finally, post-translational modifications focus of phosphorylation that modulates the transcriptional activity of the receptor, its half life and its cellular trafficking, whereas ubiquitination, sumoylation and acetylation have also been shown to play a role in regulating its degradation by the proteasome and its transcriptional properties [25].

The zebrafish as a model organism for biological research

Although traditionally used as a model organism for vertebrate development, over the last decade, the zebrafish has emerged in biomedical research as an important *in vivo* model system for studies on a variety of human diseases [78-80]. Especially the high level of similarity of its immune response to that of mammals [81] provides an excellent research platform for modeling various molecular and cellular elements of inflammation such as host-pathogen interactions during infectious diseases and immune cell migration to wound

sites [82, 83]. Zebrafish are small, easily maintained and breed well under laboratory conditions. Each female can produce hundreds of eggs per day that are fertilized externally. Upon fertilization, the embryos develop rapidly, thus providing an excellent model system for the study of embryonic development. Precursors to all major organ systems have been formed within 36 hours after fertilization, and embryos progress to the larval stage within less than 3 days [84]. Their *ex utero* development and optical transparency allow for microscopic imaging at the cellular and even subcellular level, especially when performed in combination with fluorescent labeling of specific cells or proteins [85-87].

The zebrafish offers a valuable genetic vertebrate model system for both forward- and reverse-genetic studies. The zebrafish has been used in forward-genetic studies for the unbiased identification of pathways responsible for embryonic development, heart development, axon guidance, visual behaviour, axon myelination and many more using phenotype to genotype mutant screens [88-91]. Due to their small size and large number of offspring mutant screens are relatively fast and inexpensive [88, 89]. In these screens, adult fish are subjected to chemical mutagenesis and their offspring is studied for abnormalities. This approach provides an unbiased method for assigning functions to novel or already known genes.

Reverse genetics in zebrafish is facilitated by the completion of the entire zebrafish genomic sequence and by the possibilities of genetic manipulation via microinjections of DNA, mRNA and morpholinos at 1-2 cell stage embryos [92, 93]. The latter are chemically modified antisense oligomers that provide transient knockdown of specific genes [94]. Morpholinos affect protein synthesis by blocking the translation start site of an mRNA molecule, or alter the mRNA splicing pattern by blocking specific splice acceptor or donor sites. Furthermore, an increasing number of transgenic, mutant and knockout zebrafish lines are available (e.g. knockout lines generated by TILLING [95]) as well as several zebrafish cell lines derived from embryos and adult tissues, that can be used as a complementary tool allowing more refined biochemical characterizations [96, 97].

The zebrafish as a model organism for glucocorticoid signaling

In fish, cortisol secretion is regulated by a stress axis similar to those in mammals. Whole mount RNA *in situ* hybridization studies using well-characterized markers for different hypothalamic and pituitary cell types has shown that these cell types are present in zebrafish larvae [98-100]. However, there are anatomical differences between fish and mammals. The adrenal gland in mammals is homologous to the interrenal gland in fish, a structure embedded in the anterior part of the kidney in fish. The hypothalamus and pituitary in larval stages are shaped along the anterior/posterior axis in contrast to the equivalent structures in mammals which are shaped along the dorsal/ventral axis [99]. Moreover, the zebrafish pituitary lacks a stalk-like structure and as a result it is located closer to the hypothalamus compared to the mammalian pituitary [99].

The GR has been conserved well through evolution. It is found in jawed vertebrates, and not in jawless vertebrates, like the lamprey, dating back its origin to ~ 450 million years ago [101, 102]. After the lineage leading to tetrapods (encompassing amphibians, reptiles, birds and mammals) branched off from the teleost (bony fish) lineage a gene duplication occurred in the teleost lineage [103]. Due to this genome duplication, most teleost fish genomes contain two genes encoding for GR [104]. The presence of two GRs has been demonstrated in rainbow trout [105], Burton's mouthbrooder [106], green spotted puffer [104], fugu [104], common carp [107], and sea bass [108, 109], and the

resulting isoforms are called GR1 and GR2. Both GRs are functional receptors, but EC50s in *in vitro* transactivation assays differ between GR1 and GR2 in several species. It is therefore suggested that GR1 and GR2 are active at different cortisol concentrations *in vivo* [105, 106, 110].

Surprisingly, to date only one isoform has been identified in zebrafish [77, 104, 111]. Phylogenetic analysis of the teleost GRs shows that the zebrafish GR clusters in the GR2 clade, which suggests that the GR2 gene has been conserved and the GR1 gene has been lost during the evolution of the zebrafish [77, 104]. Syntenic analysis of the genomic region surrounding the GR1 gene in several fish species confirms this hypothesis [77]. The loss of the GR1 gene appears to have occurred less than 50 million years ago, since the common carp, a close relative of the zebrafish, is known to have two GR genes [107, 112]. The zebrafish GR gene (zGR) is organized similarly to the human GR gene (Fig.1). It consists of 9 exons of which exon 2 contains the translational start site and exon 9 the translation stop site and the 3'UTR. At the protein level, a similarly high level of homology is observed [77, 104]. The DBD of the zGR shows 98.4% similarity with the DBD of the human GR, and this percentage is 86.5% for the LBD. The NTD is less well conserved and displays 39.6% of similarity between human and zebrafish [77].

The zebrafish GR β-isoform

Interestingly, the zGR gene encodes two splice variants, the zGR α- and β-isoform (Fig. 1). Like their human equivalents, the zGR α- and β-isoforms share the same NTD and DBD and only differ at the C-terminus of their LBD. However, the gene organization and splicing events leading to the expression of GRβ are different between humans and zebrafish. Whereas in humans the zGRβ-specific sequence is found in exon 9, in the zebrafish it is located in exon 8. Usage of the most 5' splice donor site in this exon results in a shorter version of exon 8 and an mRNA encoding zGRα. Usage of the most 3' splice donor site leads to an extended version of exon 8 that introduces a stop codon within that exon, resulting in a mRNA encoding zGRβ [77]. Thus, the human and zebrafish β-isoforms are generated through different splicing mechanisms and there is low homology between their β-specific sequences, but they share the same point of divergence at the protein level [77, 113]. An alternative splicing event in exon 8 similar to that leading to the expression of a GR β-isoform in zebrafish was recently discovered in mice [76].

Additionally, both the human and zebrafish GR β-isoforms exhibit the same predominantly nuclear localization, and zGRβ also acts as a dominant-negative inhibitor of zGRα-mediated transactivation in *in vitro* reporter assays [77]. Like its human equivalent, zGRβ is ubiquitously expressed, and at significantly lower levels compared to zGRα [77]. All these data point to convergent evolution of hGRβ and zGRβ, which most likely emerged due to a common biological need for regulation of specific signaling pathways [77]. Hence, zebrafish could be used as a valuable *in vivo* model system in order to elucidate the physiological significance and mode of action of the GR β-isoform.

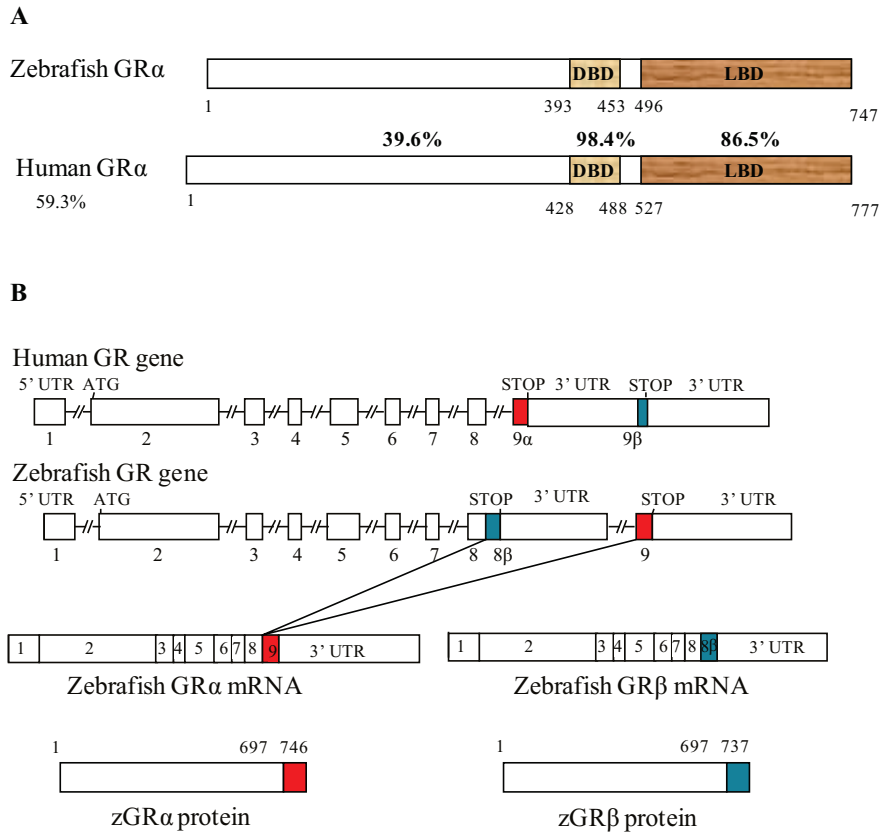

Figure 1: The zebrafish GR. **A.** The human and zebrafish GRα proteins.The percentage amino acid similarity is indicated for individual receptor domains and the full length receptor. **B**. The human and zebrafish GR genes. Both genes contain 9 exons, of which exon 1 is non-coding. A remarkable difference is the location of the sequence encoding the β-isoform-specific amino acids. In the human gene, this sequence is located in exon 9, whereas in the zebrafish gene it is found in exon 8. In zebrafish, the use of the most 5' splice donor site in this exon results in a shorter version of exon 8 and an open reading frame that extends into exon 9, resulting in mRNA encoding zGRα (GenBank Acc No. EF436284). The use of the most 3' site results in an extended version of exon 8, introducing a stop codon in exon 8, which results in zGRβ mRNA (Acc No. EF436285). The resulting GR protein isoforms are identical between amino acids 1 and 696. An additional 41 specific amino acids form the C-terminus of zGRβ which shows no homology to the 15 amino acid sequence that form the human GRβ-specific C-terminus. DBD: DNA binding domain. LBD: ligand binding domain

Glucocorticoid signaling research using the zebrafish

The zebrafish could be a valuable tool for at least two types of GC research. First, the zebrafish can be used to advance our knowledge on the molecular mechanisms underlying the effects of GR activation *in vivo*. Using techniques for transient or stable genetic manipulation in combination with imaging-based phenotypic readouts, the zebrafish can be used for analysis of how specific molecular mechanisms alter the phenotype of a living

vertebrate organism. Most of these phenotype-based assays are based on the imaging of fluorescent cells in zebrafish embryos that could be used on a relatively large number of individuals. Second, its potential could be used in studies towards the discovery of novel drugs and drug targets [114, 115]. Because of its small size and suitability for imaging studies, the zebrafish could be an ideal tool for the screening of novel glucocorticoid drugs. These screening assays could be implemented as an extra step between high-throughput drug screening assays (often performed in cell cultures) and subsequent studies in mammalian animal models like rodents. This way, compounds which appear to be ineffective in *in vivo* studies are filtered out at an early stage, limiting the number of compounds to be tested in mammalian models. In addition, using forward-genetic screens using glucocorticoid responsiveness as readout, novel drug targets may be discovered that may be exploited as a target for drugs that could increase the effectiveness of glucocorticoid treatment.

Molecular genetic tools

Several mutant zebrafish lines possibly interesting for GC research are available. A mutant zebrafish line is available that carries a mutation in the retinal homeobox gene 3 (*rx3*), resulting in a loss of corticotrope cells in the pituitary and severely reduced cortisol levels [116, 117]. In addition, other cortisol-deficient mutants lack the entire pituitary, like the fibroblast growth factor 3 mutant (*lia/fgf3* [118]) and the achaete scute-complex like 1a mutant (*pia/ascl1a* [119]). Another mutant, eyes absent 1 (*aal/eya1* [120]), only contains the lactotrope cells of the pituitary.

In addition, a few relevant morpholino studies have been performed. Transient knockdown of steroid biosynthesis using a morpholino reducing the cyp11a1 gene expression (the enzyme which converts cholesterol into pregnenolone, the first step in the steroid biosynthesis pathway) results in severe developmental defects, but which class of steroids is responsible for this effect is yet unclear [121]. In another study a morpholino approach was used to knock down GR function by blocking the splice acceptor site at the 5'end of exon 6, resulting in a GR transcript that lacks this exon [114]. The altered splicing results in an mRNA that encodes a GR protein lacking its LBD. Injection of this morpholino did not result in any obvious early developmental defects, suggesting that GR is not essential for early embryonic development [114]. This does not mean that alterations in GR function do not affect embryonic development, since GC treatment during the first days of development has been reported to result in craniofacial abnormalities, altered somitogenesis, blood pooling and pericardial and yolk sac edema [122, 123].

Expression levels of zGRα and zGRβ mRNA can be determined by qPCR [77, 111, 114, 117] and the expression pattern has been studied by in situ hybridization [77]. For detection at the protein level, western blotting [117] as well as immunohistochemistry on 1 day old embryos [124] have been performed using an anti-human GR antibody (p-20, available from Santa Cruz), which is directed against the receptor C-terminus, and is therefore specific for the GR α-isoform. Moreover, in zebrafish embryos treated with synthetic GCs such as dexamethasone, qPCR analysis revealed the transcriptional upregulation of *fkbp5, gilz,* nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha a *(nfkbiaa)* and *pepck,* and the suppression of *il8, il1b* and *tnfa,* all of which are well-known GR target genes in mammals [114, 124]. Hence, alterations in the expression of these GR target genes can be used as readouts of GR transactivation and transrepression activity *in vivo*.

Using zebrafish embryos and larvae, the immunomodulatory properties of GCs could easily be tested and characterized *in vivo*. It should be noted that zebrafish embryos and early-stage larvae only contain an innate immune system, and that cells representing the adaptive immune system (e.g. lymphocytes) start to mature at the second week of zebrafish development [125-127]. First, the actions of GR signaling in the cellular activity (e.g. trafficking, apoptosis) of zebrafish immune cells upon inflammation can be explored, based on fluorescently labeled cells. A transgenic fish line can be utilized containing the green fluorescent protein (GFP) gene driven by the myeloperoxidase (*mpo*) promoter, expressing GFP in the neutrophil granulocytes [128, 129]. These cells migrate to the site of injury after a wound has been made, and this experimental paradigm is considered as a model for acute inflammation [128]. Treatment of embryos with the synthetic GC beclomethasone results in a significant decrease in the number of neutrophils migrating to the trauma site upon amputation of a part of the tail [114]. A transgenic line expressing yellow fluorescent protein (YFP) in a subset of neutrophils [130], and a line expressing GFP in a subset of macrophages (with the GFP expression driven by the lysozyme C promoter [131]), have been used in similar assays of immune cell migration. In addition, several other transgenic lines are available in which a subpopulation of immune cells express GFP [132, 133]. Moreover, a transgenic zebrafish line that models chronic inflammation has been generated (caused by a mutation of the hepatocyte *hai1* gene), that shows accumulation of (GFP-labeled) neutrophils in the fin [134].

Second, the presence of T-cells in the thymus can be monitored. A transgenic zebrafish line can be used that expresses GFP under control of the T-cell specific tyrosine kinase promoter, resulting in GFP-labeled T cells. Treatment of embryos from this line with the GR agonist dexamethasone results in the ablation of GFP-labeled T-cells in the thymus of these embryos [135]. Another line in which GFP expression is controlled by the *rag2* promoter (resulting in GFP labeled immature T and B cells) showed similar results [135].

Third, several zebrafish infection models exist in which the status of the infection can be monitored. An increase in the proliferation of the infectious agent could be used as a measure for the immunosuppressive activity of a GR agonist. Infecting zebrafish embryos with fluorescently labeled bacteria enables the analysis of the infection in real time and *in situ*. This approach has been successfully used for *Mycobacterium marinum* and *Salmonella typhimurium* infections [136, 137].

In addition to their use in screening assays for the anti-inflammatory activity of GCs, zebrafish embryos can also be used for screening of other effects of GC treatment, like decreased bone formation which is a common side effect of GC treatment. A zebrafish model system for GC-induced osteoporosis has been developed, based on the visualization of skeletal structures of zebrafish larvae using calcium-binding dyes like calcein or alizarin red [138, 139]. As a proof of principle, treatment of 5-day-old zebrafish larvae with prednisolone, a GC that is widely used clinically, significantly reduced bone formation in this assay [140]. Using these assays at this stage of development restricts the screening to the osteoblast activity, since the first osteoclasts appear in 20 day old individuals [141].

Another common side effect of GC treatment is a decrease in circulating cortisol levels, and this effect can be studied in zebrafish as well. Total cortisol levels can be measured in homogenates from pools of zebrafish embryos of any age using an immuno-assay [111, 117]. Increased cortisol levels in response to a stressor can be detected from 97

hours post fertilization [111], and a circadian rhythm in cortisol level has been observed at 6 days post fertilization [117]. This indicates that the hypothalamus pituitary interrenal (HPI) axis is functional in zebrafish larvae, and it can be expected that GC treatment results in a decrease in circulating cortisol levels.

Scope of the present thesis

GR signaling plays an essential role in the survival and well-being of organisms. GCs are also widely used clinically in order to combat inflammatory medical conditions. GR signaling is versatile, with diverse effects and complex interactions with other molecular components. Nevertheless, its complete biological significance and exact mechanisms of regulation and action have not fully been elucidated. In the present thesis we aimed at studying the GR pathway by means of stimulation with synthetic GCs and genetic manipulation. Since the GR is a transcription factor, our main readout for GR function in most of our experimental settings was transcriptome analysis. By gathering whole transcriptome information, we aimed at unraveling the molecular pathways affected by GR signaling in different physiological conditions, thus exploring its functional role. As a model organism we employed the zebrafish, since it allows fine genetic, molecular and cellular experimental approaches and its GR pathway closely resembles that of humans. Our aim was also to further characterize the function of this versatile signaling cascade in zebrafish, in order to establish this animal model as a valid system for detailed as well as high throughput research on GR, enabling us to test hypotheses and complement results obtained from other well-established experimental animal models such as rodents.

In ***chapter 2***, we have explored the role of zGRα with respect to modulating the inflammatory response to a wound injury. For that reason, a tail fin amputation assay was employed in 3 day old zebrafish larvae which were subsequently treated with the synthetic GC beclomethasone. Amputation elicited a migratory behavior for both macrophages and neutrophils as well as induction of several immune-related signaling routes. Using cell imaging as well as whole transriptome analysis, we have studied the GC effect on the cellular trafficking of leukocytes as well as on the transcriptional rate of genes involved in molecular networks altered due to amputation.

In ***chapter 3***, we have investigated the specificity and function of both zGR α- and β-isoforms. Zebrafish embryos were injected with two splice-blocking antisense oligos (one leading to knockdown of both zGR α- and β-isoforms, and another targeting the alternative splicing of the zGR pre-mRNA in favor of the zGR β-isoform) and with zGRβ mRNA (resulting in specific zGRβ overexpression). Embryos were treated with the synthetic GC dexamethasone and transcriptome analysis was performed using microarray technology. This experimental design has allowed us to answer 3 questions. First, which specific genes are affected by zGRα under different physiological conditions. Second, which genes are specifically altered due to an intrinsic zGRβ transcriptional activity (independent of zGRα). Third, whether zGRβ exhibits a dominant-negative activity on zGRα's transcriptional properties.

In ***chapter 4***, we have embarked on a series of experimental settings in order to elucidate the biological significance of the zGR β-isoform. In particular we were interested in

answering two questions. First, whether zGRβ plays a role as a dominant-negative inhibitor on zGRα's transcriptional properties. Second, whether zGRβ exhibits an intrinsic transcriptional activity, meaning independent of zGRα. For that reason, we overexpressed zGRβ transiently by cell transfections of zGRβ plasmid and zGRβ mRNA microinjections at 1-2 cell-stage zebrafish embryos. Furthermore, we oberexpressed the zGR β-isoform stably, by generating a zebrafish zGRβ-overexpressing cell line and a GFP-zGRβ overexpressing transgenic fish line. Results were assessed by means of luciferase assays and transcriptome analysis.

In ***chapter 5***, results from all 3 experimental chapters are evaluated collectively in order to draw solid conclusions about the role and function of both zGR splice variants. In particular, the functional role of zGRα in different physiological conditions and the biological significance of the zGR β-isoform are discussed. In addition, the zebrafish embryonic model as a system for further GR signaling research is evaluated. Future experimental approaches, employing the zebrafish model, are proposed that can be undertaken in order to further elucidate the versatile and complex nature of the GR signaling. Finally, summaries of our work from our 3 previous experimental chapters, written both in English and Dutch, are also included in this chapter.

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