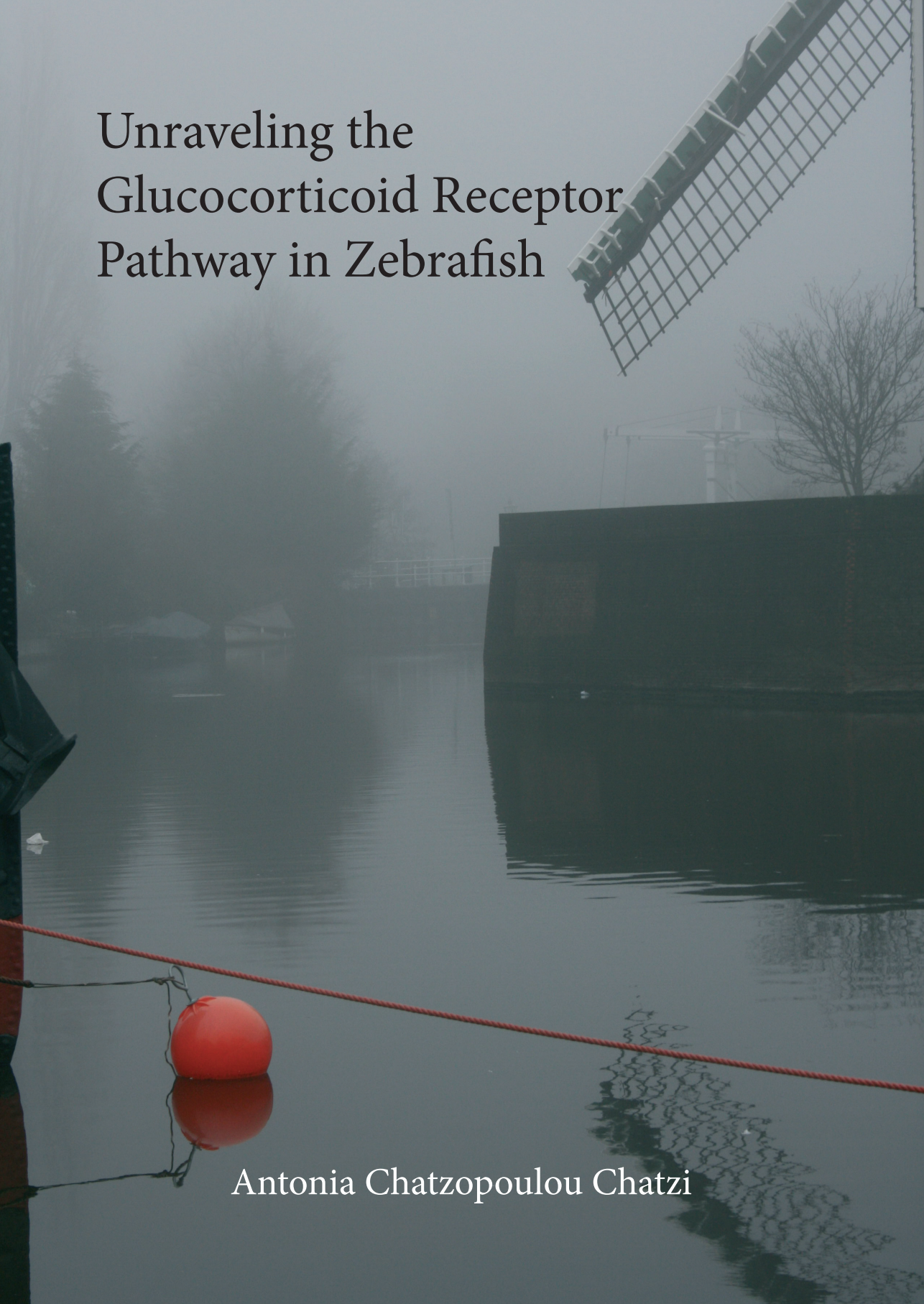
Unraveling the Glucocorticoid Receptor Pathway in Zebrafish



Antonia Chatzopoulou Chatzi

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Antonia Chatzopoulou Chatzi

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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.

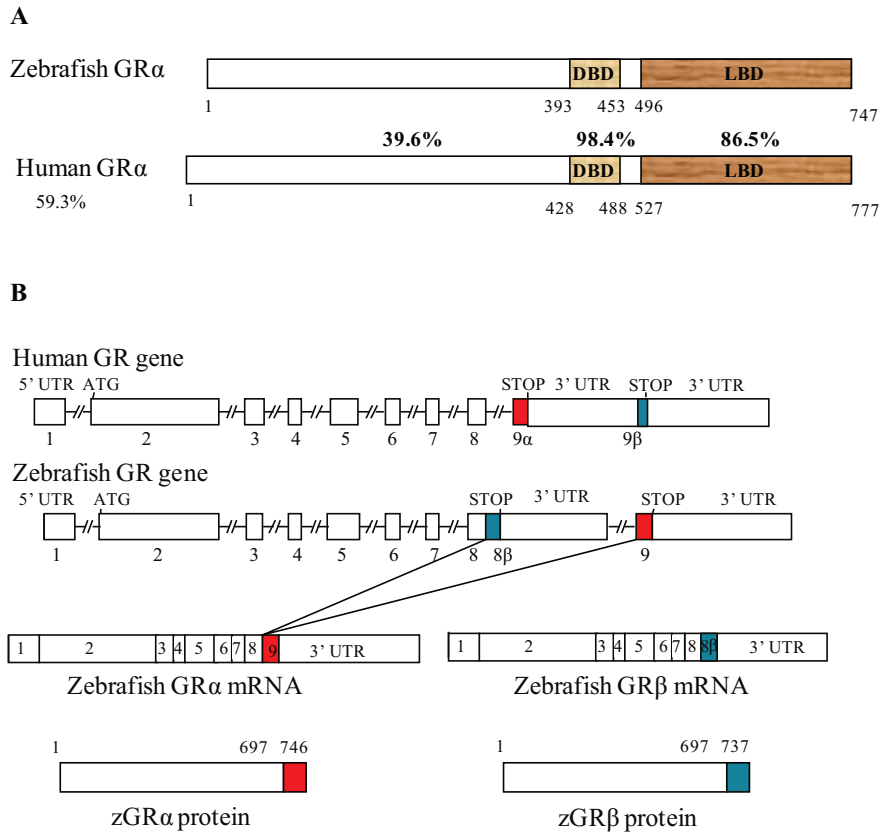

Figure 1: The zebrafish GR. **A.** The human and zebrafish GRα proteins.The percentage amino acid similarity is indicated for individual receptor domains and the full length receptor. **B**. The human and zebrafish GR genes. Both genes contain 9 exons, of which exon 1 is non-coding. A remarkable difference 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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Chapter 2

Glucocorticoid-induced attenuation of the inflammatory response in zebrafish

Antonia Chatzopoulou, Jeroen P.M. Heijmans, Herman P. Spaink, Annemarie H. Meijer, Marcel J.M. Schaaf

Abstract

Glucocorticoids are steroid hormones that are secreted by the adrenal gland upon stress. Their effects are mediated by the glucocorticoid receptor (GR) which acts as a transcription factor, regulating a wide plethora of genes. Since anti-inflammatory activity of glucocorticoids has been well established, they are widely used clinically to treat a variety of inflammatory and immune-related diseases. However, the exact specificity, mechanisms and level of regulation of different inflammatory pathways by GR signaling have not fully been elucidated. In the present study, a tail fin amputation assay was employed in 3-day-old zebrafish larvae in order to study the immunomodulatory effects of the synthetic glucocorticoid beclomethasone, using cell imaging as well as whole transriptome analysis. Our results show that amputation induced migration of both neutrophils and macrophages towards the wound site, and that beclomethasone treatment attenuated the migratory behavior of only neutrophils. In addition, the expression of many pro-inflammatory genes was induced upon amputation and this induction was suppressed by beclomethasone for virtually all induced genes. Apparently, glucocorticoid treatment has a very general dampening effect on the induction of gene expression upon amputation, without any specificity for particular pathways. These results show that the zebrafish larva model of tail fin amputation and beclomethasone treatment recapitulates the well known anti-inflammatory glucocorticoid effects, thus providing a reliable model system to further elucidate the molecular mechanisms of glucocorticoid signaling.

Introduction

Glucocorticoids (GCs) are potent anti-inflammatory agents that have been widely used clinically over the last 50 years for the treatment of many inflammatory and autoimmune diseases, graft rejection and malignancies of the immune system [1, 2]. The effects of GCs are mediated by the glucocorticoid receptor (GR), which is a nuclear receptor that is expressed almost ubiquitously in the human body and regulates a wide range of biological processes such as our metabolism, growth, reproduction, vascular tone, bone formation, immune response and brain function [3-8]. In its inactive state, the GR resides within the cytoplasm in a multiprotein complex containing chaperones and immunophilins [9]. Upon GC binding, it is released from this cytoplasmic complex and translocates to the nucleus, where it orchestrates gene expression via DNA-binding-dependent and -independent mechanisms. DNA-binding-dependent mechanisms involve GR occupancy of glucocorticoid response elements (GREs) and recruitment of transcriptional coregulators, resulting in a modulation of the transcription rate of target genes. Independent of DNA-binding, the GR can physically interact with other transcription factors via which it can either enhance the activity of these factors (e.g. in the case of STAT-5 activity on the IGF-1 promoter [6] or repress their activity (e.g. in the case of NF-κB or AP-1 activity on the promoter of a large number of cytokine genes) [3, 4, 6, 10-12]. The latter mode of action is termed *transrepression* and comprises one of the main mechanisms by which GCs exert their anti-inflammatory effects [3, 4, 9-13].

Many *in vitro* and *in vivo* studies have been performed to elucidate the cellular and molecular mechanisms underlying the effects of GR signaling on the immune system (for a review see Franchimont et al. [14]). From these studies it appeared that GCs suppress inflammation by downregulating the expression of a wide variety of pro-inflammatory

cytokines (e.g. IL1β, IL6, TNFα), chemokines (e.g. CCL1, CXCL8), enzymes (e.g. iNOS, COX-2) and adhesion molecules (e.g. ICAM-1), while the expression of several anti-inflammatory mediators is upregulated (e.g. DUSP1, IκB, IL10, TGFβ, ANXA1, GILZ) [2, 14, 15]. Furthermore, the synthesis of pro-inflammatory agents like prostaglandins, leukotrienes, proteolytic enzymes, free oxygen radicals, and nitric oxide is also inhibited by GCs [14]. Nevertheless, the exact specificity, mechanisms and level of regulation of different inflammatory pathways by the GR signaling have not fully been elucidated. For example, several studies have even revealed immunoenhancing effects of GCs, like the induction of Toll-like Receptor (TLR)2 and TLR4, the secretion of MIF (Macrophage Inhibitory Factor) and the upregulation of IL7Ra which is involved in T cell development [14, 16].

The aim of the present study is to establish a robust *in vivo* model to investigate in detail the molecular mechanism of the anti-inflammatory action of GCs. Profound understanding of the complex interplay of GR with the different components of the immune response may ultimately lead to improved GC treatment regimens, which would be of great importance, since the clinical use of GCs is currently limited by two problems: the deleterious side effects such as skin atrophy, decreased wound healing, osteoporosis, muscle atrophy, glaucoma, psychosis, diabetes mellitus and hypertension [17], and the minority of patients that shows complete or partial resistance to GC treatment [18].

Over the last decade, the zebrafish has emerged in biomedical research as an important model system for a variety of human diseases [19-21]. It is easily handled and maintained and produces big clutches of eggs, which are fertilized externally and develop rapidly [22]. Moreover, its genome has been sequenced and embryos can easily be subjected to genetic manipulations [22, 23]. Additionally, the zebrafish immune system remarkably resembles that of mammals [24], thus providing 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 [25, 26]. In the present study, zebrafish larvae are used at three days post fertilization. At this stage, two types of leukocytes are present which constitute the innate immune system, macrophages and neutrophils [27-31]. Cells representing the adaptive immune system, like lymphocytes, start to mature at the second week of zebrafish development [32-34].

Furthermore, the zebrafish is considered as a potent model organism for GC research [35-39]. Zebrafish have a single GR gene which encodes a GR protein that upon activation mediates gene transcription in a similar way as its human equivalent [35, 38, 40, 41]. This zebrafish GR gene also encodes a splice variant, zGRβ, which is the equivalent of the human GR β-isoform [39] that may play a role in resistance to glucocorticoid treatment in humans. As in all teleost fish, cortisol is the main endogenous GC in zebrafish, like in humans, and its secretion is regulated by the hypothalamus pituitary interrenal (HPI) axis, which is the equivalent to the mammalian hypothalamus pituitary adrenal (HPA) axis [36, 37, 42].

Local inflammation can be modeled in zebrafish by amputation of the tail fin of zebrafish larvae [43]. Amputation induces the expression of many pro-inflammatory mediators at the wound site and migration of the two types of leukocytes present at the larval stage, neutrophils and macrophages, towards the site of amputation [41, 43-45]. Interestingly, it has been demonstrated that this migration is inhibited by glucocorticoid treatment and therefore this model system enables studying of the anti-inflammatory action of glucocorticoids in an *in vivo* situation [41, 45].

In the present study we have used the zebrafish tail fin amputation model to study glucocorticoid effects on leukocyte migration and associated changes in gene expression at

the whole transcriptome level. Our results demonstrate that glucocorticoid treatment specifically inhibits the migration of neutrophils and not of macrophages. In addition, we show that tail fin amputation affects the expression of a wide variety of genes, among which many inflammation-related ones, and that glucocorticoid treatment attenuates the vast majority of these changes. Thus, glucocorticoids appear to dampen the inflammatory response in this model system, without any apparent specificity for particular signaling pathways.

Materials & Methods

Zebrafish strains, husbandry & egg collection

Zebrafish were maintained and handled according to the guidelines from the Zebrafish Model Organism Database (ZFIN, http://zfin.org). Fertilization was performed by natural spawning at the beginning of the light period and eggs were raised at 28.5°C in egg water (60μg/ml Instant Ocean sea salts supplemented with 0.0025% methylene blue (GUUR)). All experimental procedures were conducted in compliance with the directives of the local animal welfare committee of Leiden University.

Tail amputation & GC treatment

Three-day-old embryos were anesthetized in egg water containing 0.02% buffered aminobenzoic acid ethyl ester (tricaine, Sigma) and aligned in Petri dishes coated with 2% agarose for subsequent partial amputation of the tail fin as shown in Fig. 1. Amputation was performed using a 1mm sapphire blade (World Precision Instruments) using a Leica M165C stereo-microscope and a micromanipulator. Amputated and non-amputated embryos were pretreated for 2h with either 25μM beclomethasone (Sigma) or vehicle (0.05% DMSO) prior to amputation and again for a specified period of time after amputation (see Results section). For migration studies samples were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and stored at 4°C, whereas for gene expression analysis samples were collected in TRIzol® reagent (Invitrogen).

Myeloperoxidase staining and whole mount immunohistochemistry for visualization of macrophages and neutrophils

Embryos were fixed in 4% PFA overnight at 4°C and following washes with PBS containing 0.1% Tween 20 (PBST), the Myeloperoxidase (mpx) activity was detected using the Leukocyte Peroxidase kit (Sigma) according to the manufacturer's instructions. Mpx staining was always performed prior to L-plastin immunohistochemistry. For this purpose, embryos were washed in PBST, gradually dehydrated with methanol in PBS and stored in 100% methanol overnight at 4°C. The next day embryos were rehydrated with graded series of methanol in PBS containing 0.8% Triton X-100 (PBS-TX) and incubated with 10μg/ml Proteinase K (Roche) for 10min at 37°C. Embryos were then incubated in PBS-TX blocking buffer (containing 1% BSA) for 2h at RT and subsequently in blocking buffer containing a rabbit anti-L-plastin polyclonal antibody (provided by Dr. A. Huttenlocher [46], 1:500 dilution) overnight at 4°C. Following washes with PBS-TX, embryos were incubated again in blocking buffer for 1h at RT prior to incubation with goat anti-rabbit Alexa Fluor® 568 dye–labeled secondary antibody (Invitrogen) for 2h at RT (1:200 dilution in blocking buffer).

Imaging of the embryos was performed using a Leica MZ16FA fluorescence stereo-microscope supported by the LAS version 3.7 software. Macrophages were detected based on the red fluorescent labeling by the immunehistochemistry and neutrophils were detected based on their dark brown appearance as a result of the Mpx assay (although they are stained by both methods, the L-plastin immunolabeling is hard to detect in these cells due to the dark staining of the Mpx assay). To determine the number of cells that had migrated to the wounded area, the cells posterior to the caudal vein were counted (marked by the dashed red box in Fig. 1B). Data shown are means (±s.e.m.) of three individual experiments. In each experiment, treatment groups consisted of at least 20 larvae.

RNA isolation & cDNA synthesis

Total RNA was extracted using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions (Invitrogen). RNA was dissolved in water and denatured for 5min at 60°C. Samples were treated with DNAse using the DNA-*free*™ kit (Ambion). For microarray analysis, RNA was further purified using the RNeasy MinElute™ Cleanup kit from Qiagen and its integrity was checked with a lab-on-chip analysis using the 2100 Bioanalyzer (Agilent Technologies). For subsequent cDNA synthesis, 1µg of total RNA was added as a template for reverse transcription using the iSCRIPT™ cDNA Synthesis Kit (Biorad).

Quantitative Polymerase Chain Reaction (qPCR)

QPCR analysis was performed using the MyiQ Single-Color Real-Time PCR Detection System (Biorad). PCR reactions were performed in a total volume of 25µl containing 6.5µl diluted cDNA, 1µl forward and reverse primer (10µM) and 12.5µl of 2x iQ™ SYBR® Green Supermix (Biorad). Cycling conditions were 95°C for 3min, followed by 40 cycles of 15sec at 95°C, 30sec at 60°C and 30sec at 72°C. Ct values (cycle number at which a threshold value of the fluorescence intensity was reached) were determined for each sample. A dissociation protocol was added, determining dissociation of the PCR products from 65°C to 95°C, allowing discrimination of specific products. In all qPCR experiments, a water-control was included. Data shown are means (±s.e.m.) of three individual experiments. In each experiment, cDNA samples were assayed in duplicate. Sequences of all primers used for qPCR analysis are included in Suppl. Table 5.

Microarray design

A 4x180k microarray chip platform (customized by Agilent Technologies, (Design ID:028233)) was used in this study. This array consists of all probes already present in an earlier 45.219 custom-made array [47], and another 126.632 newly designed zebrafish probes had been added as described in [48]. A total of 16 samples (4 experimental groups from 4 replicate experiments) were processed for transcriptome analysis and were hybridized against a common reference.

Microarray amplification & labeling

Amplification and labeling of RNA was performed at the MicroArray Department (MAD) of the University of Amsterdam (Amsterdam, The Netherlands). Per sample, 0.5µg total RNA was amplified and combined with Spike A according to the Agilent Two-Color Microarray-Based Gene Expression Analysis kit (Agilent technologies). As a common reference sample an equimolar pool of all test samples was made and 0.5µg samples were amplified similarly as the test samples with the exception that Spike B was used. Amino-allyl modified nucleotides were incorporated during the aRNA synthesis (2.5mM of each

GTP, ATP, UTP (GE Healthcare), 0.75mM CTP (GE Healthcare), 0.3mM AA-CTP (TriLink Biotechnologies)). Synthesized aRNA was purified with the E.Z.N.A. MicroElute RNA Clean Up Kit (Omega Bio-Tek). The quality was inspected on the BioAnalyzer (Agilent Technologies) with the Agilent RNA 6000 kit (Agilent Technologies). Test samples were labeled with Cy3 and the reference sample was labeled with Cy5. Five µg of aRNA was dried out and dissolved in 50mM carbonate buffer pH 8.5. Individual vials of Cy3/Cy5 from the mono-reactive dye packs (GE Healthcare) were dissolved in 200µl DMSO. To each sample, 10µl of the appropriate CyDye dissolved in DMSO was added and the mixture was incubated for 1h. Reactions were quenched with the addition of 5µl 4M hydroxylamine (Sigma-Aldrich). The labeled aRNA was purified with the E.Z.N.A. MicroElute RNA Clean Up Kit. Yields of aRNA and CyDye incorporation were measured on the NanoDrop ND-1000.

Microarray hybridization, scanning & data processing

Each hybridization mixture was made up from 825ng Test (Cy3-labeled) and 825ng Reference (Cy5-labeled) material. Hybridization mixtures were using the Agilent Two-Color Microarray-Based Gene Expression Analysis kit according to the manufacturer's instructions (Agilent technologies). The samples were loaded onto the microarray chips and hybridized for 17h at 65°C. Afterwards the slides were washed and scanned (20 bit, 3µm resolution) in an ozone-free room with the Agilent G2505C scanner. Data was extracted with Feature Extraction (v10.7.3.1, Agilent Technologies) with the GE2_107_Sep09 protocol for two-color Agilent microarrays. The Agilent output from the 16 hybridizations was then imported into the Rosetta Resolver 7.2 software (Rosetta Biosoftware, Seattle, Washington) and subjected to a factorial design with a re-ratio with common reference application. Data analysis was performed setting cutoff for the p-value of $<10^{-5}$ and for fold change of either >2 or <-2.

Gene Ontology analysis

Gene Ontology analysis was performed with the PathVisio version 2 software (www.pathvisio.org [49]), setting cutoffs for p-value of $<10^{-5}$ and for fold change of either >2 or <-2. For probe annotation, the Ensembl gene ID codes (ENSDARG) were used (see http://www.ensembl.org). The Z-scores shown correspond to a standard statistical test under the hypergeometric distribution. A high Z-score corresponds to a high level of enrichment of genes from a specific pathway in the investigated gene cluster.

Statistical analysis

Statistical analyses (two-way ANOVAs with Bonferroni post-hoc tests) were performed using the GraphPad Prism version 4.00 (GraphPad Software, La Jolla, USA).

Results

The effect of GC treatment on amputation-induced leukocyte migration

Previous studies in zebrafish larvae have shown that leukocytes migrate to wound sites which represents an inflammatory response and that this response is impaired upon treatment with GCs [41, 45]. In order to study this in detail, we set up a tail fin amputation assay using 3 day post fertilization (dpf) larvae that were first exposed to either vehicle or the synthetic GC beclomethasone (25µM) for 2h. Tail fins were then amputated and vehicle or beclomethasone treatment was continued. Samples were collected at 0, 2, 4, 8,

16 and 24h post amputation (hpa) and neutrophils and macrophages were labeled and counted. To determine the number of cells migrated to the wounded area, cells posterior to the caudal vein were counted (area indicated by the dashed red box in Fig. 1B).

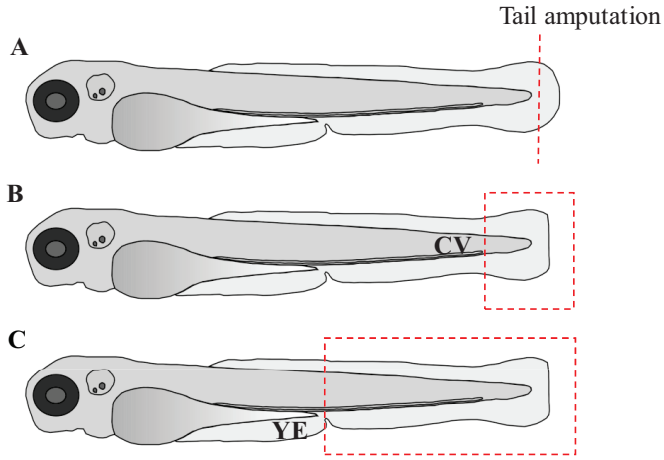

Figure 1. Schematic drawings of a zebrafish larvae at 3dpf (CV = caudal vein, YE = yolk extension). **A**. The site of the tail fin amputation. The red dashed line indicates where the cut in the tail was made. **B**. The area used to determine the number of neutrophils and macrophages that had migrated to the wounded area. The dashed box indicates the area selected for counting. (**C**) The area that is used to determine the total amount of neutrophils and macrophages present in the entire tail region. The dashed box indicates the area selected for counting.

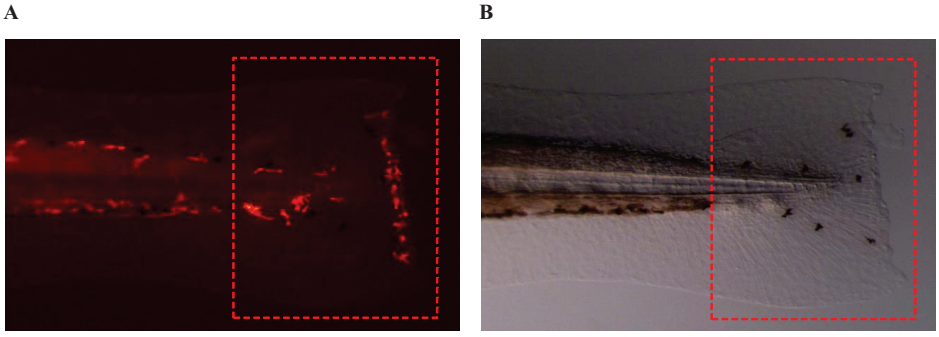

Figure 2. Leukocyte staining upon tail fin amputation in a 3dpf embryo. **A.** Staining of all leukocytes by immunohistochemistry against the pan-leukocyte marker L-plastin (shown in red). **B.** Staining of neutrophils specifically by Mpx staining (shown in black). Neutrophils are stained by both methods, but the L-plastin immunolabeling is hard to detect in these cells due to the dark staining of the Mpx assay. Therefore, the number of neutrophils was determined by counting in the cells stained by the Mpx assay (shown black in A and B) and the number of macrophages was determined by counting the number of cells stained by the L-plastin immunohistochemistry (shown red in A). Cells posterior to the caudal vein were considered to have migrated, so this area (marked by the dashed red box) was used for cell counting.

In order to label the populations of neutrophils and macrophages in 3dpf larvae we employed Myeloperoxidase (Mpx) histochemistry (specifically staining neutrophils [29]), followed by immunolabeling of L-plastin (staining all leukocytes [31]). At this stage of

development two populations of leukocytes are present: neutrophils, which are Mpx- and L-plastin-positive, and macrophages, which are Mpx-negative and L-plastin-positive [27, 29-31, 50]. The number of macrophages was therefore determined by counting the number of cells stained by the L-plastin immunostaining and not stained by the Mpx histochemistry, and the number of neutrophils was determined by counting cells stained by the Mpx assay (although they are stained by both methods, the L-plastin immunolabeling is hard to detect in these cells due to the dark staining of the Mpx assay (Fig. 2)).

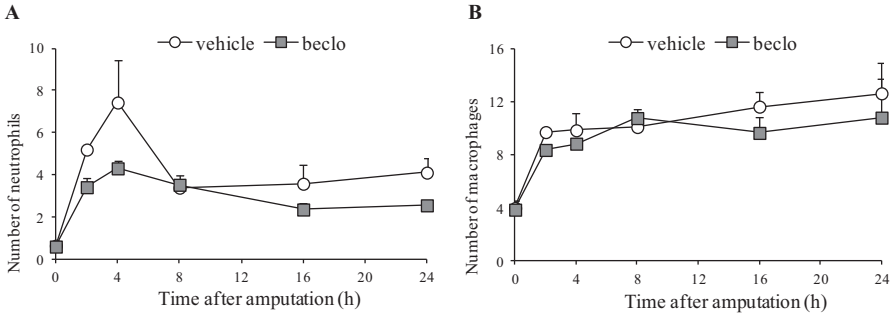

Figure 3. The effect of beclomethasone treatment on leukocyte migration upon tail fin amputation in 3dpf zebrafish larvae. **A.** The number of neutrophils in the wounded area as a function of time after amputation. Statistical analysis by two-way ANOVA revealed that both beclomethasone treatment and time had a significant effect on the number of neutrophils (both $p<0.001$), and that the neutrophil number was significantly increased at 4hpa compared to the 0hpa time point ($p<0.001$). **B.** The number of macrophages in the wounded area as a function of time after amputation. Statistical analysis by two-way ANOVA revealed a migratory response of macrophages over time ($p<0.001$), but that beclomethasone did not have an effect.

The results of this experiment revealed that both neutrophils and macrophages migrate towards the wound site, but that their migratory behavior and response to beclomethasone are remarkably different. Analysis of our data revealed a migratory response of neutrophils over time which was inhibited by beclomethasone treatment (as shown by a significant effect of time and beclomethasone treatment in a two-way ANOVA (both $p<0.001$)). Neutrophil migration reached a peak at 4hpa (7.4 ± 2.0 cells compared to 0.6 ± 0.1 at 0hpa) and rapidly decreased after this time point to 3.4 ± 0.6 at 8hpa after which it remained stable at this level until 24hpa (Fig. 2). Beclomethasone treatment had a significant inhibitory effect on the neutrophil migration at 4hpa (4.3 ± 0.4 cells in the presence of beclomethasone). For macrophages, a migratory response was observed as well ($p<0.001$), but no effect of beclomethasone treatment was observed. Macrophage migration also increased rapidly, especially in the first 2 hours (9.7 ± 0.2 at 2hpa versus 4.0 ± 0.1 0hpa), but no decline was observed afterwards. In addition, macrophages migrated more to the posterior end of the tail where they appeared to line up at the actual wound site, whereas neutrophils were more randomly located in the vicinity of the wound (for representative pictures, see Fig. 2). Based on these results, we concluded that both neutrophils and macrophages migrate towards wound sites, but that beclomethasone exhibits an inhibitory effect only on neutrophil migration.

Subsequently, higher doses of beclomethasone were tested for their inhibitory role on neutrophils and macrophages at 4hpa. These doses did not induce effects on migration different from the 25µM dose that was used in the experiment described above (data not

shown), indicating that at this dose a maximal effect had already been reached. In order to establish that beclomethasone specifically impairs the migration of neutrophils rather than their total number, cells in the entire tail fin area (posterior to the yolk extension) were counted (Fig. 1C). The results of these countings did not show any significant difference in the number of neutrophils between vehicle- and beclomethasone-treated larvae upon amputation (Suppl. Fig. 1), indicating a specific effect of beclomethasone on the neutrophil migration towards wound sites.

Design of the microarray experiment

Next we wanted to investigate the effect of beclomethasone treatment on amputation-induced changes in gene expression at the whole transcriptome level by microarray analysis. For that reason, we employed the tail fin amputation assay described above, collecting total RNA samples at 4hpa. Four experimental groups were generated: control (non-amputated) treated with vehicle (con/vehicle), control treated with beclomethasone (con/beclo), amputated treated with vehicle (4hpa/vehicle) and amputated treated with beclomethasone (4hpa/beclo). Subsequently, the RNA samples from these experimental groups were used in a microarray experiment and the data were analyzed using the Rosetta Resolver 7.2 software, setting signatures for significantly regulated probes at a p-value cutoff of $p < 10^{-5}$ and fold changes either >2 or <-2. Gene annotation and probe assignment was performed based on the Ensemble Gene ID codes (ENSDARG).

The effects of amputation on gene transcription

First, we identified 1403 probes to be significantly regulated due to amputation (comparison con/vehicle vs. 4hpa/vehicle). Gene annotation demonstrated that these probes corresponded to 585 genes, of which 410 were upregulated and 175 downregulated due to amputation. Gene ontology analysis (using PathVisio software) revealed that mainly immune-related signaling routes were affected like the Toll-like, NOD-like and RIG-I-like receptor pathway, the Prostglandin, Cytokine, Interferon, and Jak-STAT signaling pathways as well as Eicosanoid synthesis (Suppl. Table 1).

The effects of beclomethasone treatment on amputation-induced gene transcription

Subsequently, as a first readout of the effect of beclomethasone treatment on amputation-induced changes in gene expression, we studied how amputation-regulated genes generally respond to beclomethasone treatment. Therefore, for each of the 1403 probes that were significantly regulated by amputation we plotted the fold change due to beclomethasone treatment in amputated larvae (comparison 4hpa/vehicle vs. 4hpa/beclomethasone) as a function of the fold change due to amputation (comparison con/vehicle vs. 4hpa/vehicle). In the resulting scatter plot (Fig. 4) probes representing genes that are upregulated by amputation and of which this upregulation is enhanced in the presence of beclomethasone are shown in the upper right quadrant (164 probes representing 69 genes). Probes representing genes that are upregulated by amputation and of which this upregulation is attenuated by beclomethasone are presented in the lower right quadrant (876 probes, 349 genes). Interestingly, this plot demonstrates that the vast majority of probes/genes that are upregulated by amputation shows an attenuation of this upregulation in the presence of beclomethasone. This majority contains 84% of the probes and 83% of the genes showing upregulation by amputation. A similar effect was observed upon studying the probes that showed downregulation by amputation. Of these probes, 92% (363 probes representing 164 genes) displayed an attenuation of this downregulation in the presence of

beclomethasone and is shown in the upper left quadrant, whereas only a small number ended up in the lower left quadrant (32 probes, 14 genes).

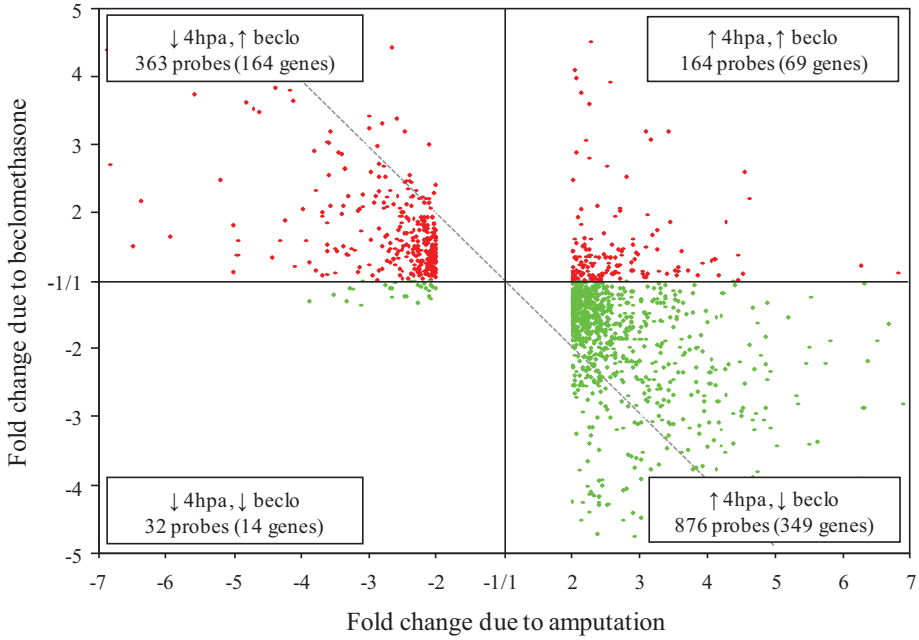

Figure 4. Scatter plot showing the effect of beclomethasone treatment on amputation-induced alterations in gene expression. For all 1403 probes showing significant regulation upon amputation (comparison con/vehicle vs. 4hpa/vehicle), the fold change due to beclomethasone treatment in amputated larvae (4hpa/vehicle vs. 4hpa/beclo) was plotted as a function of the fold change due to amputation. This plot shows that beclomethasone has a very general attenuating effect on amputation-induced gene regulation. Of the 1040 probes showing upregulation by amputation, 84% shows a suppression of this regulation in the presence of beclomethasone (lower right quadrant), and of the 385 probes showing downregulation in response to amputation, 92% shows an attenuated downregulation upon beclomethasone treatment (upper left quadrant). The grey dashed line indicates the point at which beclomethasone treatment completely abolishes amputation-induced changes, showing that in the vast majority of cases beclomethasone dampens the effects of amputation on gene expression, but does not block them entirely.

The grey dashed line in the scatter plot indicates the point at which the regulation by amputation is completely abolished by beclomethasone. Obviously, most of probes in the right lower quadrant are located above this line, indicating that in the majority of cases GC treatment attenuates the amputation-induced gene upregulation, and that it does not completely block it. In the left upper quadrant, a similar effect is shown for the probes downregulated upon amputation. The majority of probes is located below the grey line, indicating that GC treatment does not totally eliminate the amputation-induced downregulation. Taken together, these data show that beclomethasone treatment has a very general attenuating effect on amputation-induced alterations in gene expression, thereby dampening (rather than abolishing) these effects.

Next, we were interested in which individual beclomethasone-induced changes in gene regulation were statistically significant. We first studied the cluster of 410 genes

significantly upregulated by amputation, and found that 94 of these genes (23%) displayed a beclomethasone-induced attenuation of this upregulation that reached significance (Fig. 5A). We subsequently looked at the set of 175 genes downregulated by amputation and 19 genes (11%) showed a beclomethasone-induced attenuation of the downregulation that reached significance (Fig. 5B).

From the cluster of 94 genes that showed a significant upregulation by amputation that was attenuated by beclomethasone, we decided to validate by qPCR the expression of *il8, il1b, mmp9, mmp13a tnfa* and *ptgs2b.* The results demonstrated that 4 of the genes we tested (*il8, il1b, mmp9, mmp13a*) showed a significant induction due to amputation which was then significantly impaired in the presence of beclomethasone (Fig. 6). The other 2 genes (*tnfa* and *ptgs2b*) did not display significant regulation by either amputation or beclomethasone, but a trend showing upregulation due to tail fin injury and GC-mediated attenuation of this effect is present (Fig. 6).

Further gene ontology analysis on this cluster of 94 genes revealed that the majority of the signaling pathways involved were identical to those regulated by amputation (immune-related signaling cascades like the Toll-, RIG-I- and NOD-like receptor, Jak-STAT, and Cytokine signaling and Eicosanoid synthesis pathways (Suppl. Table 2)), indicating that beclomethasone treatment affects virtually all amputation-induced signalimng pathways. Since the Toll-like receptor pathway was represented by a relatively large number of genes (12) in this cluster of 94 genes, we studied the alterations in this pathway in more detail. The results showed that the only Toll-like receptor gene present in this cluster was *tlr5a.* In addition, 3 genes encoded subunits of the AP-1 complex (*fos, junb, atf3*), suggesting an important role for this transcription factor in mediating the attenuation of amputation-induced gene regulation by beclomethasone. Six genes corresponded to cytokines (*il1b, il8, il12a, ifn, tnfa, cxcl-c1c*), and two genes to other pro-inflammatory mediators (*ptgs2b, mmp9)* that are known to be induced upon activation of the Toll-like receptor pathway.

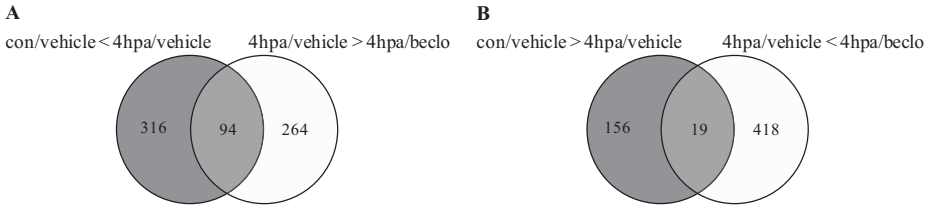

Figure 5. Venn diagrams showing the identification of the cluster of genes significantly regulated by amputation, and showing significant attenuation of this regulation in the presence of beclomethasone. **A**. Genes significantly upregulated due to amputation (410) are shown in the grey circle and genes significantly downregulated by beclomethasone treatment in amputated larvae (358) are shown in the white circle. The overlap represents the cluster of 94 genes of which the amputation-induced upregulation is attenuated by beclomethasone. **B**. Genes significantly downregulated due to amputation (175) are shown in the grey circle and genes significantly upregulated by beclomethasone treatment in amputated larvae (437) are shown in the white circle. The overlap represents the cluster of 19 genes of which the amputation-induced downregulation is attenuated by beclomethasone.

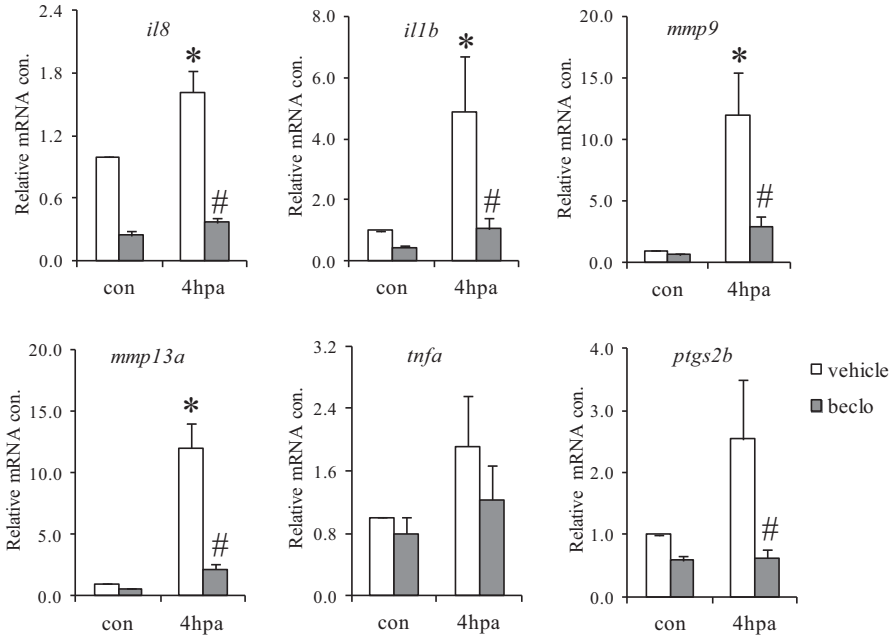

Figure 6. Validation by qPCR of 6 genes (*il8*, *il1b, mmp9*, *mmp13a*, *tnfa* and *ptgs2b)* that showed a significant upregulation by amputation that was attenuated by beclomethasone. The relative mRNA concentrations were normalized to those of *Bactin1* and the values shown are the means ± s.e.m. of four independent experiments. Statistical analysis demonstrated that beclomethasone attenuated the amputation-induced upregualtion of *il8*, *il1b, mmp9* and *mmp13a.* The other genes (*tnfa*, *ptgs2b)* did not reach significance for their induction by amputation (although the trend is present), but in the case of *ptgs2b*, beclomethasone treatment of amputated embryos led to a significant suppression compared to vehicle treated embryos. For *tnfa*, the GC-mediated attenuation did not reach significance. Asterisks (*) correspond to a statistical significance of $p < 0.05$ compared to con/vehicle groups. Key symbols (#) correspond to a statistical significance of $p < 0.05$ compared to 4hpa/vehicle groups.

The effects of beclomethasone on gene transcription

Furthermore, we investigated which genes responded to beclomethasone treatment in non-amputated larvae. A cluster of 2184 probes was identified to be significantly regulated due to beclomethasone treatment (comparison con/vehicle vs con/beclo). Gene annotation demonstrated that these probes corresponded to 815 genes, of which 606 were upregulated and 209 downregulated due to beclomethasone. Gene ontology analysis revealed specific signaling routes to be affected such as those involved in lipid metabolism (e.g. PPAR and adipocytokine signaling and bile acid synthesis pathways) alongside immune-related (e.g. NOD-like receptor and prostaglandin pathways) as well as non-immune related pathways (e.g. DNA replication and sulfur metabolism (Suppl. Table 3)).

Next, we were interested in genes which were significantly changed due to beclomethasone treatment of amputated larvae (comparison 4hpa/vehicle vs. 4hpa/beclo). We identified 2076 probes to be significantly regulated and gene annotation revealed that these probes corresponded to 795 genes, of which 437 were upregulated and 358 were

downregulated. Gene ontology analysis demonstrated that lipid-related cascades (e.g. PPAR signaling and bile acid synthesis pathways) as well as immune-related ones (e.g. Toll-like receptor, prostaglandin signaling and natural killer cell-mediated toxicity pathways) were again affected, alongside phagosome and VEGF signaling pathways (Suppl. Table 4). From these 795 genes, 113 were previously identified to be regulated by amputation and subsequently affected due to beclomethasone treatment (Fig. 5). Additionally, 241 (30%) of these genes were found to be regulated by beclomethasone in non-amputated embryos as well. This latter cluster verifies the validity of our microarray experiment and could provide a reference set of marker genes for studying GR signaling in zebrafish embryos.

Discussion

In the present study, we have used zebrafish larvae in order to study the effects of GC signaling on the inflammatory response to tail fin amputation, both at the cellular and the molecular level. It is well known that upon wounding chemotactic cues attract both neutrophils and macrophages to the wound site in order to combat microbes [51]. In our study, visual detection of macrophages and neutrophils (the former defined as L-plastin-positive and mpx-negative cells, the latter as L-plastin- and mpx-positive [27, 29-31, 50]) revealed that both cell types started to be recruited to the wound site as early as 2hpa. At this time point, macrophage recruitment reached a level which sustained for at least another 22h. Neutrophil migration reached a peak at the 4hpa time point after which their number declined, but remained elevated above basal levels for another 20h (Fig. 3). Previous studies employing similar tissue wounding in zebrafish larvae showed that macrophages [52] and neutrophils [29, 53-55] migrated to the wound sites. In two recent studies [56, 57], the time course of the recruitment of both macrophages and neutrophils to wound sites in the tail fin was studied as well and the results are in line with those from our study. In both studies, neutrophil accumulation at the wounded tissue peaked at around 6hpa, after which numbers declined to baseline, and macrophage migration increased rapidly over the first 6 hours as well and remained at a high level until 48 hpa. In our study, macrophage recruitment appears to be more rapid, reaching a plateau already at 2 hpa, but different ways to define and count the population of macrophages (L-plastin immunohistochemistry in our study versus fms:Gal4/UAS:mCherry transgenic fish [56] and neutral red staining [57]) may underlie the observed differences.

We next examined the effect of GC treatment on the migration of leukocytes towards injured sites. Our analysis showed that beclomethasone treatment had a significant inhibitory effect only on the migration of neutrophils but not on that of macrophages (Fig. 3). These results are in line with previously observed GC effects on leukocytes in 3dpf zebrafish larvae that were shown to be specifically suppressive regarding the recruitment of neutrophils towards wounded tissue but not that of macrophages [45]. Hence, the zebrafish model recapitulates the inhibitory effects of glucocorticoids on neutrophil migration towards inflamed tissues, that have been well established in mammalian models [58]. As for macrophages, studies in mammalian models have shown that GCs can suppress the migratory properties both *in vitro* [59] and *in vivo* [60]. Whether the resistance to GC treatment of macrophages observed in the zebrafish model is specific for fish in general, or the result of the developmental stage of the larvae, the time frame of the assessments, or the selected tissue remains to be investigated.

Subsequently, we evaluated the changes in gene expression that are associated with amputation and beclomethasone treatment. First, we looked for transcriptional changes 4hpa and we identified 585 genes of which the expression was significantly altered upon amputation. These genes are mainly involved in immune-related signaling routes such as that of Toll-like, NOD-like and RIG-I-like receptors, as well as Jak-STAT and cytokine signaling, and Prostaglandin, Interferon, and Eicosanoid synthesis (Suppl. Table 1). Since we used whole larvae for our transcriptional analysis, it must be noted that we do not know in which cell type the induced expression occurs. However, in other studies, it was shown that amputation-induced upregulation of pro-inflammatory genes like *mmp9* and *junb* was mainly observed in the epithelium at the wound site (Mathew 2007, Yoshinari 2009), suggesting that most of the observed amputation-induced alterations in gene expression arise in the tissue around the wound site and not in the immune cells. In a similar study by Yoshinari et al. [61], in which 2dpf embryos were tail fin amputated and samples were collected at a much later time point (16hpa), transcriptome analysis revealed that the largest fraction of regulated signaling routes were metabolic pathways (40%) and only a small fraction (2%) of signaling cascades regulated were immune-related. These results suggest that the time after injury is an important determinant for the alterations in gene transcription that can be observed. Thus, it appears that at 4 hours after injury, immune-related pathways are heavily activated at the transcriptional level, while 12 hours later amputation-induced changes in gene expression no longer reflect an inflammatory response, which is in line with the observed decline in neutrophil migration and the plateau that has been reached in macrophage recruitment at this time point. Most likely, at this stage, the transcriptional response has probably shifted towards the stimulation of regenerative processes.

Second, we were interested in how beclomethasone affected the expression of these amputation-regulated genes. Hence, we plotted for each of the 1403 probes that showed a significant regulation upon amputation the fold change due to beclomethasone treatment in amputated larvae as a function of the fold change due to amputation (Fig. 4). This analysis revealed that the vast majority (83%) of amputation-induced genes showed an attenuated induction in the presence of beclomethasone. Similarly, 94% of the amputation-downregulated genes displayed an attenuation of this downregulation in the presence of beclomethasone (Fig. 4). It must be noted that our data show that in general the transcriptional responses to tail fin injury are not completely blocked by beclomethasone, but that they are dampened. Thus, in this model GCs appear to have a suppressive effect on virtually all changes in gene transcription at 4hpa, which are mainly pro-inflammatory in nature, indicating that GCs have a general immunosuppressive effect without any apparent specificity for particular gene networks.

Ninety-four genes were identified for which both the induction by amputation and the beclomethasone-induced attenuation of this induction reached significance (and 19 genes for which the suppression upon amputation and the attenuation of this suppression by beclomethasone was significant). Gene ontology analysis revealed that within this cluster of 94 genes, immune-related signaling routes were overrepresented, like the Toll-, RIG-I- and NOD-like receptor, Jak-STAT and cytokine signaling and Eicosanoid synthesis pathways. In this gene cluster we found many well-known pro-inflammatory agents, which have been demonstrated to be suppressed upon GC treatment, such as *il8, il1b, tnfa, il11a, il12a, mmp9, mmp13a and ptgs2b* (which is the orthologue of the human *cox-2* gene) [10, 62-66]. For the majority of these genes, it has been well established that DNA-binding-independent transrepression takes place via interaction of GR and immune-related transcription factors, most notably the NF-κB and AP-1 [10, 66]. Thus, the zebrafish

model of wound-induced inflammation recapitulates the anti-inflammatory GC effects with respect to gene transcription observed in mammalian systems, and we therefore suggest that this model system could be further developed as a screening assay for novel GC drugs.

Interestingly, we also found 3 genes encoding AP-1 subunits (*fos, junb, atf3)* in the above mentioned cluster of 94 genes, indicating that, under our experimental conditions, the activity of AP-1 is inhibited by GCs through a decrease in the expression level of several AP-1 subunits. This finding supports an important role for this transcription factor in mediating the attenuation of amputation-induced gene regulation by beclomethasone in zebrafish larvae. A cross-talk (leading to transrepression of pro-inflammatory genes) between GR and AP-1 signaling has been extensively reported to occur via a physical interaction of GR and the c-Jun subunit of AP-1 complex as well as via GR-mediated inhibition of c-Jun activation [67-69]. The downregulation of the expression level observed in our study illustrates that an additional molecular mechanism of negative cross-talk between AP-1 and GR occurs *in vivo*.

Finally, 815 genes were identified that significantly responded to beclomethasone treatment in non-amputated larvae. These genes were attributed to specific signaling routes such as those involved in lipid metabolism (e.g. PPAR and adipocytokine signaling and bile acid synthesis pathways) alongside immune-related (e.g. NOD-like receptor and prostaglandin pathways) as well as non-immune related pathways (e.g. DNA replication and sulfur metabolism (Suppl. Table 3)). Similarly, we identified 795 genes to be significantly changed due to beclomethasone treatment of amputated larvae that gene ontology analysis revealed that lipid-related cascades (e.g. PPAR signaling and bile acid synthesis pathways) as well as immune-related ones (e.g. Toll-like receptor, prostaglandin signaling and natural killer cell-mediated toxicity pathways) were again affected, alongside phagosome and VEGF signaling pathways (Suppl. Table 4). A cluster of 241 genes was found to be regulated by beclomethasone in both amputated and non-amputated larvae. This latter cluster is apparently robustly regulated by GCs, and could therefore provide a reference set of marker genes for studying GR signaling in zebrafish larvae.

In summary, the zebrafish embryonic model of tail fin amputation and GC treatment constitutes a reliable system for studying GR signaling with respect to the innate immune response. In particular, we observed that both macrophages and neutrophils migrated to inflamed tissues and that this course was hindered by GC treatment only in the case of neutrophils. Furthermore, amputation resulted in activation of various immune-related signaling cascades for which GR activation had a general immunosuppressive effect, lacking any apparent specificity for particular signaling cascades. We suggest that this model could in the future be used as a screening assay for novel anti-inflammatory GC drugs.

Acknowledgments

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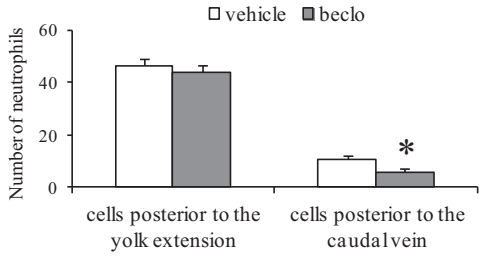
Supplemental data



Supplemental Figure 1. Neutrophils present in the entire tail region (posterior to the yolk extension, area marked by the dashed red box in Fig. 1C) compared to neutrophils present in the area close to the wound (posterior to the caudal vein, area marked by the dashed red box in Fig. 1B). Larvae were treated with either vehicle (white bars) or 25μM beclomethasone (grey bars). Statistical analysis showed that beclomethasone does not affect the number of neutrophils present in the entire tail region, but only the number of cells localized in the area close to the wound. The asterisk (*) corresponds to a statistically significant difference in area close to the wound compared to vehicle treatment ($p<0.05$).

Pathway	Genes meeting criteria	Z score
Response to infection *	39	10.31
Toll-like receptor pathway	22	7.05
Prostaglandin signaling	7	4.86
Cytokine-cytokine receptor interaction	12	3.41
Jak-STAT signaling pathway	10	3.27
Eicosanoid Synthesis	3	3.2
NOD-like receptor signaling pathway	6	3.18
RIG-I-like receptor signaling pathway	6	2.92
Pantothenate and CoA biosynthesis	2	2.68
IFN Pathway	10	2.34

Supplemental Table 1: Pathways enriched in cluster of 585 genes regulated upon amputation, determined by gene ontology analysis using PathVisio software (p-value cutoff $<10^{-5}$, fold change >2 or <-2). * Reference set based on *Mycobacterium marinum* yolk infection.

Pathway	Genes meeting criteria	Z score
Response to infection *	16	9.07
Toll-like receptor pathway	12	8.78
Eicosanoid Synthesis	2	4.73
Cytokine-cytokine receptor interaction	6	4.33
RIG-I-like receptor signaling pathway	4	3.72
NOD-like receptor signaling pathway	3	3.71
Arachidonic acid metabolism	2	3.15
Cytosolic DNA-sensing pathway	2	3.15
Jak-STAT signaling pathway	4	3.06
Pantothenate and CoA biosynthesis	1	2.88

Supplemental Table 2: Pathways enriched in cluster of 94 genes that showed an upregualtion by amputation, which was attenuated by beclomethasone, determined by gene ontology analysis using PathVisio software (p-value cutoff $<10^{-5}$, fold change >2 or <-2). * Reference set based on *Mycobacterium marinum* yolk infection.

Pathway	Genes meeting criteria	Z score
Primary bile acid biosynthesis	4	5.11
NOD-like receptor signaling pathway	8	4.49
PPAR signaling pathway	9	4.35
Prostaglandin signaling	6	3.81
Response to infection*	21	3.8
Nuclear receptors in lipid metabolism and toxicity	4	3.36
NOD pathway	7	3.14
Sulfur metabolism	2	3.08
DNA Replication	4	2.35
Adipocytokine signaling pathway	6	2.12

Supplemental Table 3: Pathways enriched in gene cluster (815 genes) regulated by beclomethasone, determined by gene ontology analysis using PathVisio software (p-value cutoff $<10^{-5}$, fold change >2 or <-2). * Reference set based on *Mycobacterium marinum* yolk infection.

Pathway	Genes meeting criteria	Z score
PPAR signaling pathway	12	5.25
Toll-like receptor pathway	21	4.98
Prostaglandin signaling	8	4.59
Natural killer cell-mediated cytotoxicity	12	4.03
Response to infection*	23	3.18
Primary bile acid biosynthesis	3	3.08
NOD pathway	8	3.01
Taurine and hypotaurine metabolism	2	2.91
Phagosome	14	2.64
VEGF signaling pathway	9	2.64

Supplemental Table 4: Pathways enriched in gene cluster (795 genes) regulated by beclomethasone in amputated larvae, determined by gene ontology analysis using PathVisio software (p-value cutoff $<10^{-5}$, fold change >2 or <-2). * Reference set based on *Mycobacterium marinum* yolk infection.

Gene name	Gene symbol	Forward primer sequence	Reverse primer sequence
Bactin1	*Bactin1*	CGAGCAGGAGATGGGAACC	CAACGGAAACGCTCATTGC
interleukin 8	*il8*	TGTGTTATTGTTTTCCTGGCATTTC	GCGACAGCGTGGATCTACAG
interleukin 1, beta	*Il1b*	CATAAACACCTTCGAGTCcG	TCTTTCCTGTCCATCTCCACCA
matrix metalloproteinase 9	*mmp9*	CATTAAAGATGCCCTGATGTATCCC	AGTGGTGGTCCGTGGTTGAG
matrix metalloproteinase 13a	*mmp13a*	ATGGTGCAAGGCTATCCCAAGAGT	GCCTGTTGTTGGAGCCAAACTCAA
tumor necrosis factor a	*tnfa*	AGACCTTAGACTGGAGAGATGAC	CAAAGACACCTGGCTGTAGAC
prostaglandin-endoperoxide synthase 2b	*ptgs2b*	TGGGCTTCGTGGTTTGCACAGG	GCGTCGGAGCCCATTTCCGT

Supplemental Table 5: Sequences of all primers used for qPCR analysis.

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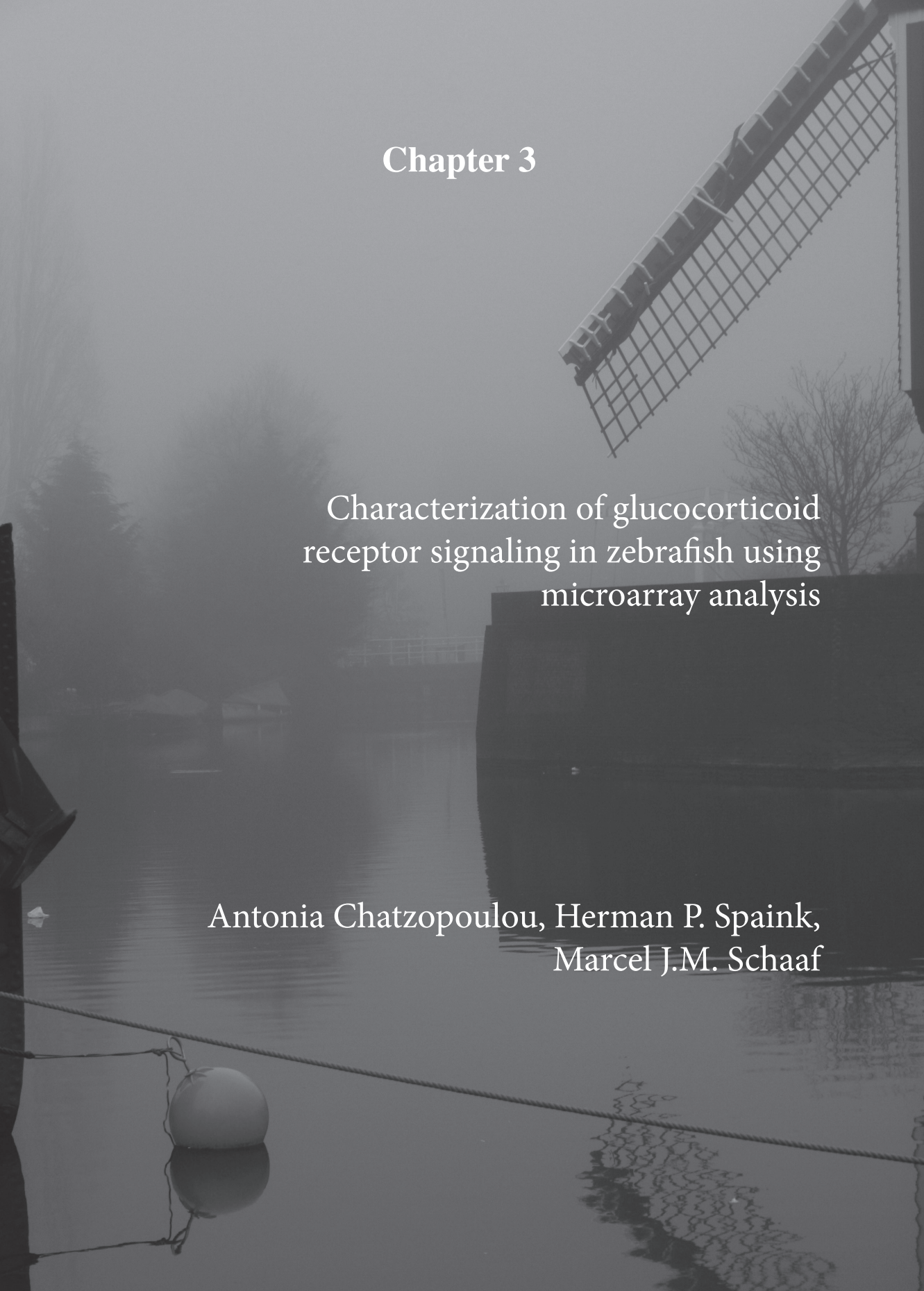

Chapter 3

Characterization of glucocorticoid receptor signaling in zebrafish using microarray analysis

Antonia Chatzopoulou, Herman P. Spaink, Marcel J.M. Schaaf

Abstract

The glucocorticoid receptor (GR) regulates gene expression upon binding to glucocorticoid (GC) hormones. In humans and zebrafish, two GR splice variants exist: the canonical GR α-isoform (GRα), acting as a GC-activated transcription factor, and the GR β-isoform (GRβ), which has been shown to have a dominant-negative effect on GRα. In the present study we have used the zebrafish model system in order to reveal genes affected by each of these two receptor isoforms. Zebrafish embryos were injected at the 1-2 cell stage with two splice-blocking antisense oligos (one leading to knockdown of both GR α- and β-isoforms, and another targeting the alternative splicing of the GR pre-mRNA in favor of the GR β-isoform) and with GRβ mRNA (resulting in specific GRβ overexpression). Embryos were treated with the synthetic GC dexamethasone and transcriptome analysis was performed using microarray technology and subsequent qPCR validation. Interestingly, our results show two distinct gene clusters that are regulated by GRα. One cluster that is regulated by GRα under basal conditions, and one that is regulated upon activation of GRα by dexamethasone treatment. In general, the first cluster of genes mainly involves cell-cycle-related genes, whereas the second cluster of genes mainly contains genes involved in metabolism. Furthermore, our data do not support a significant role for GRβ as a dominant negative inhibitor of GRα. However, GRβ could have an intrinsic transcriptional activity that would, nevertheless, require high expression levels of this splice variant.

Introduction

The glucocorticoid receptor (GR) is expressed almost ubiquitously in the human body and regulates a wide range of biological processes such as our metabolism, growth, reproduction, vascular tone, bone formation, immune response and brain function [1-6]. In its inactive state, the GR mainly resides within the cytoplasm in a multiprotein complex containing chaperones and immunophilins. The GR is activated by binding of its cognate ligands (glucocorticoids (GCs)), and upon activation, it is released from its cytoplasmic complex and translocates to the nucleus, where it orchestrates gene expression via DNA-binding-dependent and -independent mechanisms. DNA-binding-dependent mechanisms involve GR binding to glucocorticoid response elements (GREs), where it recruits transcriptional coregulators and thus, directs (positively or negatively) the transcription rate of target genes. Independent of DNA-binding, the GR can also physically interact with other transcription factors (e.g. NF-κB and AP-1), via which it can either synergistically induce gene expression or repress activation of genes. The latter mode comprises one of the classical ways by which GCs exert their anti-inflammatory effects, which is the basis of their intensive clinical use in the treatment of immune-related diseases like asthma and rheumatoid arthritis [1, 2, 7-11].

When the human GR was cloned, two splice variants were discovered, named as hGRα and hGRβ, which derive from alternative splicing within the most 3' exon, encoding the C-terminal end of the ligand binding domain (LBD). [12, 13]. Hence, the hGR α-isoform (777 amino acids, representing the canonical receptor) is able to interact with GCs, whereas the hGR β-isoform (742 amino acids) has a shorter LBD with a unique C-terminal 15 amino acid sequence, which renders it unable to bind GCs [12-14]. Experimental data have provided evidence for a dominant-negative effect of hGRβ on hGRα's transcriptional

properties [15-18] as well as intrinsic transcriptional activity (independent of hGRα) [19, 20]. However, the biological significance of hGRβ is still debated, mainly due to inconsistent results regarding its transcriptional role, [21-27] its low expression levels [15, 28, 29] and low level of evolutionary conservation [30, 31].

Over the last decade, the zebrafish has emerged in biomedical research as an important model system for a variety of human diseases [32-34]. It is easily handled and maintained and produces big clutches of embryos, which are fertilized externally and develop rapidly [35]. Moreover, its genome has been sequenced and embryos can easily be subjected to genetic manipulations [35, 36]. Furthermore, the zebrafish has been considered as a potent model organism for GC research [37-40]. As humans, zebrafish have a single GR (zGR), and cortisol is the main endogenous GC. Its secretion is regulated by the hypothalamus pituitary interrenal (HPI) axis, which is the equivalent to the mammalian hypothalamus pituitary adrenal (HPA) axis [38, 39, 41]. Upon secretion of cortisol or treatment with synthetic GCs, the activated zGRα can mediate gene transcription in a similar way as the hGRα [37, 40, 42, 43]. Interestingly, zebrafish express both zGR splice variants, since the zGR gene gives rise to a zGR α- and a zGR β-isoform as a result of alternative splicing [30, 44]. The zGRβ is highly similar to its human equivalent in structure, subcellular localization and expression level [30]. However, the gene organization and splicing events underlying the expression of GRα and GRβ are different between humans and zebrafish, suggesting that zGRβ and hGRβ have evolved independently [30].

In the present study we have used the zebrafish as an *in vivo* model to study the GR signaling pathway at the whole-organism level. A genetic manipulation approach of the zGR in zebrafish embryos was used in order to reveal responsive genes specific for the zGR α- and β-isoform in the absence of exogenous GCs (thus, studying zGR signaling under basal cortisol levels) as well as in the presence of exogenous GCs (thus, studying zGR signaling under elevated GC levels). We availed ourselves of the use of splice-blocking morpholino antisense oligomers, which are widely used for studying gene function via impeding normal splicing, thus leading to knockdown of genes or altered regulation of alternative splicing during embryonic development [45]. By using a morpholino for knocking down both zGRα and zGRβ and another that directs splicing in favor of the zGRβ transcript, in combination with injection of zGRβ mRNA, we aimed to identify specific clusters of target genes for both GR variants.

Materials & Methods

Zebrafish strain, husbandry & egg collection

Wild type adult ABxTL zebrafish were used in this study. Livestock was maintained and handled according to the guidelines from http://zfin.org. Fertilization was performed by natural spawning (single crossings) at the beginning of the light period and eggs were raised at 28°C in E3 egg water medium containing 60μg/ml Instant Ocean sea salts supplemented with 0.0025% methylene blue (GURR). All experimental procedures were conducted in compliance with the directives of the animal welfare committee of Leiden University.

zGRβ mRNA synthesis & injections

PCS2+zGRβ plasmid was linearized with NsiI (New England Biolabs) and used as a template for the synthesis of zGRβ mRNA using the mMESSAGE mMACHINE SP6 kit from Ambion. Newly synthesized mRNA was purified using the RNeasy MinElute™ Cleanup kit (Qiagen). RNA was denatured at 60°C for 5min and its integrity was checked on an agarose gel. Prior to injections, zGRβ mRNA was denatured at 60°C for 5min and diluted at a final concentration of 5ng/μl in 1x Danieu's buffer (58mM NaCl, 0.7mM KCl, 0.4mM $MgSO_4$, 0.6mM $Ca(NO_3)_2$, 5mM HEPES at pH 7.6) containing 0.05% phenol red (Sigma). Approximately 1nl of zGRβ mRNA solution was injected into the yolk of 1-2 cell stage fertilized eggs.

Morpholinos preparations and injections

In this study 3 different morpholinos were used (purchased from GeneTools), all of which prepared and stored according to the manufacturer's instructions. Sequence for Standard Control morpholino (SC-MO): CCTCTTACCTCAGTTACAATTTATA. Sequence for morpholino targeted against the splice acceptor site of exon 8 of the zebrafish glucocorticoid receptor gene (MO1): TTTAGGAGAAGCATCATTACGTCGT. Sequence for moprholino targeted against the splice donor site of exon 2 of the zebrafish glucocorticoid receptor gene (MO2): GGTCTTGATGGCTTACCTGGAATAG. Prior to injections, morpholinos were diluted at a final concentration of 0.05mM in 1x Danieu's buffer (58mM NaCl, 0.7mM KCl, 0.4mM $MgSO_4$, 0.6mM $Ca(NO_3)_2$, 5mM HEPES at pH 7.6) containing 0.05% phenol red (Sigma). Approximately 1nl of each morpholino solution was injected into the yolk of 1-2 cell stage fertilized eggs.

RT-PCR analysis of modified splicing of the glucocorticoid receptor upon morpholino injections

One hundred ng of DNAase treated total RNA samples were used as a template for one-step RT-PCR analysis of zebrafish glucocorticoid receptor RNA sequences using the SuperScript® III One-step RT-PCT System with Platinum® Taq DNA Polymerase kit (Invitrogen) according to the manufacturer's manual (setting annealing temperature at 60°C). Four different primer pairs were used: Pair 1: forward primer: TGTGAACA-GATGTTGAAGATCTCCA, reverse primer: AAACATCCGTCTAGAGGCAA, Pair 2: forward primer: TGTGAACAGATGTTGAAGATCTCCA, reverse primer: CTGGTGA-AAGAGCAGCGGTTTA, Pair 3: forward primer: AGCTGTCGTCGGTCTGCAGAAA, reverse primer: GCAGGTTAGGGTAATTAGGCAAGT, Pair 4: forward primer: CAGG-GGTCATCAAACAGGAGAA, reverse primer: GACAGATCTTGTGTGTGCCAGTCTT.

Glucocorticoid treatment of injected embryos

Twenty-five hours post fertilization embryos injected with either SC-MO, MO1, MO1 or zGRβ mRNA (in the latter case, SC-MO was co-injected together with zGRβ mRNA for optimal comparison with the other groups) were placed in 2% agarose-coated Petri dishes, where they were incubated with 100μM dexamethasone (Sigma) in E3 medium for 6h at 28°C. Samples were collected in TRIzol® reagent (Invitrogen) and total RNA was isolated according to the manufacturer's instructions (Invitrogen).

RNA isolation & cDNA synthesis

Total RNA was extracted using the TRIzol® reagent (Invitrogen) according to the manufacturer's instructions (Invitrogen). RNA was dissolved in water and denatured for

5min at 60°C. Samples were treated with DNAase using the DNA-*free*™ kit (Ambion). For microarray analysis, RNA was further purified using the RNeasy MinElute™ Cleanup kit from Qiagen and its integrity was checked with a lab-on-chip analysis using the 2100 Bioanalyzer (Agilent Technologies). For subsequent cDNA synthesis, at least 250ng of total RNA was added as a template for reverse transcription using the iSCRIPT™ cDNA Synthesis Kit (Biorad). Non reverse-transcribed samples were also generated to be used as negative controls during the quantitative polymerase chain reaction step.

Quantitative Polymerase Chain Reaction (qPCR)

qPCR analysis was performed using the MyiQ Single-Color Real-Time PCR Detection System (Biorad). PCR reactions were performed in a total volume of 25µl containing 5µl of cDNA, 1µl forward and reverse primer (10µM) and 12.5µl of 2x iQ™ SYBR® Green Supermix (Biorad). Cycling conditions were 95°C for 3min, followed by 40 cycles of 15 sec at 95°C, 30 sec at 60°C and 30sec at 72°C. Ct values (cycle number at which a threshold value of the fluorescence intensity was reached) were determined for each sample. A dissociation protocol was added, determining dissociation of the PCR products from 65°C to 95°C, allowing discrimination of specific products. In order to determine the efficiency of the amplification reaction, serial dilutions of cDNA were used and standard curves were generated plotting the Ct values against the relative cDNA concentration. The linear slope of the standard curve for each primer pair was determined and the efficiency was calculated based on the following formula: $Efficieny = 10^{-(\frac{1}{slope})}$. This was done for the target gene and a reference gene. Finally, the ΔCt value was determined by subtracting the Ct value of the experimental sample from the Ct value of a control sample, and the fold change of gene expression was calculated using this formula: $Foldchange = \frac{\left(Efficiency_{target}\right)^{\Delta Ct_{target}}}{\left(Efficiency_{reference}\right)^{\Delta Ct_{reference}}}$. In all qPCR experiments, a non reverse transcribed sample and a water-control were included. All cDNA samples were assayed in duplicate. Values shown are means ± s.e.m of three individual experiments. Sequences of all primers used for qPCR analysis are included in Suppl. Table 4.

Microarray design

A 4x180k microarray chip platform (customized by Agilent Technologies, (Design ID:028233)) was used in this study. This array consists of all probes already present in an earlier 45.219 custom-made array [46], and another 126.632 newly designed zebrafish probes had been added as described in [47]. A total of 24 samples (8 experimental groups from 3 replicate experiments) were processed for transcriptome analysis. On each 4x180k slide, 4 random samples from different replicate experiments were hybridized against a common reference.

Microarray amplification & labeling

Amplification and labeling of RNA was performed at the MicroArray Department (MAD) of the University of Amsterdam (Amsterdam, The Netherlands). Per sample, 0.5µg total RNA was amplified and combined with Spike A according to the Agilent Two-Color Microarray-Based Gene Expression Analysis kit (Agilent technologies). As a common reference sample an equimolar pool of all test samples was made and 0.5µg samples were amplified similarly as the test samples with the exception that Spike B was used. Amino-allyl modified nucleotides were incorporated during the aRNA synthesis (2.5mM of GTP,

ATP, and UTP (GE Healthcare), 0.75mM CTP (GE Healthcare), 0.3mM AA-CTP (TriLink Biotechnologies)). Synthesized aRNA was purified with the E.Z.N.A. MicroElute RNA Clean Up Kit (Omega Bio-Tek). The quality was inspected on the BioAnalyzer (Agilent Technologies) with the Agilent RNA 6000 kit (Agilent Technologies). Test samples were labeled with Cy3 and the reference sample was labeled with Cy5. Five µg of aRNA was dried out and dissolved in 50mM carbonate buffer pH 8.5. Individual vials of Cy3/Cy5 from the mono-reactive dye packs (GE Healthcare) were dissolved in 200µl DMSO. To each sample, 10µl of the appropriate CyDye dissolved in DMSO was added and the mixture was incubated for 1h. Reactions were quenched with the addition of 5µl 4M hydroxylamine (Sigma-Aldrich). The labeled aRNA was purified with the E.Z.N.A. MicroElute RNA Clean Up Kit. Yields of aRNA and CyDye incorporation were measured on the NanoDrop ND-1000.

Microarray hybridization, scanning & data processing

Each hybridization mixture was made up from 825ng Test (Cy3-labeled) and 825ng Reference (Cy5-labeled) material. Hybridization mixtures were using the Agilent Two-Color Microarray-Based Gene Expression Analysis kit according to the manufacturer's instructions (Agilent technologies). The samples were loaded onto the microarray chips and hybridized for 17h at 65°C. Afterwards the slides were washed and scanned (20 bit, 3µm resolution) in an ozone-free room with the Agilent G2505C scanner. Data was extracted with Feature Extraction (v10.7.3.1, Agilent Technologies) with the GE2_107_Sep09 protocol for two-color Agilent microarrays. The Agilent output from the 16 hybridizations was then imported into the Rosetta Resolver 7.2 software (Rosetta Biosoftware, Seattle, Washington) and subjected to a factorial design with a re-ratio with common reference application. Data analysis was performed setting cutoff for the p-value of $<10^{-10}$ and for fold change of either >2 or <-2.

Gene Ontology analysis

Gene Ontology analysis was performed with the PathVisio version 2 software (www.pathvisio.org [4]), setting cutoffs for p-value of $<10^{-10}$ and for fold change of either >2 or <-2. For probe annotation, the Ensembl gene ID codes (ENSDARG) were used (see http://www.ensembl.org). The Z-scores shown correspond to a standard statistical test under the hypergeometric distribution. A high Z-score corresponds to a high level of enrichment of genes from a specific pathway in the investigated gene cluster.

Statistical analysis

Statistical analyses (t-tests and ANOVAs with Bonferroni post-hoc tests) were performed using the GraphPad Prism version 4.00 (GraphPad Software, La Jolla, USA). For results in Fig. 2 and Fig. 9 statistical analysis was performed on $\log_{10}$ transformed data.

Results

Analysis of zGR splice-blocking morpholino effects

In order to downregulate the expression of the zGRα transcript and upregulate that of zGRβ, we designed a splice-blocking morpholino targeted against the splice donor site of exon 8 (MO1), thus directing the alternative splicing of the zGR pre-mRNA towards

increased production of the GR β-isoform and simultaneously towards a decreased production of the GR α-isoform (Fig. 1A).

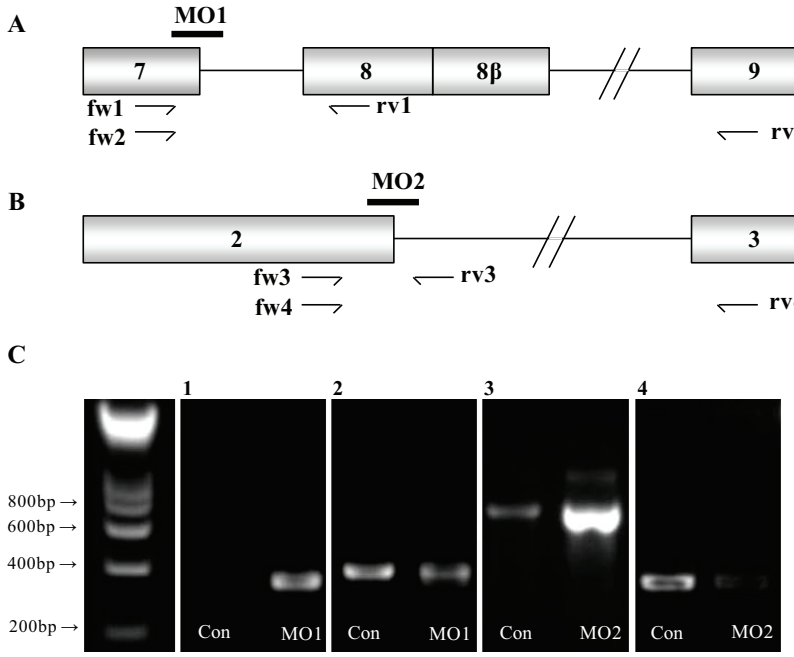

Figure 1. Schematic overview of the location of the target sites of zGR splice-blocking morpholinos MO1 (**A**) and MO2 (**B**). Primers used to analyze the effect of the morpholinos on the splicing pattern are indicated as well. Grey boxes represent the exons (numbers indicated) and black lines connecting the boxes represent the introns. **C.** Agarose gel showing products of RT-PCR analysis of RNA samples from 31hpf embryos injected with either MO1 (panel 1 and 2) or MO2 (panel 3 and 4). Panel 1 shows a 360bp product after using primers fw1 and rev1. Increased production of this band was shown in MO1-injected samples. Panel 2 shows a 400bp product after using primers fw2 and rev2. The MO1-injected sample shows a decreased production of this amplicon. Panel 3 shows a 789bp product after amplification using primers fw3 and rev3. The MO2-injected sample shows an increased production of this amplicon. Panel 4 shows a 361bp product using primers fw4 and rev4. The MO2-injected sample shows a decreased generation of this PCR product compared to control.

Zebrafish embryos at the 1-2 cell stage were injected with 0.05pmol of MO1 and no obvious morphological changes were observed in 1 day old injected embryos (data not shown). At 31h post fertilization (hpf) the effect of the morpholino injection on the splicing of the zGR pre-mRNA was analyzed by RT-PCR (Fig. 1C, panels 1 and 2). Two primer pairs were used: pair 1 amplifies a 360bp region between exon 7 and exon 8β (a sequence unique to the zGRβ transcript), and pair 2 results in a 400bp amplicon corresponding to the region between exon 7 and exon 9 (a sequence unique to the zGRα transcript). Using pair 1, an increased production of the 360bp PCR amplicon was observed in MO1-injected samples compared to controls, reflecting a MO1-induced shift in alternative splicing towards generation of the zGRβ mRNA (Fig. 1C, panel 1). RT-PCR analysis using pair 2 showed a decreased production of the 400bp amplicon in MO1-injected samples compared to the controls, reflecting a MO1-induced decrease in

alternative splicing towards generation of the zGRα mRNA (Fig. 1C, panel 2). Quantitative PCR (qPCR) analysis of RNA samples from 31hpf MO1-injected embryos showed a dramatic increase (approximately 30-fold) in the generation of the zGRβ splice variant compared to standard control morpholino (SC-MO)-injected embryos and a substantial decrease (approximately 15-fold) in the synthesis of zGRα mRNA (Fig. 2A and B). These results show that blocking the splice donor site of exon 8 using MO1 is effective and leads to a substantial upregulation of the expression of the zGRβ transcript, whereas the expression of zGRα mRNA is decreased.

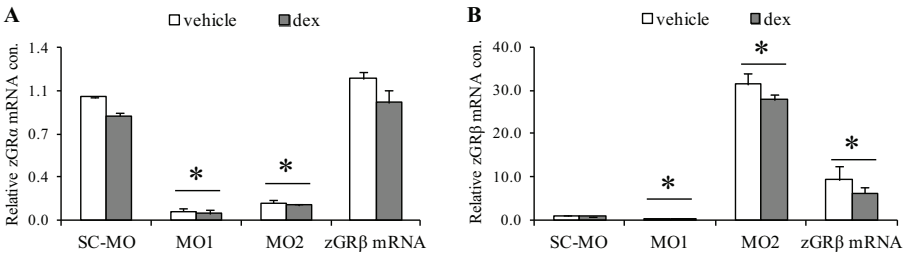

Figure 2. Analysis by qPCR of the effect of injection of morpholinos and zGRβ mRNA on the expression of zGRα and zGRβ transcripts. Zebrafish embryos were injected with either SC-MO, MO1, MO2 or zGRβ mRNA. Injected embryos at 25hpf were treated with dexamethasone (dex) for 6h and total RNA samples were collected for qPCR analysis. For all genes analyzed with qPCR, specific primers were used (sequences shown in Suppl. Table 4). The relative mRNA concentrations were normalized to those of *Bactin1* and the values shown are the means ± s.e.m. of three independent experiments. **A.** Expression of zGRα mRNA. Both morpholino treatments resulted in a significant downregulation of the zGRα mRNA concentration compared to SC-MO injections whereas no significant difference between SC-MO and zGRβ mRNA injected samples was detected. Dex did not affect the expression of the zGRα mRNA. **B.** Expression of zGRβ mRNA. MO2 caused a downregulation of the zGRβ mRNA concentration, whereas MO1 injections resulted in an upregulation of the zGRβ mRNA concentration compared to SC-MO injections. Similarly, zGRβ mRNA injections resulted in an increase in the zGRβ mRNA concentration compared to SC samples. No effect on the expression of the zGR β-isoform due to dex treatment was detected. Asterisks (*) correspond to a statistical significance of $p < 0.001$ compared to SC-MO injected groups.

In order to downregulate the expression levels of both zGRα and zGRβ, we designed a splice-blocking morpholino against the splice donor site of exon 2 of the zGR gene (MO2, Fig. 1B). This morpholino will cause retention of intron 2 in both zGRα and zGRβ mRNA, and the resulting transcript encodes a GR protein that is truncated at amino acid 367, due to the introduction of a translational stop codon, which is encoded by nucleotides 2-4 of intron 2. This truncated GR lacks the DNA-binding domain (amino acids 394-454) and the C-terminal ligand-binding domain so it can be considered inactive. Zebrafish embryos were injected at the 1-2 cell stage with 0.05pmol MO2 and no obvious morphological changes were observed in 1 day old injected embryos (data not shown). At 31hpf, the effect of MO2 injection on the splicing of the zGR pre-mRNA was analyzed by RT-PCR (Fig. 2D and E). Two primer pairs were used: pair 3 amplifies a 798 base pair (bp) region between the 5' end of exon 2 and the 3' end of the adjacent intron 2, whereas pair 4 results in a 361bp amplicon corresponding to the 5' end of exon 2 and the 3' end of exon 3 (Fig. 1B). Using pair 3, we observed an increased production of the 798bp PCR

product in MO2-injected samples compared to controls, reflecting increased retention of the intron 2 sequence due to an effective block of the splice donor site of exon 2 by MO2 (Fig. 1C, panel 3). Pair 4 showed an increased amplification of the 361bp product in MO2 injected samples compared to controls, also reflecting increased intron 2 retention (Fig. 1C, panel 4). Analysis of MO2-injected embryos showed a significant downregulation of the expression of the transcripts of both zGRα and zGRβ compared to SC-MO injected embryos, probably reflecting a decreased stability of zGR mRNA that contains the intron 2 sequence (Fig. 2A and B). Together, these results show that MO2-mediated inhibition of the splice donor site of exon 2 is effective and leads to the generation of zGRα and zGRβ mRNA that encompasses intron 2 and therefore encodes a truncated GR protein.

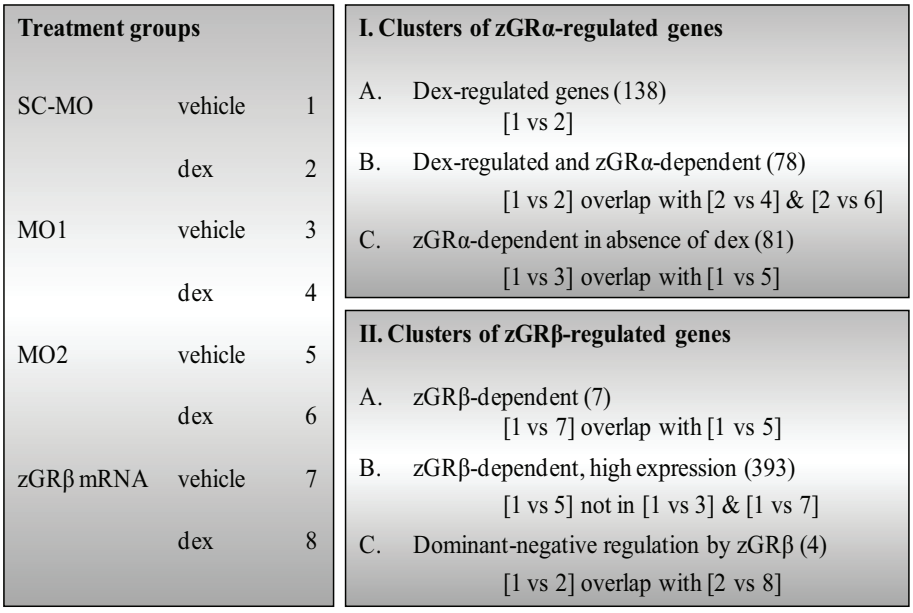

Figure 3. Overview of the microarray results on zGRα- and zGRβ-mediated effects on transcription, analyzed using Rosetta Resolver 7.2 software. The left panel indicates the eight different treatment groups, and the right panels each indicate three clusters of either zGRα- (top panel) or zGRβ-regulated (bottom panel) genes. For details on the different comparisons, see text. The number of genes (shown in parentheses) refers to annotated genes found to be significantly regulated at a p-value cutoff of $<10^{-10}$ and fold changes either >2 or <-2. Annotation and probe assignment was performed based on the Ensemble Gene ID codes (ENSDARG).

Experimental design of the microarray experiment

We used 1 day old zebrafish embryos and performed a whole transcriptome analysis in order to study the GR signaling pathway upon altering of the expression levels of both the zGR α and β-isoforms and treatment with the synthetic GC dexamethasone (dex). Zebrafish embryos at the 1-2 cell stage were injected with either 0.05pmol SC-MO (control injections), 0.05pmol MO1, 0.05pmol MO2, or 5pg zGRβ mRNA. Injection of zGRβ mRNA was performed to specifically increase the zGRβ mRNA concentration (Fig. 2), and 0.05pmol SC-MO was co-injected in this group for proper comparisons to the other groups. The next day injected embryos were treated with either dex or vehicle for 6h.

Thus, 8 groups were generated: SC-MO/vehicle, SC-MO/dex, MO1/vehicle, MO1/dex, MO2/vehicle, MO2/dex, zGRβ mRNA/vehicle, and zGRβ mRNA/dex. Data were analyzed using the Rosetta Resolver 7.2 software, setting signatures for significantly regulated probes at a p-value cutoff of $p<10^{-10}$ and fold changes either >2 or <-2. An overview of all treatment groups and a summary of the results are presented in Fig. 3.

Microarray analysis of zGRα-mediated gene transcription

First, the regulation of gene transcription due to dex treatment was studied, and for this purpose the SC-MO/veh and SC-MO/dex groups were compared. We identified 479 probes to be significantly regulated and these were attributed to 138 annotated genes, of which 128 were found to be up-regulated and only 10 to be down-regulated upon dex treatment. By means of qPCR, the induction of 4 genes (*rhcgb*, *agtxb*, *hsd11b2,* and *slc5a1*) found in our microarray analysis to be dex-upregulated (with at least two probes showing this effect) was validated and verified (Fig. 4). Using the PathVisio software for attributing gene ontology to the cluster of significantly regulated probes, specific molecular pathways were revealed to be influenced by dex treatment. Of these, the 10 pathways with the highest Z-scores are listed in Suppl. Table 1, and an enrichment of pathways involved in metabolism is obvious from this list.

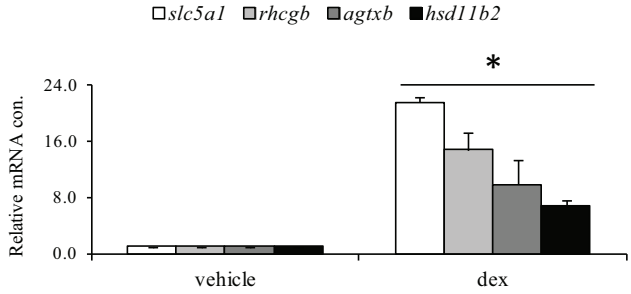

Figure 4. Validation by qPCR of gene regulation by dex identified by microarray analysis. Zebrafish embryos of 1dpf were treated with 100μM dex for 6h and total RNA samples were processed for qPCR analysis. For all genes shown, the mRNA concentration was significantly higher in the dex-treated group compared to the vehicle group. These data provide confirmation of our microarray analysis for dex-mediated gene transcription. Validation of microarray results by qPCR was performed on the same RNA samples as those used for microarray. Asterisks (*) correspond to a statistical significance of $p < 0.005$.

In order to study whether gene regulation by dex is dependent on the zGRα expression level we compared this cluster of dex-regulated genes with those found to be significantly changed due to MO1 and MO2 in the presence of dex (comparison SC-MO/dex vs. MO1/dex and SC-MO/dex vs. MO2/dex respectively). A number of 78 genes were identified to be present in all 3 clusters (Fig. 5), which means that the observed regulation by dex is dependent on the presence of zGRα. Of these genes, 71 were dex-induced and 7 were suppressed upon dex treatment, and all genes verified by qPCR to be dex-regulated (*slc5a1, rhcgb, agtxb* and *hsd11b2* (Fig. 4)) were present. These 78 genes account for 57% of the genes in the dex-regulated cluster, indicating that the majority of dex-regulation observed in our microarray is significantly compromised upon morpholino knockdown of zGRα (it must be noted that effects of MO1 can also result from an upregulation of zGRβ expression, and this point will be discussed below). This result

demonstrates the validity of our experimental approach and provides a corroborated set of marker genes for studying GR signaling in zebrafish embryos.

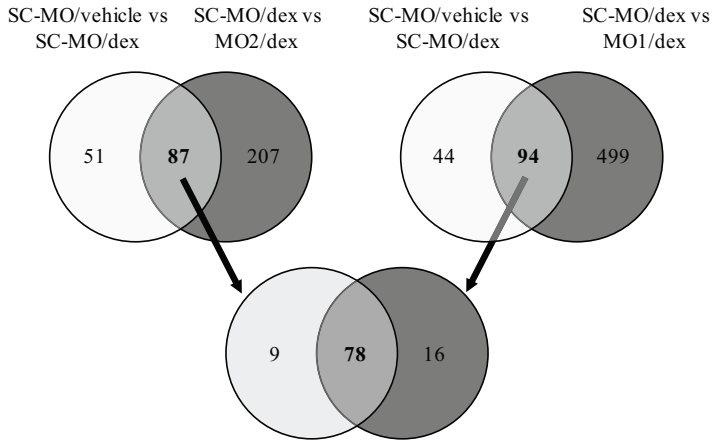

Figure 5. Venn diagrams showing the identification of the cluster of dex-regulated and zGRα-dependent genes. First, the overlap between genes regulated by dex (SC-MO/vehicle vs SC-MO/dex, white circle) and genes regulated by MO2 in the presence of dex (SC-MO/dex vs MO2/dex comparison, grey circle) was determined (87 genes). Subsequently, the overlap between genes regulated by dex (SC-MO/vehicle vs SC-MO/dex, white circle) and genes regulated by MO1 in the presence of dex (SC-MO/dex vs MO1/dex comparison, grey circle) was determined (94 genes). The overlap between these two clusters was determined, yielding the cluster of dex-regulated and zGRα-dependent genes (78 genes).

Subsequently, we studied the effect of zGRα expression in the absence of dex on gene transcription regulation by comparing the SC-MO/veh and MO2/veh groups, and this analysis showed that MO2 injection significantly affected 604 probes which represented 185 annotated genes, 133 of which are upregulated and the rest 52 are downregulated (Fig 6). In order to analyze these zGRα-specific target genes more stringently, we identified probes which were regulated by MO1 as well, since this morpholino decreases the expression of zGRα too (in addition to increasing the zGRβ expression). We identified 1401 probes to be significantly regulated as a result of MO1 injection, and they were attributed to 482 annotated genes, 321 of which are upregulated and the rest 161 are downregulated. A number of 81 genes were regulated by both MO1 and MO2 (Fig. 6). This cluster of genes regulated by both MOs accounts for 44% of the MO2-affected genes and 17% of MO1-affected genes. Validation by qPCR was performed for 4 of these genes and all of them were shown to be significantly upregulated due to both morpholinos in this experiment (Fig. 7). Further analysis using the PathVisio software for attributing gene ontology to regulated probes unveiled specific molecular pathways to be stimulated due to suppression of zGRα in the absence of exogenous GCs (Suppl. Table 2). Among those, we distinguished the p53 pathway with 8 of its components to be regulated (and 2 of them (*tp53* and *casp8*) to have been verified as zGRα-regulated genes by qPCR (Fig. 7)). This cluster of genes we consider as zGRα-dependent, and surprisingly it is completely distinct from the dex-dependent cluster we described in the previous section, since there is no overlap at all between these clusters. This indicates that in the absence of dex a different

set of genes is regulated by zGRα than upon dex treatment, suggesting that zGRα acts upon a different set of genes when activated by basal GC levels (presence of endogenous cortisol only) than upon activation by a pharmacological dose of an exogenous GC like dex.

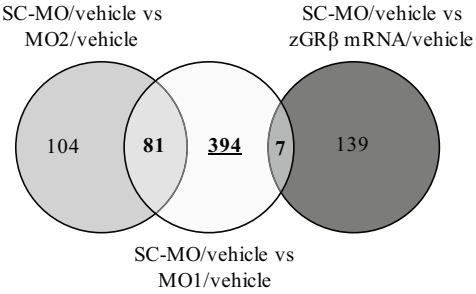

Figure 6. Venn diagram showing the identification of clusters of genes that are zGRα and β-dependent. The left (light grey) circle contains the cluster of genes found to be significantly regulated by MO2 injection in the absence of dex (SC-MO/vehicle vs MO2/vehicle comparison). The middle (white) circle contains the genes found to be significantly regulated by MO1 injection in the absence of dex (SC-MO/vehicle vs MO1/vehicle). The overlapping region between these two circles contains the cluster of 81 gene regulated upon MO1 and MO2 injection (in the absence of dex). These genes, we consider as zGRα-dependent. The right (dark grey) circle encompasses the genes found to be regulated upon GRβ mRNA injection (SC-MO/vehicle vs zGRβ mRNA/vehicle). The overlap between the right and middle circle contains the 7 genes which are regulated by both zGRβ mRNA and MO1 injections in the absence of dex. These genes we consider as zGRβ-dependent. The 394 genes which are exclusively regulated by MO1 we consider as regulated by zGRβ, but requiring higher zGRβ overexpression levels than those obtained with zGRβ mRNA injections.

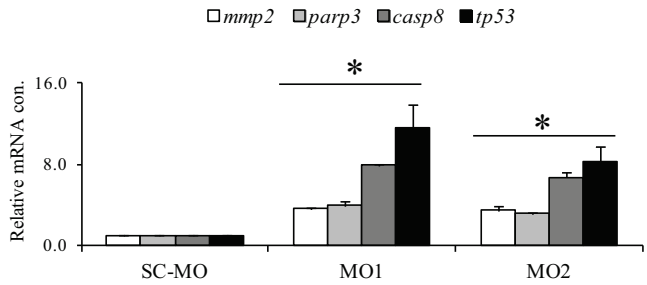

Figure 7. Validation by qPCR of MO-regulated genes identified by microarray analysis. The transcription rates of all genes examined were significantly higher in both MO1- and MO2-injected groups compared to the SC-MO group. These data provide confirmation of the results from our microarray analysis for these genes (zGRα-dependent transcription in the absence of dex). Asterisks (*) correspond to a statistical significance of $p < 0.005$.

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Microarray analysis of zGRβ-mediated gene transcription

Usage of MO1 alters the splicing of the zGR pre-mRNA towards generation of zGRβ, thereby downregulating the expression level of zGRα mRNA (Fig. 2A and B). Since the expression levels of both isoforms are affected, we cannot attribute any specific gene expression changes to either one. For that reason we first analyzed the effect of injecting MO1 in combination with that of injecting zGRβ mRNA, which results in specific zGRβ

overexpression (Fig. 6). Injection of zGRβ mRNA resulted in 353 significantly regulated probes that corresponded to 146 annotated genes (comparison SC-MO/vehicle vs zGRβ mRNA/vehicle). In order to find genes specifically affected due to zGRβ upregulation, we compared this zGRβ mRNA-regulated cluster with the MO1-regulated cluster. This analysis revealed that only 7 genes were affected by both injection of MO1 and zGRβ mRNA (Fig. 6). This number accounts for <5% of the zGRβ mRNA-affected genes and <1.5% of MO1-affected ones. Validation by qPCR was performed for 2 of these genes together with 1 gene from the zGRβ mRNA-regulated cluster and none of the observed effects could be verified (Suppl. Fig. 1). The low level of overlap between MO1-affected genes and zGRβ mRNA-affected genes and the failure to validate the observed regulation of several genes by qPCR makes the relevance of this small cluster of supposedly zGRβ-regulated genes questionable.

When we compared MO1-regulated genes to MO2- and zGRβ mRNA-regulated ones, we identified a pool of uniquely MO1-affected genes (Fig. 6). We found 1035 probes that were uniquely regulated and they were attributed to 394 annotated genes. Validation by qPCR was performed on 5 of these genes and 2 of them showed to be significantly regulated by MO1 (Fig. 8). Further analysis of this cluster of uniquely MO1-regulated genes by using the PathVisio software for attributing gene ontology to regulated probes revealed particular pathways to be specifically affected due to shifting splicing towards increased zGRβ production (Suppl. Table 3). This gene cluster could be explained as evidence for an intrinsic transcriptional activity of zGRβ (although regulation in this case probably requires higher zGRβ expression levels than the levels observed upon zGRβ mRNA injections), but might also be a result of a more efficient knockdown of zGRα by the morpholino.

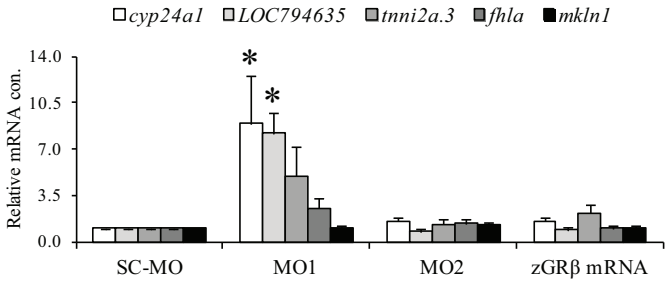

Figure 8. Validation by qPCR of unique MO1-regulated genes identified by microarray analysis. The mRNA concentration levels of *cyp24a1* and *LOC794635* were significantly higher only in MO1 injected group compared to SC one. The rest of the genes tested did not show any differential regulation. Asterisks (*) correspond to a statistical significance of $p < 0.01$.

Finally, in order to examine a possible dominant-negative effect of zGRβ, we studied whether dex-induced regulation of gene transcription was affected by an altered expression level of zGRβ. Therefore, we considered the cluster of dex-regulated genes and compared it with the cluster of zGRβ mRNA-regulated genes in the presence of dex (comparison SC-MO/dex vs zGRβ mRNA/dex). The overlap between these clusters, being the cluster of genes of which transcriptional regulation upon dex treatment was significantly compromised in the zGRβ mRNA-injected group, consisted of only 4 genes, which constitutes <3% of the dex-affected gene cluster (Fig. 9). Further validation by qPCR on 3 of these genes (expression of *il17a/f2* gene was not detectable by qPCR)

showed that only the expression of *si:ch211-234p6.12* was significantly affected by zGRβ mRNA injections whereas for the other two genes tested (*si:ch211-284e13.2* and *zgc: 163121*), no such effect was observed (Fig. 10). Thus, our analysis shows that only a very small fraction of dex-regulated genes is regulated by zGRβ in a dominant-negative way.

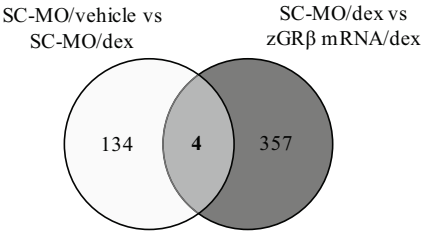

Figure 9. Venn diagram showing the analysis of the possible dominant-negative acivity of zGRβ. The white circle contains dex-regulated genes (SC-MO/vehicle vs SC-MO/dex) and the grey circle encompasses the zGRβ mRNA-regulated genes (SC-MO/dex vs zGRβ mRNA/dex). The overlap contains 4 genes affected by dex treatment, and displaying a decreased level of dex-mediated regulation due to zGRβ overexpression. Thus, in our microarray experiment, zGRβ was shown to have a dominant-negative effect on the transcription of 4 genes.

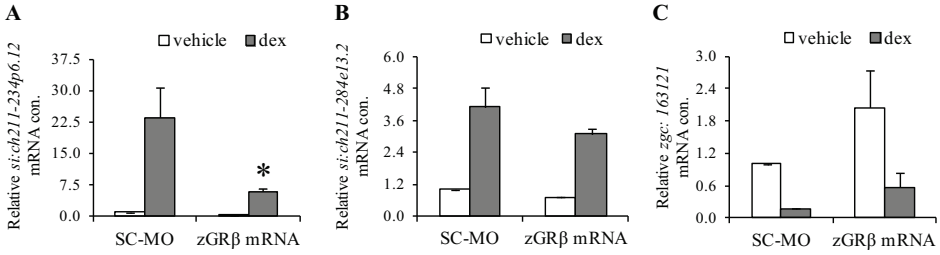

Figure 10. Validation by qPCR of the dominant-negative effect of zGRβ. **A.** The dex-mediated upregulation of *si:ch211-234p6.12* gene was significantly compromised due to zGRβ mRNA injection compared to SC-MO injected group. **B**. The dex-mediated upregulation of *si:ch211-284e13.2* gene was not significantly altered due to zGRβ mRNA injection compared to SC-MO injected group. **C.** The dex-mediated downregulation of *zgc: 163121* gene was not significantly altered due to zGRβ mRNA injection compared to SC-MO injected group. These results demonstrate that zGRβ exhibits a dominant-negative effect only on the *si:ch211-234p6.12* gene. Asterisk (*) indicates a statistical significance of $p < 0.001$.

Discussion

Recently, the zebrafish embryo model has emerged as a potent *in vivo* system for gene knockdown studies using morpholinos. These agents efficiently block either the translation of expressed transcripts or their proper splicing, thus enabling functional characterization of gene activity at the whole organism level [45, 49]. In the present study, we have employed this reverse-genetic approach in zebrafish embryos in order to study the GR signaling pathway. In particular, we aimed to reveal specific target genes of the zGR α-

and β-isoform under basal conditions as well as after dex treatment. For this purpose, two different (splice-blocking) morpholinos were used. One that directs alternative splicing of the zGR pre-mRNA towards increased zGRβ expression, thereby decreasing the expression of the canonical zGR α-isoform (MO1), and another that functionally knocks down both the zGR α- and β-isoform (MO2). In addition, zGRβ mRNA injections were used to specifically increase zGRβ expression. The efficacy of the morpholino treatments was tested by PCR, showing that the splice pattern was altered as expected.

Neither the morpholino treatments nor injections of zGRβ mRNA caused any obvious morphological abnormalities during embryonic development. This observation is consistent with other studies, in which different splice-blocking morpholinos were used for a functional knockdown of zGR [50, 51]. In mice, knockout of GR has been shown to be lethal several hours after birth, mainly as a result of compromised lung development [52]. This apparent paradox could be due to the fact that our splice-blocking morpholino treatment does not result in a complete knockout and does not affect maternally deposited mRNAs (so morpholinos start to act after the onset of zygotic transcription (~4-5hpf). Alternatively, zebrafish development may be more resistant to fluctuations in GR availability, for example because of the absence of lungs. Despite the absence of any obvious morphological disruptions, our genetic manipulation of zGRα and zGRβ transcripts led to significant transcriptional changes as shown by microarray analysis and further qPCR validation.

Before studying genes regulated upon our genetic manipulation, we first investigated genes of which the expression is affected by ligand activation of zGRα. For this purpose, we compared the control (SC-MO)-injected group treated with vehicle with the control-injected group treated with dex, and we identified 138 genes that were significantly regulated upon dex treatment. From these genes, 4 (*slc5a1, agxtb, rhcgb* and *hsd11b2*) were tested by qPCR and the regulation observed in the microarray was verified for all 4 (Fig. 4). Moreover, gene ontology analysis (Suppl. Table 1) showed that metabolic pathways (e.g. cytochrome P450-dependent metabolism, citrate cycle, glutathione metabolism, steroid hormone biosynthesis) are particularly induced upon dex treatment, alongside other pathways well-known to be affected by GC signaling like the insulin [53] and prostaglandin signaling pathway [54]. Our dex-responsive gene set, obtained in an *in vivo* model, could complement data derived from previous studies in which researchers also availed themselves of microarray technology in order to identify GR target genes and characterize their transcriptional regulation [55-58]. In some of these studies, gene ontology analysis also revealed GR activation to affect metabolic pathways as we found in our data set [59, 60].

Moreover, further examination showed that for the majority (57%, 78 genes) of these 138 dex-regulated genes the induction upon dex treatment was significantly reduced due to suppression of zGRα expression upon either MO1 or MO2 injections (Fig. 5). The rest of the dex-regulated genes could either be less sensitive to zGRα availability (most likely due to different promoter affinities for GR occupancy [61-63]) or represent false positive results. Thus, this analysis provided us with a corroborated set of 78 genes which are regulated upon dex treatment and of which this regulation is zGRα-dependent. In addition, it confirmed the validity of our microarray experiment.

Next, we examined genes influenced due to specific zGRα knockdown (Fig. 6) by both MO1 and MO2 (in the absence of dex). We identified 81 genes to be significantly regulated due to zGRα suppression. From these genes we tested 4 by qPCR and zGRα-dependency was verified for all 4 of them (Fig. 7). Gene ontology analysis unveiled specific pathways to be influenced by zGRα under basal conditions. These pathways

concern (among others) apoptosis, the Androgen receptor signaling, the MAPK signaling, and the cell cycle (Suppl. Table 2). From this list, we distinguished the p53 pathway with 7 of its components to be induced (and 1 to be suppressed) due to knockdown of zGRα. From this particular signaling cascade we confirmed by qPCR the induction of *casp8* and *tp53* (Fig. 7). Thus, under basal conditions, zGRα appears to suppress the p53 pathway and this finding is in line with the results of many other studies, both in zebrafish and in other systems. In a microarray study by Pikulkaew et al. [51], the use of a morpholino targeted against the zebrafish GR also led to a substantial induction of *tp53* and *casp8* at 10hpf [51]. Another interesting observation comes from a study by Danilova et al. [64], who showed that long-term (1-3 days) dex treatment suppressed *tp53* levels that were initially elevated due to *rpl11* gene mutation. In mammalian systems, GR and p53 can physically interact and impair each other's transcriptional activities (e.g. via sequestration in the cytoplasm [65-67]) as well as act synergistically [68-70]. Additionally, there are data on a negative correlation between p53 and GR levels [71, 72]. Since most of this research has been done using cell lines, the zebrafish could provide a powerful *in vivo* model in order to elucidate the molecular mechanism of interaction between GR and p53 and explore the biological outcome at the whole organism level, e.g. alterations in the level of apoptotic cell death during development.

Furthermore, we investigated whether the gene cluster of 138 dex-regulated genes overlaps with the cluster of 81 genes identified as zGRα-dependent in the absence of dex. Surprisingly, we found no overlap between these clusters, indicating that zGRα regulates a different set of target genes under basal conditions than upon activation by dex. Upon dex treatment various metabolic pathways are induced, whereas morpholino knockdown of the receptor activates immune-releated pathways and the MAPK signaling cascade (Suppl. Tables 1 and 2). These findings put forward the idea of two distinct gene sets; one set to be regulated under basal conditions for maintenance of homeostasis (e.g. in our case by suppressing p53 pathway) and the other to be regulated upon a stressful stimulus in order to restore homeostasis (e.g. in our case by inducing metabolic routes) [61]. In this respect, it can be hypothesized that the potency of zGRα to induce transcription differs between target genes, which may be for example due to different binding affinities for ligand-bound receptors of the GREs. Hence, some genes could already be activated upon zGRα activation by basal cortisol levels, whereas other genes may need increased levels of activated zGRα, like those observed after dex treatment in our work [61-63]. In support of this notion, a recent study by Reddy et al. [73] showed variation in the sensitivity of genes to siRNA treatment against the GR. Further examination of the GRE architecture of genes from these distinct gene clusters could explain their differential response to zGRα signaling and therefore contribute to a better understanding of GRα-mediated transcription under various physiological conditions.

Subsequently, we looked into target genes specific for zGRβ in the absence of dex, in order to reveal a possible intrinsic transcriptional activity of this isoform. Therefore, we investigated whether there was any overlap between the cluster of MO1- and zGRβ mRNA-affected genes (in the absence of dex). This analysis resulted in a cluster of only 7 genes regulated due to zGRβ overexpression by both MO1 and zGRβ mRNA (Fig. 6). Validation by qPCR on 2 of those genes failed to verify the zGRβ-mediated regulation observed in the microarray. This failure to verify the regulation in combination with the very low number of genes affected by both MO1 and zGRβ mRNA strongly suggests that this cluster represents false positive hits.

A surprisingly large cluster of 394 genes was identified that was regulated exclusively by MO1 (Fig. 6). The lack of regulation of these genes by MO2 suggests that

they are not regulated by zGRα but exclusively by zGRβ, whereas the lack of regulation upon zGRβ mRNA injection suggests that regulation of these genes requires relatively high zGRβ levels, since qPCR analysis showed that MO1 led to a significantly higher zGRβ upregulation than that observed upon zGRβ mRNA injections (Fig. 2). Validation by qPCR confirmed the induction of 2 genes, *Cyp24a1* and *LOC794635* (out of 5 genes tested, Fig. 9), which are involved in Vitamin D_3 metabolism and the immune response respectively [74, 75]. Applying gene ontology analysis to this cluster of uniquely MO1-regulated probes revealed specific signaling cascades to be regulated like that of TGF-beta, delta-notch, Id, noncanonical wnt, and pentose phosphate as well as fructose and mannose metabolism (Suppl. Table 3). This gene cluster could reflect specific target genes for zGRβ, thus providing evidence for an intrinsic transcriptional activity for this receptor. Regulation in this case probably requires higher zGRβ expression levels than the levels observed upon zGRβ mRNA injections. Alternatively, it could be explained as a gene cluster specific for zGRα but as a result of a more efficient knockdown by MO1 compared to MO2.

Next, we examined a possible dominant-negative activity of zGRβ and identified only 4 genes for which the dex-induced gene transcription was significantly compromised due to zGRβ mRNA injection, accounting for <3% of the cluster of dex-affected genes. Validation by qPCR of these 4 genes confirmed a dominant-negative effect on 1 of those (*si:ch211-234p6.12*, encoding an unknown protein). These data most likely reflect the absence of a significant inhibitory role of zGRβ on the transcriptional properties of zGRα. Nevertheless, we cannot rule out that very specific and limited effects (as in the case of *si:ch211-234p6.12)* can be attributed to a dominant-negative function of zGRβ. With respect to this notion, in mice it was shown that GRβ's dominant-negative activity was specific for a subset of genes, but still 4 out of 6 genes tested showed a dominant-negative effect, suggesting a much lower level of specificity than observed in our study for zGRβ [31]. Alternatively, the dominant-negative activity of zGRβ may require higher expression levels in order to exert more wide-spread effects, as previously hypothesized for its intrinsic transcriptional activity. Collectively, our findings on zGRβ do not point to a very profound effect for this receptor, but suggest particular requirements such as high expression levels and gene specificity for zGRβ's transcriptional properties. This conclusion is in line with controversial data obtained from overexpression studies of the human GRβ, which revealed inconsistencies concerning its inhibitory effects among different experimental setups [16-18, 21, 22, 26, 27], as well as little overlap between hGRβ target genes found in different cell types [19, 20].

In summary, our microarray analysis of the zGR signaling in 1dpf zebrafish embryos identified two distinct zGRα-dependent gene clusters. One that is affected at basal levels of zGRα activation and another that is regulated after stimulation with exogenous glucocorticoids. Additionally, zGRβ may exhibit intrinsic transcriptional activity that requires a high expression level of this splice variant. Finally, zGRβ does not appear to have a profound inhibitory role in the zGRα's transcriptional properties.

Acknowledgments

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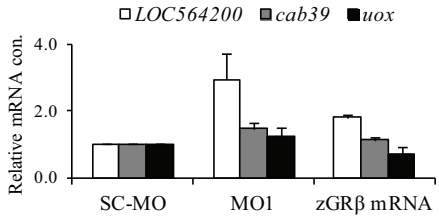
Supplemental data



Supplemental Figure 1. Verification by qPCR of zGRβ mRNA and MO1-mediated intrinsic transcriptional activity on *LOC564200, cab39* and *uox* identified by microarray analysis. Groups assessed were SC-MO/vehicle vs MO1/vehicle and zGRβ mRNA/vehicle. None of the genes tested showed any differential regulation upon either MO1 or zGRβ mRNA injections.

Pathway	Genes meeting criteria	Z scores
Drug metabolism - cytochrome P450	3	5.6
Metabolism of xenobiotics by cytochrome P450	3	5.37
Prostaglandin signaling	3	4.97
Glutathione metabolism	3	4.79
NOD pathway	3	3.71
Insulin signaling pathway	5	3.51
Toll like receptor pathway	5	3.47
Adipocytokine signaling pathway	3	3.4
Citrate cycle (TCA cycle)	2	3.26
Steroid hormone biosynthesis	2	3.07

Supplemental Table 1: Molecular pathways revealed by PathVisio gene ontology analysis of dex-regulated genes.

Pathway	Genes meeting criteria	Z scores
p53 signaling pathway	8	10.83
G1 to S cell cycle control	4	6.13
Androgen Receptor Signaling Pathway	4	4.38
MAPK signaling pathway	4	3.26
RIG-I-like receptor signaling pathway	3	3.19
Apoptosis	3	3.12
Cell cycle	4	3.08
Glycosaminoglycan biosynthesis - chondroitin sulfa	1	2.93
NOD-like receptor signaling pathway	2	2.72
Toll-like receptor signaling pathway	3	2.7

Supplemental Table 2: Molecular pathways revealed by PathVisio gene ontology analysis for morpholinos-regulated genes (see Fig. 6).

Pathway	Genes meeting criteria	Z scores
TGF-beta	9	4.92
Id Signaling Pathway	4	3.61
Toll like receptor pathway	8	3.45
noncanonical wnt pathway	7	3.2
Nodal signaling pathway	5	3.14
neural crest development	4	2.96
Fructose and mannose metabolism	3	2.64
Pentose Phosphate Pathway	1	2.64
Delta-Notch Signaling Pathway	4	2.59
IFN Pathway	6	2.57

Supplemental Table 3: Molecular pathways revealed by PathVisio gene ontology analysis for genes exclusively regulated by MO1 (see Fig. 6).

Official gene name	Gene symbol	Forward primer sequence	Reverse primer sequence
Bactin1	*Bactin1*	CGAGCAGGAGATGGGAACC	CAACGGAAACGCTCATTGC
FK506 binding protein 5	*fkbp5*	TCTGCCAGCACAAGATTCGTGAGC	GACCCTGCTTATTCTGATCGGAAA
nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	*zGRβ transcript*	GATGAACTACGAATGTCTTA	GCAACAGACAGCCAGACAGCTCACT
nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	*zGRα transcript*	AACTGGCAACGGTTCTATCAGCTCA	TTCTGGTGAAAGAGCAGCGG
matrix metalloproteinase 2	*mmp2*	TTCTTGGACTGCTGTTCCcG	TGTGACCATCACCATTAAGGCTA
poly (ADP-ribose) polymerase family, member 3	*parp3*	CCAAGGACAAATTCACTTCcG	GGTTCCTTGTGCCCTTCACCT
caspase 8, apoptosis-related cysteine peptidase	*casp8*	TGGTAAACCAGTTGAAATCCGAGA	TTGCTAGTGTTCTGCATCcG
tumor protein p53	*tp53*	CGGATGGAGATAACTTGGcG	TTCCCTGTAATTTGCTCGCTGAT
si:ch211-234p6.12	*si:ch211-234p6.12*	TGGAGAGAGAAAGATGGCcG	TGGAGACAGGTGAACCATTGCT
zgc: 163121	*zgc: 163121*	TCAACTCCAGCATTGAGGAAGC	AGGCTCCATAGACTGTAGTTCcG
si:ch211-284e13.2	*si:ch211-284e13.2*	AGACAATGCAGAGTGCTGCTGG	TTGAACCCGTGCCAACACTGCC
calcium binding protein 39	*cab39*	GTACCAATGAGAAGGAGCcG	GAGCAGGCCGCTGTTGTAGAG
metallo-beta-lactamase domain-containing protein 1-like	*LOC564200*	GCTGAAGATGACGGCGCGTT	ACGGACCGCCTGTGTCCACTAA
urate oxidase	*uox*	ATCCGGCGTGAAGGGAACCA	TTTGGCAAGAGCGTGGACGGTG

solute carrier family 5 (sodium/glucose cotransporter), member 1	*slc5a1*	GTAAAGCTGTCTGTGGAG CcG	CCACCACAAGTTTGGGA TAAGC
hydroxysteroid 11-beta dehydrogenase 2	*hsd11b2*	AACCCCAGGTGCGATACT AcG	AACCTGTCGCTGATGCT GAGTG
rhesus blood group, C glycoprotein b	*rhcgb*	GTGCTTCGTCTGGCAGGTG T	GTGTCTGCCTCCTCGTTA TACcG
alanine-glyoxylate aminotransferase b	*agxtb*	TGAAGCATCACCAGATCG AGTTT	GTATCCCATGAGCCCAA TCcG
cytochrome P450, family 24, subfamily A, polypeptide 1	*cyp24a1*	TTCACCGTCCGTGCCGTTC A	AGGCAGGGTGTAATCGC CCA
muskelin 1, intracellular mediator containing kelch motifs	*mkln1*	AGCTCTTGCCCTCCGCTCT GTT	TGTGTGCGCTGGGCGTA TGT
complement C4-like	*LOC794635*	AGCATCGCTCGTGCCACC AA	TCCAGGCTGACAGTGCT TGGGT
four and a half LIM domains a	*fhla*	AGTGCAAGCAGCCTATCC GCA	TTGCAGCGAACGCAGTG CTTG
troponin I, skeletal, fast 2a.3	*tnni2a.3*	ATGACATCGAGCCGTAGG CACC	GCATGGACAGAGGAGC GCAGTT

Supplemental Table 4: Sequences of all primers used qPCR analysis.

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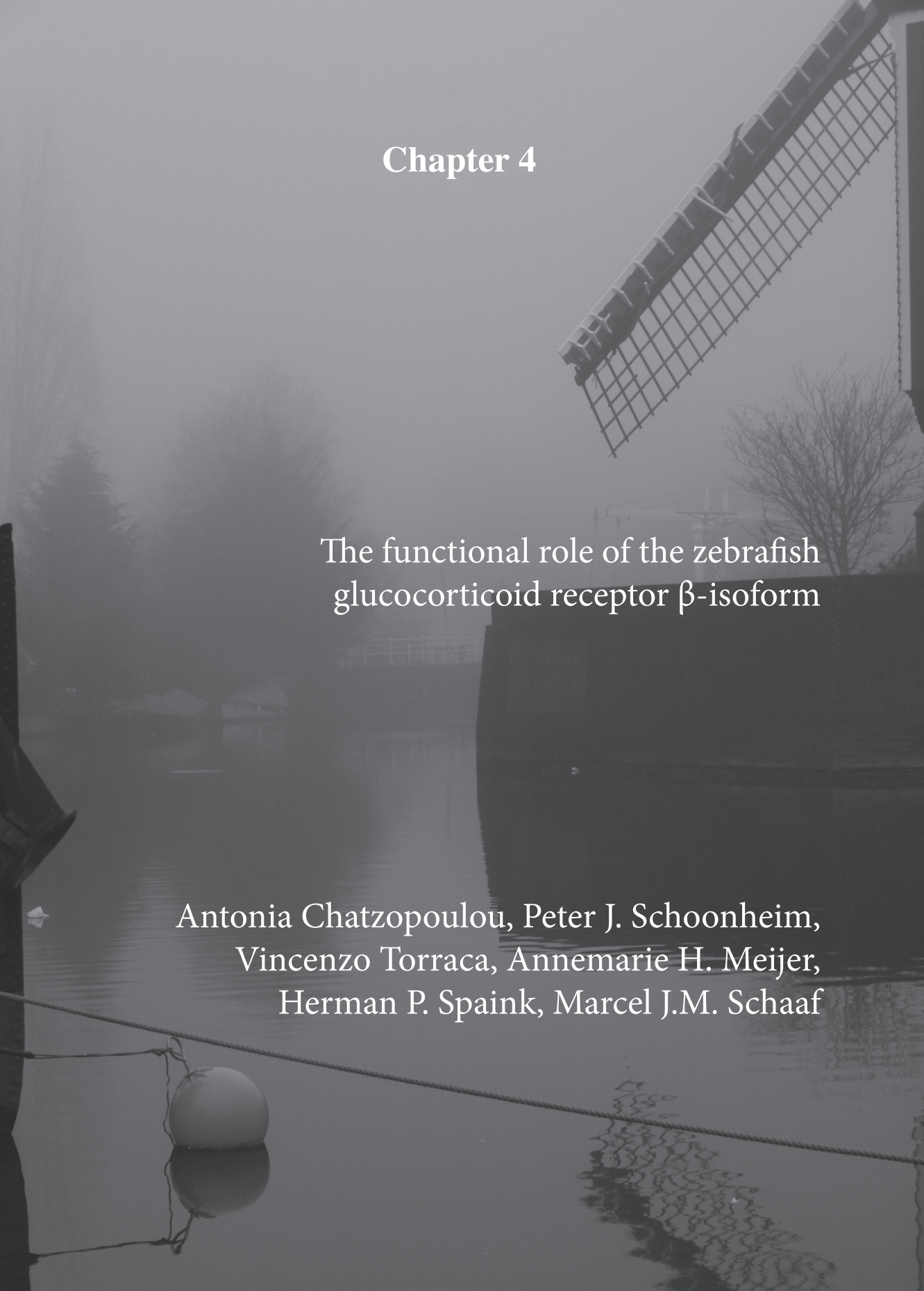

Chapter 4

The functional role of the zebrafish glucocorticoid receptor β-isoform

Antonia Chatzopoulou, Peter J. Schoonheim, Vincenzo Torraca, Annemarie H. Meijer, Herman P. Spaink, Marcel J.M. Schaaf

Abstract

In humans, two splice variants of the glucocorticoid receptor (GR) exist: the canonical human GR α-isoform (hGRα), acting as a ligand-activated transcription factor, and the human GR β-isoform (hGRβ), which has been shown to have a dominant-negative effect on hGRα. Several studies have shown that elevated hGRβ levels are associated with resistance to anti-inflammatory glucocorticoid treatment. In the present study we have used the zebrafish model system in order to elucidate the functional role of the GR β-isoform *in vivo*. Luciferase reporter assays in transiently transfected COS-1 cells demonstrated a dominant-negative effect of the zebrafish GR β splice form (zGRβ) on the transactivation properties of the GRα isoform (zGRα). However, no such effect was observed in zebrafish PAC2 cells using induction of the *fkbp5* gene as readout. In addition, upon zGRβ mRNA injections in 1-2 cell stage zebrafish embryos, no effect was observed on the zGRα-induced transactivation of the *fkbp5*, *pepck* and *nfkbiaα* genes. Subsequently, we generated a transgenic fish line with inducible zGRβ overexpression. Transcriptome analysis by microarray and subsequent qPCR validation using this line did not reveal any regulatory effect of zGRβ (either as a dominant-negative inhibitor of zGRα or as a transcription factor independent of zGRα). Based on these results, we suggest that the zebrafish GR β-isoform does not have a regulatory role in transcription and that splicing of the GR pre-mRNA into a messenger encoding an alternative splice variant could instead represent a physiological mechanism to downregulate the levels of the canonical receptor variant

Introduction

The glucocorticoid receptor (GR) is expressed almost ubiquitously in the human body and regulates a wide range of biological processes such as our metabolism, growth, reproduction, vascular tone, bone formation, immune response and brain function [1-6]. The GR is activated by binding of its cognate ligands (glucocorticoids (GCs)), and upon activation it acts as a transcription factor. In its inactive state, the GR mainly resides within the cytoplasm in a multiprotein complex containing chaperones and immunophilins. Upon glucocorticoid binding, the GR is released from its cytoplasmic complex and translocates to the nucleus, where it orchestrates gene expression via DNA-binding dependent and independent mechanisms. DNA-binding dependent mechanisms involve GR binding to glucocorticoid response elements (GREs), where it recruits transcriptional coregulators and thus, directs (positively or negatively) the transcription rate of target genes. The mode of GR action by which DNA binding leads to upregulation of gene expression is known as *transactivation*. Independent of DNA-binding, the GR can also physically interact with other transcription factors (e.g. NF-κB and AP-1), via which it can either synergistically induce gene expression or repress activation of genes. The latter mode of action is called *transrepression* and comprises one of the classical ways by which GCs exert their anti-inflammatory effects [1, 2, 7-11].

Cloning of the human GR gene revealed the occurrence of two splice variants, named as hGRα and hGRβ, which derive from alternative usage of an acceptor splice site within the last coding exon (exon 9) [12, 13]. The two isoforms are identical between amino acids 1 and 727, thus sharing the same N-terminal domain and DNA binding domain (DBD), and only differ in the C-terminal part of their ligand binding domain

(LBD). The GR α-isoform (777 amino acids) is able to interact with GCs, and represents the canonical receptor. The hGR β-isoform (742 amino acids) has a shorter LBD with a unique C-terminal 15 amino acid sequence, which renders it unable to respond to GCs [12-14].

Almost ten years after the discovery of hGRβ, it was shown (using *in vitro* reporter assays) that this isoform had a pronounced dominant-negative inhibitory effect on hGRα's transactivational properties [15-17]. Moreover, hGRα-mediated transrepression of *in vitro* reporter assays was also reported to be inhibited by hGRβ [17]. These findings were soon coupled to clinical data demonstrating a positive correlation between high expression levels of the β-isoform and GC resistance of patients suffering from immune-related disorders, like asthma [18-23], ulcerative colitis [24-26], leukemia [27-29] and rheumatoid arthritis [30, 31]. Further cell-based research supports an inhibitory role for hGRβ on both hGRα-induced transactivation and transrepression of endogenous genes (MKP-1, myocilin, fibronectin, TNFα and IL6 [23, 32, 33]), as well as on hGRα-mediated regulation of cell death, proliferation and phagocytosis [34-36]. Moreover, recent data support the notion that hGRβ can have its own intrinsic transcriptional activity. Overexpression of zGRβ has been demonstrated to attenuate NF-κB and AP-1 induction of luciferase reporter constructs [37], as well as GATA3-mediated activation of IL5 and IL13 promoters of luciferase genes [38]. Additionally, transcriptome analyses of cultured cells showed that hGRβ can direct gene transcription independently of hGRα activation [39, 40].

Despite several studies indicating a dominant negative function or intrinsic transcriptional activity of the GR β-isoform, its physiological relevance has often been questioned. First, in many studies the dominant-negative role of hGRβ on hGRα's transcriptional properties could not be demonstrated in *in vitro* reporter assays [37, 38, 41-46]. Second, despite the fact that hGRβ is expressed in almost all human tissues, its expression levels are significantly lower compared to hGRα [15, 16, 34, 47-49]. This raises doubts about its *in vitro* dominant negative effect, since in most studies this required transfection of a 10-molar excess of GRβ expression vector compared to hGRα [15-17].

Until recently, functional studies of GRβ were complicated by the fact that expression of this isoform was only found in humans, and that rodents contained a mutation in the splice site required for GRβ expression [50]. It was therefore remarkable that we discovered the occurrence of a GR β-isoform in zebrafish [51]. Like humans, zebrafish contain a single GR gene that contains 9 exons and gives rise to two splice variants, the GR α- and β-isoform. Like their human equivalents, both zebrafish GR α- and β-isoforms share the same N-terminal domain and DBD and only differ at the C-terminus of their LBD. In contrast, the gene organization and splicing events leading to the expression of GRβ are different between humans and zebrafish. Whereas in humans the β-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 an mRNA encoding zGRβ [51]. Thus, the human and zebrafish β-isoforms derive via 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 [51, 52]. Interestingly, an alternative splice event in exon 8 similar to that leading to the expression of a GR β-isoform in zebrafish was recently discovered in mice [53].

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 [51]. Like its human equivalent, zGRβ is ubiquitously expressed, and at significantly lower levels compared to zGRα [51]. 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 [51].

In the present study we have used the zebrafish as an *in vivo* model to elucidate the biological significance of GRβ. Over the last decade, the zebrafish has emerged as a major contributor to biomedical research for an array of human diseases [54, 55]. It is easily handled and maintained and produces big clutches of embryos, which are fertilized externally and develop rapidly [56]. Moreover, its genome has been sequenced and embryos can easily be subjected to genetic manipulations [56, 57].

The scope of this study was to investigate the role of zGRβ in gene transcription either as a dominant negative inhibitor of zGRα or as a distinct transcription factor independent of zGRα. We have utilized both *in vitro* and *in vivo* approaches. For the *in vitro* studies, we performed luciferase assays in COS-1 cells transiently transfected with zGRβ. Additionally, we used the zebrafish PAC2 cell line, in which we have stably overexpressed zGRβ and studied the transcription rate of several endogenous target genes. For *in vivo* experiments, we have overexpressed zGRβ by means of mRNA injections in embryos, and have generated a transgenic zebrafish line with an inducible zGRβ expression construct. While a clear dominant-negative effect of zGRβ was observed on luciferase assays in COS-1 cells, our data do not reveal any regulatory effect of zGRβ on the expression of endogenous genes. Therefore, our study does not support the proposed functions of the GR β-isoform as a dominant negative inhibitor of zGRα or as a transcription factor independent of zGRα, but rather suggest an alternative function of alternative splicing, such as downregulation of the GRα expression level.

Materials & Methods

Cell cultures

COS-1 cells (African green monkey kidney cells were cultured in DMEM (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin and streptomycin (Invitrogen). Cells were grown at 37°C and 5% CO_2. PAC2 zebrafish cells were cultured in Leibovitz's medium (Invitrogen), supplemented with 15% fetal bovine serum (Invitrogen) and 1% penicillin and streptomycin (Invitrogen). Cells were grown at 28°C.

Luciferase reporter assays in COS-1 cells

COS-1 cells were seeded into 24-well plates ($3x10^4$ cells/well). Transfection was performed 24h later using the *Trans*IT®-COS Transfection Kit (Mirus Bio) according to the manufacturer's instructions. For the experiments in which the transactivation activity of zGRα was assayed, 200ng MMTV-luciferase reporter construct was transfected, with a range of PCS+zGRα plasmid concentrations (1-300ng) and/or 100ng pCS2+zGRβ expression vector (previously described in [51]), together with 2ng CMV-renilla (Promega). For the experiments in which the transrepression activity was assayed, cells were transfected with 50ng of a κB-luciferase reporter construct (containing 5 κB response elements (Stratagene)) and 50ng of a human p65 expression vector (pCMV4-p65, described in [58]), together with a range of pCS2+zGRα concentrations (0-1000ng) in the presence and absence of 100ng pCS2+zGRβ. The total amount of transfected DNA was

always kept equal among groups by transfecting empty pCS2+ vector. Twenty-four hours after transfection, cells were treated with 100nM dexamethasone (Sigma) and 24h later, they were assayed for luciferase activity using the Dual-Luciferase® Reporter Assay System (Promega). Bioluminescence was detected using a Wallac 1450 MicroBeta Luminometer. For each sample, the luciferase activity was normalized to the renilla activity. Per sample, measurements were performed in duplicate, and data shown are averages ± s.e.m. of three individual experiments.

Generation of the PAC2-zGRβ cell line

For the generation of a stable cell line overexpressing zGRβ, the pCS2+zGRβ plasmid [51] and a neomycine resistance plasmid were transfected into PAC2 zebrafish cells using the Amaxa® Cell line Nucleofector kit V and the Nucleofector™ II device set at T27 programme (Lonza). Four days after transfection, cells were subjected to selection for antibiotic resistance by supplementing their culture medium with 500µg/ml of geneticin (G418, Invitrogen). Resistant cells were propagated to establish the PAC2-zGRβ cell line.

Glucocorticoid treatment of PAC2 cells for gene expression analysis

PAC2 wild type and PAC2-zGRβ cells were seeded in 6 well plates ($2x10^5$ cells/well) the day before treatment with either 10µM betamethasone 17-valerate (Sigma) or vehicle (<2% DMSO) in complete culture medium for 3h. Samples were collected in TRIzol® reagent (Invitrogen) and total RNA was isolated following the supplier's manual (Invitrogen).

RNA isolation & cDNA synthesis

Total RNA was extracted using the TRIzol® reagent (Invitrogen) according to the manufacturer's instructions (Invitrogen). RNA was dissolved in water and denatured for 5 min at 60°C. Samples were treated with DNAase using the DNA-*free*™ kit (Ambion). For microarray analysis, RNA was further purified using the RNeasy MinElute™ Cleanup kit from Qiagen and its integrity was checked with a lab-on-chip analysis using the 2100 Bioanalyzer (Agilent Technologies). For subsequent cDNA synthesis, at least 200ng of total RNA was added as a template for reverse transcription using the iSCRIPT™ cDNA Synthesis Kit (Biorad). Non reverse-transcribed samples were also generated to be used as negative controls during the quantitative polymerase chain reaction step.

Quantitative Polymerase Chain Reaction (qPCR)

qPCR analysis was performed using the MyiQ Single-Color Real-Time PCR Detection System (Biorad). PCR reactions were performed in a total volume of 25µl containing 2µl cDNA, 1µl forward and reverse primer (10µM) and 12.5µl of 2x iQ™ SYBR® Green Supermix (Biorad). Cycling conditions were 95°C for 3 min, followed by 40 cycles of 15 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C. Ct values (cycle number at which a threshold value of the fluorescence intensity was reached) were determined for each sample. A dissociation protocol was added, determining dissociation of the PCR products from 65°C to 95°C, allowing discrimination of specific products. In order to determine the efficiency of the amplification reaction, serial dilutions of cDNA were used and standard curves were generated plotting the Ct values against the relative cDNA concentration. The linear slope of the standard curve for each primer pair was determined and the efficiency was calculated based on the following formula: $Efficieny = 10^{-(\frac{1}{slope})}$. This was done for the target gene and a reference gene. Finally, the ΔCt value was determined by subtracting the Ct value of the experimental sample from the Ct value of a control sample, and the fold

change of gene expression was calculated using this formula: $Foldchange = \frac{\left(Efficiency_{t\arg et}\right)^{\Delta Ct_{t\arg et}}}{\left(Efficiency_{reference}\right)^{\Delta Ct_{reference}}}$. In all qPCR experiments, a non-reverse transcribed sample and a water-control were included. All cDNA samples were assayed in duplicate. Values shown are means ± s.e.m of three individual experiments. Sequences of all primers used for qPCR analysis are included in Suppl. Table 4.

Zebrafish strains, husbandry & egg collection

Wild type adult ABxTL zebrafish and the transgenic lines *Tg*(*hsp70*:*Gal4)* (provided by Dr. H. Baier, University of California, San Francisco, USA) and *Tg(UAS:GFP-zGRβ)* (see 'Generation of the *Tg(UAS:GFP-zGRβ)* transgenic line' later in this section) were used in this study. Livestock was maintained and handled according to the guidelines from http://zfin.org. Fertilization was performed by natural spawning (single crossings) at the beginning of the light period and eggs were raised at 28°C in E3 egg water medium containing 60µg/ml Instant Ocean sea salts supplemented with 0.0025% methylene blue (GURR). All experimental procedures were conducted in compliance with the directives of the animal welfare committee of Leiden University.

zGRβ mRNA synthesis & injections

pCS2+zGRβ plasmid was linearized with NsiI (New England Biolabs) and used as a template for the synthesis of zGRβ mRNA using the mMESSAGE mMACHINE SP6 kit from Ambion. Newly synthesized mRNA was purified using the RNeasy MinElute™ Cleanup kit (Qiagen). RNA was denatured at 60°C for 5min and its integrity was checked on an agarose gel. Prior to injections, zGRβ mRNA was denatured at 60°C for 5min and diluted at a final concentration of 5ng/µl in 1x Danieu's buffer (58mM NaCl, 0.7mM KCl, 0.4mM $MgSO_4$, 0.6mM $Ca(NO_3)_2$, 5mM HEPES at pH 7.6) containing 0.05% phenol red (Sigma). Approximately 1nl of zGRβ mRNA solution was injected into the yolk of 1-2 cell stage fertilized eggs.

Glucocorticoid treatment of zGRβ mRNA injected embryos

Twenty-eight hours post fertilization embryos injected with zGRβ mRNA were dechorionated and placed in 2% agarose-coated Petri dishes, where they were incubated with 100µM dexamethasone (Sigma) in E3 medium for 6h at 28°C. Samples were collected in TRIzol® reagent (Invitrogen) and total RNA was isolated according to the manufacturer's instructions (Invitrogen).

Generation of the UAS:GFP-zGRβ expression vector

The zGRβ coding sequence was amplified out of the pCS2+zGRβ plasmid [51] using the CCCTCGAGCACCATGTACCCATACGATGTTCCAGATTATGCTATGGATCAAGG-AGGCTGGA forward and CCGGATCCTCAGTTTGAAAACTGCCTTTGAA reverse primer. This primer pair also introduced the Xho and BamH1 restriction sites flanking the 5' and 3' zGRβ ORF, respectively, together with an HA tag upstream of zGRβ. The resulting amplicon was, then cloned into a pME-MCS vector ([59], provided by Dr. K. Kawakami (National Institute of Genetics, Japan). The HA-zGRβ-ME-MCS was further used as template for PCR amplification using the TCAGAATTCACCATGCTATGGATC-AAGGAGGACTGG forward and CTAAAGGGAACAAAAGCTGGAGCTC reverse primer. The forward primer omitted the HA tag, while introducing an EcoRI site at the 5' end of the zGRβ sequence, whereas the reverse primer was designed downstream of a

unique NotI restriction site at the 3' end of the zGRβ sequence. The resulting amplicon was cloned into the 14xUAS E1b Tol2 transposon-based vector (provided by Dr. H. Baier, University of California, San Francisco, USA). Furthermore, the destabilized green fluorescent protein (d1GFP) sequence was amplified from pd1EGFP-N1 vector (purchased from Clontech) using the TCAGAATTCATGGTGAGCAAGGGCGAGG forward and TCAGAATTCCGTTGTACAGCTCGTCCATGCC reverse primer, introducing EcoRI restriction sites at both ends of the ORF of d1GFP as well as a deletion of the stop codon at the 3' end. The resulting amplicon was cloned into the 14xUAS:zGRβ construct, upstream of and in frame with the zGRβ coding sequence.

Generation of the Tg(UAS:GFP-zGRβ) transgenic line

The plasmid pCS2FA-transposase (provided by Dr. K. Kawakami) was linearized with NotI (New England Biolabs) and further used as a template for the mRNA synthesis using the mMESSAGE mMACHINE SP6 kit from Ambion [60]. Newly synthesized mRNA was purified using the RNeasy MinElute^{TM} Cleanup kit from Qiagen and its integrity was checked on a 1.5% agaroze electrophoresis gel.

The UAS:GFP-zGRβ construct and the Tol2 transposase mRNA were diluted at a final concentration of 30 and 50ng/μl respectively, in 1x Danieu's buffer (58mM NaCl, 0.7mM KCl, 0.4mM $MgSO_4$, 0.6mM $Ca(NO_3)_2$, 5mM HEPES at pH 7.6) containing 0.05% phenol red [60]. 1nl of mixed UAS:GFP-zGRβ DNA and transposase mRNA solution was microinjected at the single-cell stage eggs. Positive F0 founders were outcrossed again with wild type fish and their offspring were raised to adulthood. F1 adult fish were genotyped and positive F1 fish were subsequently used in crossings with the *Tg*(*hsp70*:*Gal4)* line [61].

Heat shock and glucocorticoid treatment of transgenic embryos

The *Tg*(*hsp70*:*Gal4)* and *Tg(UAS:GFP-zGRβ)* lines were crossed and their offspring were heat-shocked at 1dpf in Petri dishes filled with pre-warmed (37°C) E3 egg water medium in a 37°C incubator for 2.5h. The next day, embryos were screened for GFP expression and wild types as well as their GFP expressing siblings were subjected again to the heat shock protocol. Three day old hatched wild types and their GFP expressing siblings were checked again for GFP signal and treated with either vehicle (<2% DMSO) or 25μM beclomethasone for 6h. Samples were collected in TRIzol® reagent (Invitrogen) for subsequent RNA isolation.

Microarray design

A 4x180k microarray chip platform (customized by Agilent Technologies, (Design ID:028233)) was used in this study. This array consists of all probes already present in an earlier 45.219 custom-made array [62], and another 126.632 newly designed zebrafish probes had been added as described in [63]. A total of 16 samples (4 experimental groups from 4 replicate experiments) were processed for transcriptome analysis. On each 4x180k slide, 4 samples from the different experimental groups within the same replicate were hybridized against a common reference.

Microarray amplification & labeling

Amplification and labeling of RNA was performed at the MicroArray Department (MAD) of the University of Amsterdam (Amsterdam, The Netherlands). Per sample, 0.5μg total RNA was amplified and combined with Spike A according to the Agilent Two-Color Microarray-Based Gene Expression Analysis kit (Agilent technologies). As a common

reference sample an equimolar pool of all test samples was made and 0.5µg samples were amplified similarly as the test samples with the exception that Spike B was used. Amino-allyl modified nucleotides were incorporated during the aRNA synthesis (2.5mM of each GTP, ATP, UTP (GE Healthcare), 0.75mM CTP (GE Healthcare), 0.3mM AA-CTP (TriLink Biotechnologies)). Synthesized aRNA was purified with the E.Z.N.A. MicroElute RNA Clean Up Kit (Omega Bio-Tek). The quality was inspected on the BioAnalyzer (Agilent Technologies) with the Agilent RNA 6000 kit (Agilent Technologies). Test samples were labeled with Cy3 and the reference sample was labeled with Cy5. Five µg of aRNA was dried out and dissolved in 50mM carbonate buffer pH 8.5. Individual vials of Cy3/Cy5 from the mono-reactive dye packs (GE Healthcare) were dissolved in 200µl DMSO. To each sample, 10µl of the appropriate CyDye dissolved in DMSO was added and the mixture was incubated for 1h. Reactions were quenched with the addition of 5µl 4M hydroxylamine (Sigma-Aldrich). The labeled aRNA was purified with the E.Z.N.A. MicroElute RNA Clean Up Kit. Yields of aRNA and CyDye incorporation were measured on the NanoDrop ND-1000.

Microarray hybridization, scanning & data processing

Each hybridization mixture was made up from 825ng Test (Cy3-labeled) and 825ng Reference (Cy5-labeled) material. Hybridization mixtures were using the Agilent Two-Color Microarray-Based Gene Expression Analysis kit according to the manufacturer's instructions (Agilent technologies). The samples were loaded onto the microarray chips and hybridized for 17h at 65°C. Afterwards the slides were washed and scanned (20 bit, 3µm resolution) in an ozone-free room with the Agilent G2505C scanner. Data was extracted with Feature Extraction (v10.7.3.1, Agilent Technologies) with the GE2_107_Sep09 protocol for two-color Agilent microarrays. The Agilent output from the 16 hybridizations was then imported into the Rosetta Resolver 7.2 software (Rosetta Biosoftware, Seattle, Washington) and subjected to a factorial design with a re-ratio with common reference application. Data analysis was performed setting cutoff for the p-value of $<10^{-5}$ and for fold change of either >2 or <-2.

Statistical analysis

Statistical analyses (t-tests and two-way ANOVAs with Bonferroni post-hoc tests) were performed using the GraphPad Prism version 4.00 (GraphPad Software, La Jolla, USA).

Results

Dominant-negative activity of zGRβ in luciferase assays in COS-1 cells

Previously, we have demonstrated that zGRβ has a dominant-negative effect on zGRα-mediated transactivation of an MMTV-luciferase construct in COS-1 cells [51]. In the present study, we aimed at studying this effect on a broader range of zGRα/zGRβ ratios and exploring whether zGRβ has a dominant-negative effect on zGRα-mediated transrepression as well. For the studies on transactivation, COS-1 cells were transiently transfected with an MMTV-luciferase reporter construct and a range of zGRα expression vector amounts in the presence and absence of 100ng zGRβ plasmid. Upon transfection, cells were incubated with 100nM dexamethasone (dex) for 24h and assayed for luciferase activity. The results show that, as expected, the luciferase signal was increased upon increasing concentrations of zGRα (Fig. 1A). In the presence of zGRβ, the luciferase activity was strongly reduced, even when equal amounts of the zGRα and zGRβ

expression vector were transfected. These data demonstrate that zGRβ exhibits a dominant-negative effect on the transactivation properties of zGRα in these reporter assays.

In order to study the effect of zGRβ on the transrepression activity of zGRα, COS-1 cells were transiently transfected with a κB-luciferase construct, an expression vector for the human p65 subunit of the NF-κB transcription factor complex and a range of zGRα expression vector amounts in the presence and absence of 100ng zGRβ plasmid. Upon transfection, cells were incubated with 100nM dex for 24h and assayed for luciferase activity. The results, shown in Fig. 1B, reveal that with increasing concentrations of zGRα, NF-κB-mediated luciferase activation was dramatically repressed, reflecting the transrepression activity of zGRα. In these experiments, the presence of zGRβ did not affect the observed luciferase activity levels, indicating that zGRβ does not have a dominant-negative inhibitory role on the transrepression activity of zGRα.

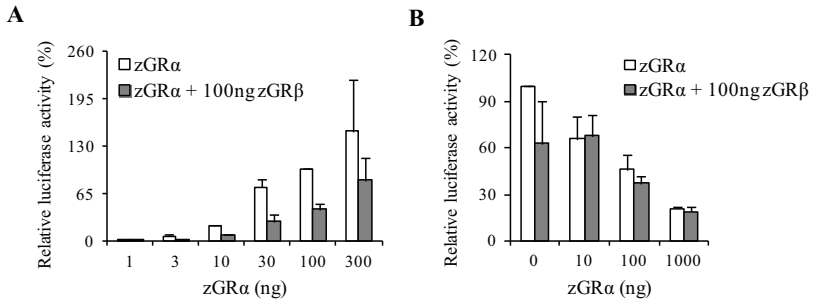

Figure 1. A. Dominant-negative activity of zGRβ on transactivation analyzed by luciferase assays. COS-1 cells were transiently transfected with an MMTV-luciferase reporter construct and a range of zGRα expression vector amounts in absence (white bars) and presence (grey bars) of 100ng zGRβ, and incubated with 100nM dex for 24h. The relative luciferase activity values (setting the 100ng zGRα transfected group value as 100%) shown are the means ± s.e.m. of three independent experiments. Statistical analysis by ANOVA revealed an effect of zGRβ expression ($p=0.001$). **B.** Lack of dominant-negative activity of zGRβ on transrepression analyzed by luciferase assays. COS-1 cells were transiently transfected with a κB-luciferase construct, an expression vector for the human p65 subunit of the NF-κB transcription factor complex and a range of zGRα expression vector amounts in the absence (white bars) and presence (grey bars) of 100ng zGRβ plasmid, and incubated with 100nM dex for 24h. The relative luciferase activity values (setting the 0ng zGRα transfected group value as 100%) shown are the means ± s.e.m. of three independent experiments. Statistical analysis by ANOVA demonstrated no significant effect of zGRβ expression.

Dominant-negative activity of zGRβ in PAC2 cells

Transiently transfected reporter constructs contain simple promoters and are not embedded into chromatin structure. In order to study the effect of zGRβ on endogenous promoters *in vitro*, we generated a PAC2 cell line that stably overexpresses the zebrafish GR β-isoform (PAC2-zGRβ). In PAC2-zGRβ cells, the zGRβ mRNA expression level was 260 fold higher than in wild type PAC2 cells (PAC2-wt), whereas the expression of zGRα was not different between these two cell lines (Fig. 2A and B). That renders zGRβ as the predominant GR splice variant in the PAC2-zGRβ line, with a 10-fold higher absolute

mRNA abundance than that of zGRα (Suppl. Fig. 1). Treatment with GCs did not significantly alter the expression of either splice variant (Fig. 2A and B).

The zGRα signaling in those two cell lines was evaluated upon incubation with 10μM betamethasone-17-valerate (BV) for 3h. Hence, we investigated the expression profile of a well-known endogenous GR-target gene, *fkbp5* [64], by means of qPCR. Upon BV treatment, a more than 10-fold induction of *fkbp5* was observed in both PAC2-wt and PAC2-zGRβ cells, and that was not statistically different between the two lines examined (Fig. 2C). Thus, zGRβ does not exhibit any dominant-negative activity on zGRα-induced transactivation of the *fkbp5* gene in PAC2 cells. Several other known GR target genes (*gilz, slc5α1, agtxb, hsd11b2, pepck, nfkbiαα*) were tested for induction upon BV treatment in PAC2 cells. None of these genes showed increased mRNA expression upon treatment, leaving *fkbp5* as the only gene that could be used to study the effect of zGRβ on the transactivation properties of zGRα in these cells.

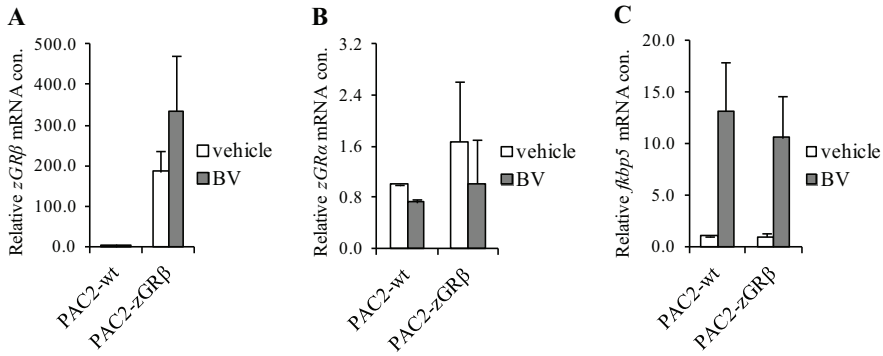

Figure 2. A. Stable overexpression of the zebrafish GR β-isoform in PAC2 cells. Cells were stably transfected with a zGRβ expression vector for the generation of the PAC2-zGRβ line. The zGRβ expression level of this cell line was determined by qPCR. The data show that the PAC2-zGRβ cell line highly overexpresses (> 250 fold) the zGR β-isoform compared to its parental wild type line. Stimulation of both lines with 10μM BV for 3h did not significantly affect zGRβ mRNA expression. **B.** Expression levels of zGRα mRNA in PAC2-wt and PAC2-zGRβ cells treated with either 10μM BV or vehicle for 3h. Statistical analysis by ANOVA demonstrated that there is no significant effect on the expression of the zGR α-isoform due to either BV treatment or overexpression of the zGR β-isoform. **C.** Expression levels of *fkbp5* mRNA in PAC2-wt and PAC2-zGRβ cells, determined by qPCR. Cells from both lines were compared for their ability to upregulate the expression of *fkbp5* upon treatment with either 10μM BV or vehicle for 3h. Statistical analysis revealed that *fkbp5* induction due to BV treatment does not differ between the two lines examined. In all figures mRNA expression measurements were normalized to those of *bactin1* and the relative mRNA concentration values are the means ± s.e.m. of three independent experiments.

Injections of zGRβ mRNA in zebrafish embryos

In order to assess the zGRβ effect on transactivation *in vivo*, we injected 1-2 cell stage zebrafish embryos with 5pg zGRβ mRNA. The next day we treated them with 100μM dex for 6h, and total RNA was isolated from the embryos and processed for qPCR analysis of three GRα target genes: *fkbp5*, *pepck* and *nfkbiaa*. Dex treatment of control embryos resulted in approximately 100 (± 10.6 s.e.m.), 3.5 (± 0.9 s.e.m.) and 1.6 (± 0.25 s.e.m.) fold induction of *fkbp5*, *pepck* and *nfkbiaa* mRNA, respectively (Fig. 3A, B and C). zGRβ-injected embryos treated with dex showed similar induction levels of these genes (Fig. 3A, B and C). These results confirm *in vivo* the lack of a dominant-negative zGRβ effect on

fkbp5 as already observed in PAC2 cells, and additionally demonstrate no dominant-negative effect of zGRβ on the induction of *pepck* and *nfkbiaa* gene expression by zGRα.

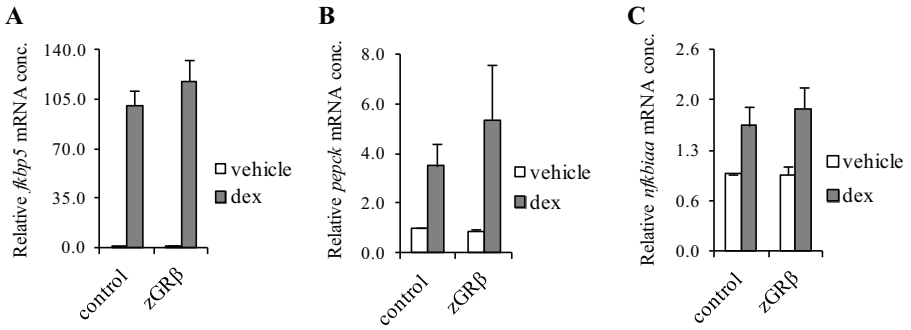

Figure 3. Expression levels of the GR target genes *fkbp5* (A)*, pepck* (B)*,* and *nfkbiaa* (C) in zGRβ mRNA injected embryos, determined by qPCR. Embryos were injected at the 1-2 cell stage with 5pg zGRβ mRNA. At 28 hours post fertilization, they were treated with 100μM dex for 6h. Expression levels were normalized to those of *bactin1* and the relative mRNA concentration values shown are the means ± s.e.m. of three independent experiments. Statistical analysis by ANOVA demonstrated that *fkbp5, pepck,* and *nfkbiaa* induction upon dex treatment were not affected by zGRβ mRNA injection. These data show that zGRβ does not exhibit any dominant-negative effect on the zGRα-induced transactivation of any of the genes tested in 1 day post fertilization zebrafish embryos.

Characterization of a Tg(UAS:GFP-zGRβ) transgenic line

Subsequently a fish line was generated which stably overexpressed zGRβ, using the Gal4/UAS expression system. We generated a *Tg(UAS:GFP-zGRβ)* line, in which the GR β-isoform is fused to a green fluorescent protein (GFP) at its N-terminus and is downstream of a 14xUAS promoter that responds to the Gal4 transcription factor. This line was crossed with the *Tg*(*hsp70*:*Gal4)* line (in which Gal4 expression is controlled by a heat shock-inducible promoter), yielding embryos in which the zGRβ expression was induced upon a heat shock. Embryos at 1 and 2 day post fertilization (dpf) were heat shocked and the GFP signal was readily detectable within a few hours, reflecting expression of the GFP-tagged zGR β-isoform. Expression was clearly present in the muscles, yolk and eyes of 3dpf larvae (Fig. 4B). Further magnification suggested that GFP-zGRβ is localized in the nuclei of the cells (Fig. 4C), which was confirmed by co-staining with Sytox Orange (data not shown). This observation is in line with previous data showing that a YFP-tagged zGRβ expressed in COS-1 cells resulted in a more nuclear localization compared to YFP-zGRα in the same cell type [51].

In order to confirm that the fusion protein was properly expressed, we performed a western blot analysis on samples from fluorescent and non-fluorescent embryos using an anti-GFP antibody. We could identify a specific band at the expected size (approximately 108kDa) which was lacking from control embryos (data not shown). In addition, using an expression vector for YFP-zGRβ we showed that the YFP tag does not affect zGRβ's dominant-negative activity in COS-1 cells, indicating that this type of tag does not interfere with the dominant-negative activity of the zGR β-isoform (Suppl. Fig. 2).

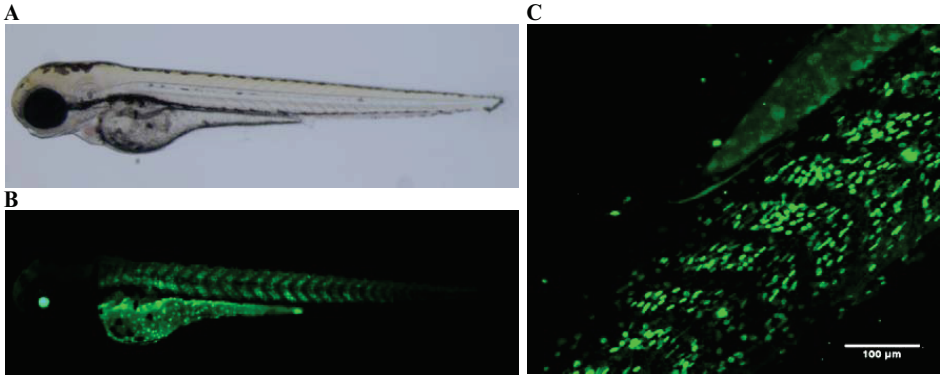

Figure 4. Images of a 3dpf zebrafish embryo generated by crossing the *Tg*(*hsp70*:*Gal4)* and *Tg(UAS:GFP-zGRβ)* transgenic lines. Embryos were heat shocked at 37°C for 2.5h at 1 and 2dpf. **A.** Transmitted light microscopy image showing representative embryo, indicating that transgenesis and heat shock treatment do not alter the morphology of zebrafish embryos. **B.** Fluorescence microscopy image showing expression of the GFP-zGRβ fusion protein. The green fluorescent signal is clearly present in the muscles, on the yolk sac and in the eyes. **C.** Larger magnification fluorescence microscopy image showing expression of GFP-zGRβ in muscle tissue. The image shows that GFP-zGRβ is localized in the nuclear cell compartment.

Microarray analysis of zGRβ transcriptional activity

Next it was explored at the whole transcriptome level whether zGRβ plays a role either as a dominant-negative inhibitor of the zGR α-isoform or as an intrinsic transcription factor (i.e. independent of zGRα), using a custom-designed Agilent 180k zebrafish microarray platform [63]. The *Tg*(*hsp70*:*Gal4)* line was crossed with the *Tg(UAS:GFP-zGRβ)* line. The resulting embryos were identified, upon heat shock, based on the observed fluorescence as either wild type (WT, lacking fluorescence) or zGRβ-overexpressing (zGRβ, showing green fluorescence). Both populations were treated with either 25µM beclomethasone (beclo) or vehicle for 6h at 3dpf. Thus, 4 groups were generated: WT/vehicle, WT/beclo, zGRβ/vehicle, zGRβ/beclo. Total RNA samples from 4 biological replicates (16 samples in total) were processed for the microarray study using a common reference design. Data were analyzed using Rosetta Resolver 7.2 software, setting signatures for significantly regulated probes at a p-value cutoff of $p<10^{-5}$ and fold changes either >2 or <-2. Figure 5 gives a schematic overview of different microarray group comparisons that were performed to analyze beclo- and zGRβ-mediated effects on transcription.

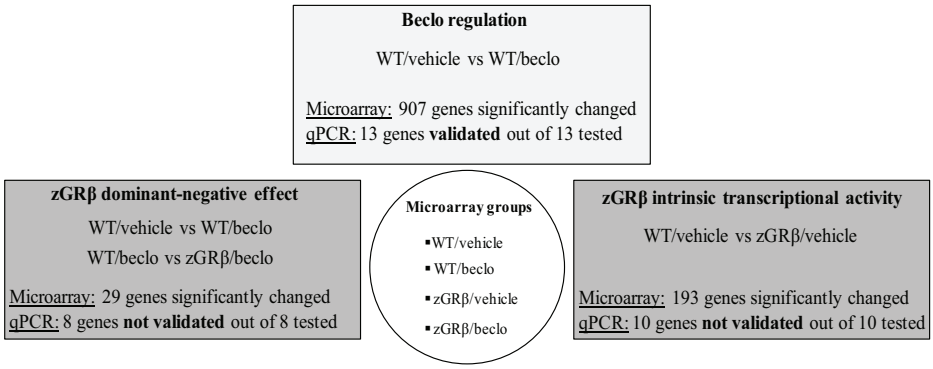

Figure 5. Schematic overview of microarray analysis and subsequent qPCR validation of beclo and zGRβ effects using a 180k zebrafish microarray platform. The *Tg*(*hsp70*:*Gal4)* line was crossed with the *Tg(UAS:GFP-zGRβ)* line and the resulting embryos were heat shocked at 37°C for 2.5h at 1 and 2dpf. At 3dpf, embryos were sorted based on their GFP signal and treated with 25µM beclo for 6h. Thus, 4 groups were generated: WT/vehicle, WT/beclo, zGRβ/vehicle, zGRβ/beclo. Total RNA samples were processed for transcriptome analysis by microarray. Results for beclo- as well as zGRβ-mediated effects on transcription were analyzed using Rosetta Resolver 7.2 software. Numbers of genes refer to annotated genes found to be significantly regulated at a p-value cutoff of $p<10^{-5}$ and fold changes either >2 or <-2. Beclo regulation of genes is defined as significant up- or down-regulation based on the WT/beclo versus WT/vehicle group comparison. The dominant-negative activity of zGRβ is defined as a significant up- (or down-) regulation by beclo, and a decrease (or increase) in the GRβ/beclo group as compared to the WT/beclo group (see also Fig. 7). The intrinsic transcriptional activity of zGRβ is defined as significant up- or down-regulation based on the WT/vehicle versus zGRβ/vehicle group comparison. Annotation and probe assignment was based on the Ensemble Gene ID codes (ENSDARG).

As a first step, we studied genes regulated upon beclo treatment in the wild type fish (WT/vehicle vs WT/beclo comparison). We identified 1805 probes corresponding to genes significantly upregulated by beclo treatment and 703 probes corresponding to downregulated ones. Of these 2508 total probes, 1592 could be attributed to an annotated gene (in total, 907 beclo-regulated genes). For 13 randomly chosen genes (10 up-regulated, 3 down-regulated, Suppl. Table 1) which were represented in this cluster by at least two probes (enabling less false-positive discoveries), validation by qPCR was performed, and a significant effect of beclo treatment was confirmed for all genes tested (Fig. 6).

We, then, took the latter subset of probes representing beclo-regulated genes and compared the expression of these genes between the WT/beclo and the zGRβ/beclo group, in order to examine a dominant-negative effect of zGRβ. For 30 of the 1805 probes corresponding to beclo-upregulated genes, a downregulation was observed upon overexpression of zGRβ (in the presence of beclo), indicating a dominant-negative effect of zGRβ. In addition, for 35 of the 703 probes corresponding to genes downregulated by beclo treatment, an upregulation was observed upon overexpression of zGRβ (in the presence of beclo), also indicating a dominant-negative effect of zGRβ (Fig. 7). Thus, in our microarray experiment, zGRβ appeared to exhibit a significant inhibitory role in zGRα signaling for 65 probes, which accounts for only 2.6% of beclo-regulated ones.

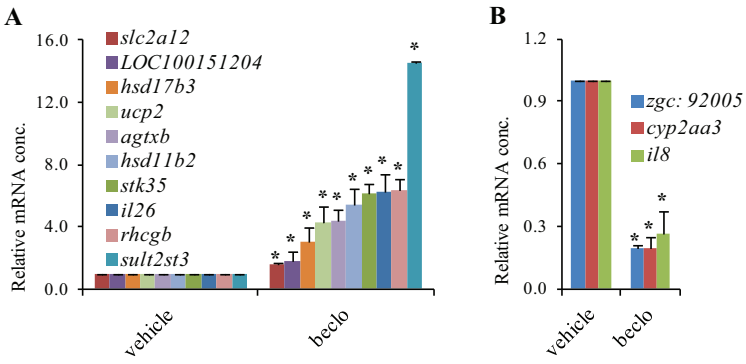

Figure 6. Validation by qPCR of the effect of beclo treatment on the expression of several genes identified as beclo-responsive in the microarray analysis. Expression rates of all genes were normalized to those of *bactin1* and relative mRNA concentration values are the means ± s.e.m. of three independent experiments. **A.** Validation for genes identified as up-regulated by beclo treatment in the microarray study. Statistical analysis demonstrated that for all genes the mRNA expression was significantly higher in the beclo-treated group compared to the vehicle-treated one. **B.** Validation for genes identified as down-regulated by beclo treatment in the microarray study. Statistical analysis demonstrated that for all genes the mRNA expression was significantly lower in the beclo-treated group compared to the vehicle-treated one. Asterisks (*) correspond to a statistically significant difference between the beclo- and vehicle-treated group ($p<0.05$). These data confirm the effects of beclo treatment observed in the microarray analysis.

Within the cluster of 65 probes that indicated a dominant-negative activity of zGRβ, 34 probes could not be attributed to an annotated gene. The other 31 probes could be attributed to 29 genes, since 27 genes were represented by one probe and only two genes (*LOC100151204* and *crybb2*) by two probes (Table 1). This is not a result of these genes being represented by a low number of probes on the array, since only one of these 27 annotated genes is represented by one probe on the 180k array whereas expression of the rest can be detected by multiple probes. *LOC100151204* together with 7 randomly chosen genes from the pool of 29 annotated genes with putative dominant-negative regulation were used for validation of the microarray results by qPCR. In this validation, we could not verify any significant zGRβ dominant-negative effect on any of the genes tested (Suppl. Table 2).

Subsequently, we investigated whether zGRβ has intrinsic transcriptional activity, able to regulate the expression of target genes, independent of zGRα activity. For that reason we compared the WT/vehicle and zGRβ/vehicle groups and we identified 258 probes corresponding to up-regulated transcripts due to the zGRβ overexpression and 223 probes corresponding to down-regulated ones.

Of these 481 regulated probes, 271 could not be attributed to an annotated gene. The other 210 probes corresponded to 193 genes. As expected, the GR gene itself was present among the genes up-regulated in the zGRβ transgenic line, and this up-regulation was detected with 12 probes. In addition, the *hsp47* gene (5 probes) and the *LOC100151204* and *zgc: 111983* genes (2 probes) were represented by more than 1 probe (Table 1). The *hsp47* and *zgc: 111983* genes and eight randomly selected genes were used for validation. Again, qPCR analysis failed to verify any significant zGRβ effect on all genes tested, (Suppl. Table 3).

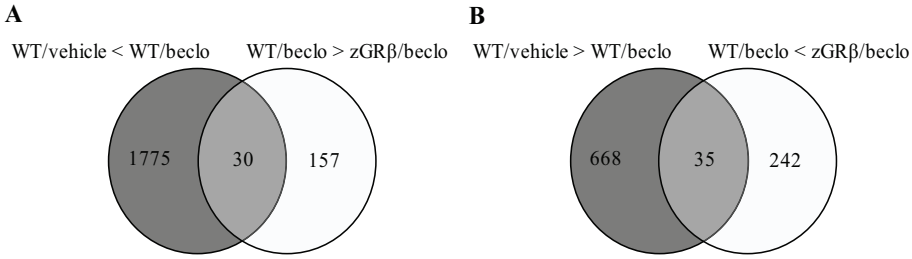

Figure 7. Analysis of the dominant-negative activity of zGRβ observed in the microarray. **A.** Venn diagram depicting the pool of probes corresponding to transcripts upregulated in the WT/beclo group compared to the WT/vehicle group (grey circle, 1805 probes), and the pool of probes corresponding to transcripts downregulated in the zGRβ/beclo compared to the WT/beclo group (white circle, 187 probes). The overlap between these pools (30 probes) represents transcripts which were significantly upregulated upon beclo treatment, reflecting a possible dominant-negative effect of zGRβ on transactivation properties of zGRα. **B.** Venn diagram depicting the pool of probes corresponding to transcripts downregulated in the WT/beclo group compared to the WT/vehicle group (grey circle, 703 probes), and the pool of probes corresponding to transcripts downregulated in the zGRβ/beclo compared to the WT/beclo group (white circle, 277 probes). The overlap between these pools (35 probes) represents transcripts which were significantly downregulated upon beclo treatment, reflecting a possible dominant-negative effect of zGRβ on the transrepression properties of zGRα.

Differences between the clusters of beclo- and zGRβ-regulated genes

As presented previously, we were not able to verify by qPCR the regulation of genes due to zGRβ overexpression (dominant-negative or intrinsic transcriptional activity), whereas gene regulation due to beclo treatment was confirmed in all cases studied. This prompted us to further investigate differences between the clusters of beclo- and zGRβ-regulated genes.

In our custom-made 180k microarray platform, the annotated (provided by ENSEMBL, www.ensembl.org) genes are represented by either one or multiple probes, with the largest number of them being represented by two probes (Table 1). Our analysis revealed that approximately 33% of the beclo-regulated genes were represented within the cluster of beclo-regulated genes by more than one probe (Table 1), which is a dramatically higher percentage than that found in the subset of genes regulated by GRβ in a dominant-negative way (7%), or as an intrinsic transcription factor (2% , Table 1). Limiting the analysis to genes that show regulation for at least two probes would have left very few zGRβ-regulated genes (6 genes in total, 2 for zGRβ dominant-negative activity and 4 for zGRβ intrinsic transcriptional activity), whereas still a significant number of beclo-regulated genes (302 genes) would have been found in the array. The disadvantage of this analysis is that effects on genes that are represented by only one probe on the array (19% of genes represented on the 180k chip) are overlooked. Second, the p-values corresponding to the (significant) regulation of the individual probes were studied, and for each subset of regulated probes (beclo-regulation, zGRβ dominant-negative effect, zGRβ intrinsic transcriptional activity) the distribution of these p-values was plotted (Fig. 8A). Interestingly, zGRβ-regulated probes (both zGRβ intrinsic transcriptional and dominant-negative activity), mainly show higher p-values, whereas beclo-regulated probes show a more even distribution over a wider range of p-values (Fig. 8A). Obviously, using a lower p-value as significance threshold would decrease the number of false positive results. For example, using a significance threshold of $p=10^{-10}$, zGRβ would show a dominant-negative

effect on only 11 probes (previously, that number was 65) and an intrinsic transcriptional activity on only 105 probes (previously, it was 481). In contrast, beclo treatment would significantly still affect 1809 probes (previously they were up to 2508). Lowering p-values shows that the cluster of beclo-regulated probes is altered much less that those of zGRβ-regulated ones, since 72% of the beclo-affected probes previously regulated at a cutoff of $p=10^{-5}$ are still regulated at a cutoff of $p=10^{-10}$. As for zGRβ, the equivalent numbers correspond to less than 17% for its dominant-negative effect and almost 22% for its intrinsic transcriptional activity. However, clusters deriving from a lower p-value could still contain a large number of false positives, since from the pool corresponding to zGRβ dominant-negative effect already two genes have been shown to be false positive hits by qPCR (Suppl. Table 2), and from the pool corresponding to zGRβ intrinsic transcriptional activity four genes have already been shown to be false positives by qPCR (Suppl. Table 3).

	180k array	**Regulation by beclo**	**Regulation by zGRβ**	
			Dominant-negative effect	**Intrinsic transcriptional activity**
Total no. probes	**175975**	**2508**	**65**	**481**
probes without annotation	81534	916	34	271
probes with gene annotation	94441	1592	31	210
Total (annotated) genes	**23837**	**907**	**29**	**193**
genes with 1 probe	4539 (19%)	605 (66.7%)	27 (93%)	189 (98%)
genes with 2 probes	4578 (19.2%)	127 (14%)	2 (7%)	2 (1%)
genes with 3 probes	3920 (16.4%)	78 (8.6%)		
genes with 4 probes	3008 (12.6%)	47 (5.2%)		
genes with 5 probes	2334 (9.8%)	21 (2.3%)		1 (0.5%)
genes with 6 probes	1728 (7.2%)	14 (1.5%)		
genes with 7 probes	1238 (5.2%)	8 (0.9%)		
genes with 8 probes	852 (3.6%)	1 (0.1)		
genes with 9 probes	528 (2.2%)	2 (0.2%)		
genes with 10 probes	330 (1.4%)	4 (0.4%)		
genes with 11 probes	222 (0.9%)			
genes with 12 probes	126 (0.5%)			1 (0.5%)
genes with >12 probes	434 (1.8%)			

Table 1: Overview of microarray results. Shown are the numbers of probes and annotated genes represented on the microarray and the numbers of probes and annotated genes showing significant regulation (p-value cutoff of 10^{-5} and fold changes either >2 or <-2). Annotation of probes was performed based on the Ensembl Gene ID codes (ENSDARG). Percentages correspond to the number of genes represented by a certain number of probes relative to the total number of annotated genes. The analysis shows that within the cluster of (beclo- and zGRβ-) regulated probes the majority of corresponding annotated genes is represented by one probe, despite the fact that most of these can be detected by more than one probe on the array. Furthermore, approximately 33% of beclo-regulated genes are represented by more than one probe, which is dramatically more than that observed for zGRβ-regulated genes (2%).

In conclusion, a more stringent analysis of the microarray data (increasing significance threshold and excluding genes showing regulation for only one probe) dramatically decreases the size of the clusters of zGRβ-regulated genes, whereas it has a much smaller impact on the cluster of beclo-regulated ones. This suggests that the relative number of false-positives among the zGRβ-regulated genes is considerably larger than among the beclo-regulated genes, which was confirmed by our qPCR validation that verified the up- or downregulation of several beclo-regulated genes, but failed to validate any zGRβ effect on several genes observed in the microarray analysis.

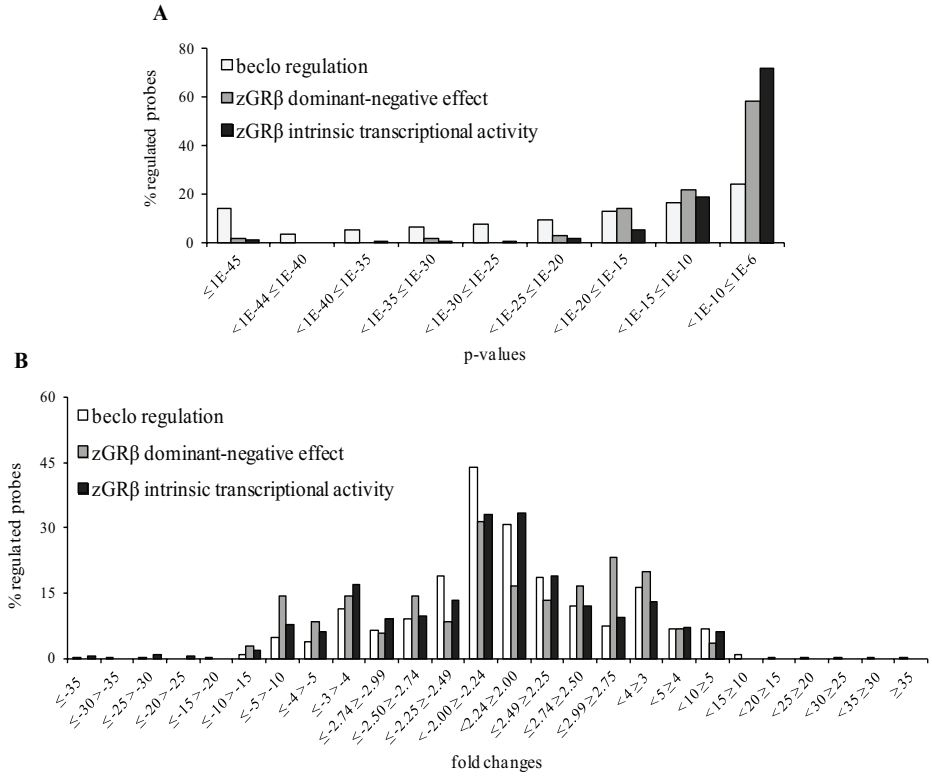

Figure 8. Distribution of significantly regulated probes (cutoff set at p-value $<10^{-5}$ and fold change of either >2 or <-2) from our microarray expression data analysis for beclo as well as zGRβ-mediated effects on transcription. **A.** Percentage (of the total number of regulated probes) of signature probes which fall into a certain range of p-values for beclo regulation (probes corresponding to transcripts up- or down-regulated in the WT/beclo group compared to the WT/vehicle group), zGRβ dominant-negative effect (subset of beclo-regulated probes corresponding to transcripts up- or down-regulated in the zGRβ/beclo group compared to the WT beclo group (see Fig. 6)) and zGRβ intrinsic transcriptional activity (probes corresponding to transcripts up and down-regulated in the WT/vehicle group compared to the zGRβ/vehicle group). For beclo regulation (white bars), there is a substantial probe presence over a wide range of p-values even lower than 10^{-20}. In contrast, for zGRβ-mediated effects (grey and black bars), their significantly regulated probes are mainly in p-values higher that 10^{-20}. **B.** Percentage (based on the total number of up- or down-regulated probes) of signature probes which fall into a certain range of fold changes. The data show that for both beclo and zGRβ-regulated probes, fold changes mainly fall into a 3/-3 range.

Discussion

In the present study we have used the zebrafish as a model organism to investigate the regulatory role of the zebrafish GR β-isoform on gene transcription. We first looked at its dominant-negative effect on both transactivation and transrepression properties of the GR α-isoform. By means of luciferase reporter assays using transient transfections in COS-1 cells we demonstrated that zGRβ strongly inhibits zGRα's transactivation properties even when we transfected equal amounts of zGRα and zGRβ expression vectors. This dominant-negative activity had already been observed in our laboratory using a 1:10 zGRα/zGRβ ratio [51], and recapitulates data obtained in similar reporter assays in which the human [15, 17, 52, 65] and mouse [53] GRβ showed a dominant-negative effect at 1:10 and 1:2 α/β transfection ratio, respectively. In contrast, we did not observe an effect of zGRβ on the transrepression activity of zGRα. These data are in line with most of the literature on the human GR β-isoform, since in most experimental approaches employing reporter assays and transient transfections in various cell lines (e.g. COS-1, A549, Jurkat T-cells) the human GRβ was also reported not to exhibit any dominant-negative activity on hGRα transrepression properties [37, 38, 44, 46], even at a 1:20 α/β transfection ratio in COS-7 cells [43]. In contrast, one study has demonstrated a specific dominant-negative effect on transrepression rather than transactivation using reporter assays [45] and a dominant-negative effect on the dex-induced repression of TNFα and IL6 was demonstrated by siRNA knockdown of hGRβ expression in human monocytes [33].

The reporter constructs used in our luciferase assays have simple promoters and are not embedded into chromatin like the promoters of endogenous gene. Therefore, we next addressed the question whether zGRβ also exhibits dominant-negative properties on the zGRα-induced transactivation of endogenous genes. From studies in human and mouse cell cultures there is a solid body of evidence that GRβ inhibits GRα-induced transactivation of a number of endogenous genes, like MKP-1 in human monocytes [33] and BAL macrophages [23], myocilin and fibronectin in human trabecular meshwork eyes cells [32], CANT1, NEK2, TWSGI1, and LATS2 in Hela cells [40], and GILZ, PDK4, and G6Pase in mouse MEF cells [53].

In the present study, we started our investigation on endogenous genes by first establishing a cell-based system that subsequently could allow us to further characterize the molecular properties of zGRβ. We chose the PAC2 zebrafish cell type which expresses both zebrafish GR isoforms and is easily subjected to transfection protocols. Thus, we generated a PAC2 cell line that stably overexpressed zGRβ (on average ~260 fold higher at the mRNA level compared to wild type). We determined that zGRβ is expressed more than 10 times higher in these cells than zGRα, rendering the zGR β-isoform as the predominant GR receptor at the mRNA level in these cells. The GRβ protein has been shown to be more stable than GRα in humans [66] and zebrafish [51], so it can be assumed that the higher zGRβ expression level compared to zGRα observed in our cells at the mRNA level is translated to the protein level as well.

In this zGRβ-overexpressing cell line we investigated whether zGRβ had a dominant-negative activity on the zGRα-induced transactivation of *fkbp5* (a well-known GRα target gene [67]). Surprisingly, we did not observe any effect of zGRβ overexpression on the induction of *fkbp5* by zGRα. Thus, it appears that zGRβ does not exhibit a general dominant-negative activity on zGRα-induced transactivation. Interestingly, recent studies in mice reported a lack of GRβ overexpression effect on GRα-induced *fkbp5* transcription, whereas a dominant-negative activity on other, mostly metabolism-related genes, was observed [53]. Unfortunately, due to a limited

responsiveness of our PAC2 line to GC treatment, we were not able to assess the zGRβ counteractive effect on other GRα-target genes.

Previously, we had observed that 1dpf zebrafish embryos are responsive to GC treatment, as shown by an upregulation of the expression of the GR target genes *pepck*, *nfkbiaa*, and *fkbp5* [64]. Therefore we further investigated a possible dominant-negative effect of zGRβ *in vivo* by injecting mRNA encoding the zGR β-isoform into 1-2 cell stage embryos. Like in the PAC2 cells, overexpression of the zGR β-isoform did not suppress zGRα-mediated gene induction, as shown on all three genes tested (Fig. 3).

Subsequently, in order to get a genome-wide and unbiased view of the zGRβ transcriptional properties, we performed a transcriptome analysis upon zGRβ overexpression *in vivo*. We generated the *Tg(UAS:GFP-zGRβ)* transgenic line that we crossed with the *Tg*(*hsp70*:*Gal4)* one. To our knowledge, this is the first attempt to identify novel pathways of zGRβ influence within the physiological content of a living organism. Therefore, using our *in vivo* transgenic embryo model combined with a microarray approach, we expected to elucidate the biological significance of the zGR β-isoform. Our results showed an effect of zGRβ overexpression on 481 probes (independent of zGRα activity), and 65 probes displayed a dominant-negative effect of zGRβ, out of a total of 180,000 probes present on the array. However, after careful analysis of the microarray data we concluded that at least the vast majority of these data represent false positive results. The distributions of p-values and number of probes per gene showing regulation within the subsets of zGRβ-regulated genes (either independent of zGRα or dominant-negative) was remarkably different from those found for the cluster of probes showing regulation by beclo treatment (Fig. 8A and Table 1), raising questions about the confidence level of the results on zGRβ regulation. Indeed, using qPCR of a randomly selected set of genes showing regulation by zGRβ in the microarray we could not verify the effect of zGRβ on any of the selected genes. Using qPCR on genes showing regulation by beclo could verify the results from the microarray on all selected genes, supporting the validity of our study. These results led us to suggest that the effects of zGRβ on transcription in our transgenic embryo model are absent. Combined with the negative results obtained in PAC2 cells and upon zGRβ mRNA injection in embryos, these data suggest that, if existing, any effect of zGRβ on transcription is probably limited to a very small subset of genes or highly specific for a certain tissue or condition, and is obscured in our experimental systems.

The lack of an effect of the GR β-isoform in zebrafish presented in this study follows the trend of controversy over the significance of the GR β-isoform in humans. After more than 15 years of research, a consistent view on a possible dominant-negative activity on either transactivation or transrepression is still lacking. The observations on the intrinsic transcription factor activity of hGRβ [39, 40] have recently added more inconsistent data, since a large set of genes appeared to be oppositely regulated by hGRβ in different studies [14]. Our work on the functional role of zGRβ does not support a physiologically significant role for the GR β-isoform, and we conclude that the effect of this type of GR splice variant on transcription is anything but universal or generic. Nevertheless, gene-, cell type-, condition-, or species-specific effects can obviously not be ruled out, and these effects may explain the large inconsistencies in the literature on the GR β-isoform.

Finally, C-terminal splice variants of nuclear receptors are widespread [68], and most of these splice variants have been shown to display dominant-negative activity on the transactivational properties of their canonical equivalent in reporter assays. However, the presented studies on the zGR β-isoform demonstrate that dominant-negative activity observed in reporter assays is not necessarily followed by a similar effect *in vivo*. We

suggest that splicing of a pre-mRNA into a messenger encoding an alternative splice variant could instead be a physiological way to downregulate the levels of the canonical receptor variant. In such a case, the GR β-isoform does not have an active inhibitory role but its expression would result in less GRα available to mediate glucocorticoid signaling.

Acknowledgements

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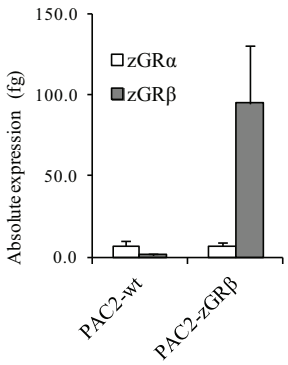
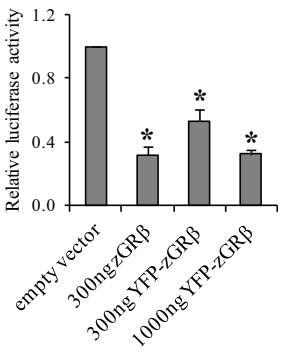
Supplemental data



Supplemental Figure 1. In order to measure the absolute mRNA concentration for zGRα and zGRβ, 500ng of total RNA samples from both PAC2-wt and PAC2-zGRβ cells were analyzed by qPCR for zGRα and zGRβ mRNA expression. Absolute amounts (± s.e.m.) of both transcripts were calculated based on standard curves. Analysis showed that in PAC2-wt cells, the average amount of zGRα is 7.08 ± 2.55fg per 500ng total RNA and 6.95 ± 2.63fg in PAC2-zGRβ. As for zGRβ, its mRNA concentration in PAC2-wt cells is 1.35 ± 0.34fg, per 500ng total RNA whereas in PAC2-zGRβ, it is dramatically higher: 95.05 ± 35.85fg. These results clearly demonstrate that in PAC2-wt cells, there is a more than 5-fold excess of zGRα mRNA as compared to zGRβ mRNA. In stark contrast, in PAC2-zGRβ cells, zGRβ mRNA becomes the dominant receptor isoform transcript, since it is expressed almost 14 times higher than zGRα mRNA.



Supplemental Figure 2. Dominant-negative activity of tagged and non-tagged zGRβ on transactivation analyzed by luciferase assays. COS-1 cells were transiently transfected with MMTV-luciferase reporter gene and zGRα plasmid together with either empty vector, 300ng zGRβ, 300ng or 1000ng YFP-zGRβ expression vectors and incubated with 100nM dex for 24h. Relative luciferase activity values are the means ± s.e.m. of three independent experiments. The analysis shows that in the presence of both tagged and non-tagged zGRβ, zGRα-mediated activation of luciferase gene is significantly suppressed. These data demonstrate that the YFP tag does not impair the dominant-negative activity of zGRβ on zGRα-induced transactivation in *in vitro* luciferase reporter assays. Asterisks (*) correspond to a statistical significance of $p < 0.001$ as calculated by a Bonferroni's multiple comparison test between empty vector group and each other one present in the upper graph.

Gene name	Gene symbol	Gene ID	Probes regulated by beclo	Probes/gene on the array	Fold change (microarray)	P-value (microarray)	Fold change (qPCR validation)	P-value (qPCR validation)
Genes upregulated by beclo								
sulfotransferase family 2 cytosolic sulfotransferase 3	*sult2st3*	777792	5	7	16.27 15.68 15.33 13.52 11.62	<1E-45 <1E-45 <1E-45 <1E-45 1.17E-9	14.53	<0.05
LOC100151204 cytochrome P450 2F2-like	*LOC100151204*	*100151204*	2	3	2.90 2.71	9.22E-19 <1E-45	1.80	<0.05
interleukin 26	*il26*	553971	4	10	8.49 7.67 5.92 4.07	7.6E-27 9.48E-22 6.72E-11 1.15E-6	6.24	<0.05
serine/threonine kinase 35	*stk35*	568080	5	6	5.40 5.31 4.95 4.91 4.76	1.9E-20 5.19E-38 4.96E-32 1.63E-16 <1E-45	6.17	<0.05
uncoupling protein 2	*ucp2*	555812	10	12	4.20 3.95 3.90 3.82 3.81 3.66 3.63 3.59 3.52 2.60	2.56E-33 8.36E-25 2.2E-31 1.51E-30 2.39E-26 4.67E-25 2.17E-27 2.72E-19 <1E-45 2.44E-7	4.29	<0.05
hydroxysteroid 11-beta dehydrogenase 2	*hsd11b2*	334098	6	6	3.83 3.80 5.72 4.99	<1E-45 4.23E-11 <1E-45 <1E-45	5.37	<0.05

					4.96	<1E-45		
					4.95	<1E-45		
rhesus blood group C glycoprotein b	*rhcgb*	792274	5	8	3.96	3.6E-37	6.30	<0.05
					3.8	3.54E-31		
					3.36	<1E-45		
					2.32	1.55E-16		
					4.18	<1E-45		
solute carrier family 2 (facilitated glucose transporter) member 12	*slc2a12*	393510	4	6	3.3	6.12E-21	1.63	<0.05
					2.47	<1E-45		
					2.3	<1E-45		
					2.15	1.22E-15		
hydroxysteroid (17-beta) dehydrogenase 3	*hsd17b3*	393335	5	7	2.81	<1E-45	3.06	<0.05
					2.74	1.71E-36		
					2.66	3.64E-44		
					2.43	1.03E-36		
					2.38	<1E-45		
alanine-glyoxylate aminotransferase b	*agxtb*	79378	5	6	5.64	<1E-45	4.42	<0.05
					5.51	<1E-45		
					5.42	<1E-45		
					4.21	4.12E-36		
					6.50	<1E-45		
Genes downregulated by beclo								
zgc:92005	*zgc:92005*	445477	3	6	-5.47	<1E-45	-5.26	<0.05
					-5.83	<1E-45		
					-11.79	<1E-45		
cytochrome P450 family 2 subfamily AA polypeptide 3	*cyp2aa3*	386638	2	3	-2.94	1.14E-29	-5.26	<0.05
					-3.06	<1E-45		
interleukin 8	*il8*	100002946	6	6	-2.93	2.81E-14	-3.70	<0.05
					-2.94	2.07E-25		
					-3.19	3.28E-23		
					-4.47	9.69E-24		
					-2.06	6.39E-29		
					-2.71	5.86E-10		

Supplemental Table 1: Details of 13 genes found by microarray analysis to be significantly (p-value $<10^{-5}$ and a fold change of either > 2 or <-2) regulated by beclo treatment and subsequently subjected to qPCR validation (Fig. 5). Fold changes refer to beclo-mediated fold induction or suppression per probe based on WT/vehicle vs WT/beclo comparison for both microarray and qPCR analysis.

Gene name	Gene symbol	Gene ID	Probes regulated by beclo	Probes/gene on the array	Fold change (microarray)	P-value (microarray)	Fold change (qPCR validation)	P-value (qPCR validation)
Genes upregulated by beclo								
LOC100151204 cytochrome P450 2F2-like	*LOC100151204*	100151204	2	4	**2.90** / *-3.97* **2.71** / *-3.56*	**9.22E-19** / *1.4E-12* **<1E-45** / *4.6E-14*	**1.8** / *2.9*	**<0.05** / *>0.05*
FGFR1 oncogene partner 2	*fgfr1op2*	335511	1	5	**3.86** / *-5.56*	**3.93E-8** / *3.69E-12*	**-1.15** / *-1.11*	**>0.05** / *>0.05*
novel immune-type receptor 2e	*Nitr2e*	368526	1	3	**3.33** / *-2.52*	**3.35E-8** / *4.43E-6*	**-1.05** / *-1.25*	**>0.05** / *>0.05*
zinc finger, CCHC domain containing 9	*zcchc9*	386639	1	3	**3.11** / *-3.59*	**9.27E-6** / *1.92E-6*	**-1.01** / *1.05*	**>0.05** / *>0.05*
si:dkey-14d8.21	*si:dkey-14d8.21*	558317	6	14	**3.15** / *-2.03*	**1.37E-21** / *7.99E-7*	**1.62** / *1.56*	**>0.05** / *>0.05*
zgc:165670	*zgc:165670*	100073345	2	3	**2.98** / *-2.03*	**1.6E-31** / *2.48E-13*	**1.82** / *2.56*	**<0.05** / *>0.05*
Genes downregulated by beclo								
chemokine (C-C motif) receptor 12.3	*ccr12.3*	563803	1	1	**-6.45** / *8.69*	**6.8E-10** / *1.9E-12*	**-1.35** / *-1.11*	**>0.05** / *>0.05*
protocadherin 2 gamma 5	*pcdh2g5*	554053	1	3	**-2.55** / *4.45*	**4.7E-6** / *1.3E-10*	**1.10** / *-1.05*	**>0.05** / *>0.05*

Supplemental Table 2: Details of 8 genes found by microarray analysis to be significantly (p-value $<10^{-5}$ and a fold change of either >2 or <-2) regulated by a dominant negative zGRβ effect and subsequently subjected to qPCR validation. Numbers in **bold** refer to beclo-mediated fold change per probe based on WT/vehicle vs WT/beclo comparison for both microarray and qPCR. Numbers in *italics* refer to zGRβ-mediated fold change per probe based on WT/beclo vs zGRβ/beclo comparison for both microarray and qPCR analysis.

Gene name	Gene symbol	Gene ID	Probes regulated by zGRβ	probes/gene on the array	Fold change (microarray)	P-value (microarray)	Fold change (qPCR validation)	P-value (qPCR validation)
acyl-CoA thioesterase 11a	*Acot11a*	554842	1	7	9.02	3.6E-8	1.12	>0.05
G protein-activated inward rectifier potassium channel 1-like	*LOC557095*	557095	1	5	5.52	1E-13	1.12	>0.05
Zinc finger, AN1-type domain 1	*zfand1*	445257	1	3	-4.95	3.7E-6	-1.01	>0.05
Cytochrome P450, family 17, subfamily A, polypeptide 1	*cyp17a1*	399692	1	6	-29.94	<1E-45	-1.19	>0.05
melatonin receptor 1C	*mtnr1c*	30661	1	3	-7.23	5.4E-9	-1.04	>0.05
serine/threonine kinase 33	*stk33*	565828	1	6	-4.15	5.1E-6	-1.18	>0.05
trh1 protein	*trh1*	259250	1	3	-3.26	1.3E-17	-1.01	>0.05
heat shock protein 47	*hsp47*	30449	5	9	-2.75 -2.89 -3.04 -2.96 -2.84	2.2E-6 7.1E-6 7E-6 1.4E-6 3.3E-6	-1.59	>0.05
zgc:111983	*zgc:111983*	550501	2	11	-2.31 -25.43	2.1E-7 <1E-45	-1.2	>0.05
synaptotagmin VIII	*Syt8*	570237	1	4	-3.29	1.1E-7	-1.19	>0.05

Supplemental Table 3: Details of 10 genes found by microarray analysis to be significantly (p-value $<10^{-5}$ and a fold change of either >2 or <-2) regulated by zGRβ and subsequently subjected to qPCR verification. Fold changes refer to zGRβ-mediated fold induction or suppression per probe based on WT/vehicle vs zGRβ/vehicle comparison for both microarray and qPCR analysis.

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Gene name	Gene symbol	Forward primer sequence	Reverse primer sequence
Zinc finger, AN1-type domain 1	*zfand1*	TGGCTGAACTAGACATTGGAAAGC	AACAGCTGCTACACACGAATGGT
acyl-CoA thioesterase 11a	*Acot11a*	GGCTTCCGTGAACACCGTCAAT	CGCAAACTTGCCTTAGGAGTCTG
G protein-activated inward rectifier potassium channel 1-like	*LOC557095*	GCCCCCGAGAAGAAAAGGAA	CGGCCGTTCTTGTCCACAAA
Cytochrome P450, family 17, subfamily A, polypeptide 1	*cyp17a1*	GTTCCACATGGAAGTTTCACCG	GAACCTTCTCCAAACATGCAAAGA
melatonin receptor 1C	*mtnr1c*	GATCTTCACCACCGTGGCTG	TGCATTCCTCAGTTTCTTGTTCC
serine/threonine kinase 33	*stk33*	TAGAGATGATGCGTCAGTTCCG	GTGCAGTCCCTCGCTCGTCT
trh1 protein	*trh1*	CGATGAAAGCGAGCCTGATG	TGCGCTCTTCTTCTTCTCcG
heat shock protein 47	*hsp47*	GGAGTGGGACACAGAGGGAAAC	TCCTCATCTTCTCACTGCcG
zgc:111983	*zgc:111983*	AGCCACAACAGCTTCAACCG	TTGTGGCTCCAGATGCAGTG
synaptotagmin VIII	*Syt8*	TTTCTCTCCGCTATGTCCCG	CTGAAGAGCCTACCTGGTCCATC
Cytochrome P450 2F2-like	*LOC100151204*	GTTGACTGCACTGGTTTTGTTGTG	CGCCGAATTCTCACTTGCAAG
FGFR1 oncogene partner 2	*fgfr1op2*	CCCGTTGCTGCTGATAAGGA	TGGCATCAGCCAGAACCTTCTC
novel immune-type receptor 2e	*Nitr2e*	TCACATTTGGAAACAGCACAGACC	CAGACTCGATGTTCACTCCACCAG
zinc finger, CCHC domain containing 9	*zcchc9*	TAATTCCATCACTCTGGGCCG	CACATGCACGTCCTCATGGTC
si:dkey-14d8.21	*si:dkey-14d8.21*	CTGCAAAGCTCACCACCAGAC	GATATACTGTTGTGCAGTGCcG
zgc:165670	*zgc:165670*	CTGAACGAAGCCTTCCGTGTT	TCCCAATGAGAACAGCACcG
chemokine (C-C motif) receptor 12.3	*ccr12.3*	TGAAGGCAGCATCGTAACCG	TGAATCGAATCACATCGGCTTT
protocadherin 2 gamma 5	*pcdh2g5*	CCTCAGATATTATACCCCTCTCCG	CAGCTTTGGGCACCATCTCG
Bactin1	*Bactin1*	CGAGCAGGAGATGGGAACC	CAACGGAAACGCTCATTGC
FK506 binding protein 5	*fkbp5*	TCTGCCAGCACAAGATTCGTGAGC	GACCCTGCTTATTCTGATCGGAAA
phosphoenolpyruvate carboxykinase 1	*pepck*	CAGGGCGATCTGGCGTCTCT	CTGCTGTCGATGAACTCCcG
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha ALPHA	*nfkbiaa*	CTTGGGCTAAAGTAGTCACcG	GATGGCAAGGTGCAGATACGTG
nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	*zGRbeta*	GATGAACTACGAATGTCTTA	GCAACAGACAGCCAGACAGCTCACT
nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	*zGRa*	AACTGGCAACGGTTCTATCAGCTCA	TTCTGGTGAAAGAGCAGCGG
zgc:92005	*zgc:92005*	AGGAGGGATACGGGAGACcG	CGTCTATCTCACGGTGGATGTG
cytochrome P450, family 2, subfamily AA, polypeptide 3	*cyp2aa3*	CCGTGTGTTAGAGGAAAGCcG	CACTGGCTTGCCTTCATCTTTG
interleukin 8	*il8*	TGTGTTATTGTTTTCCTGGCATTTC	GCGACAGCGTGGATCTACAG
sulfotransferase family 2, cytosolic sulfotransferase 3	*sult2st3*	CTTCGTGGAGTCTTGTGCcG	TACTGACGACACGATCCAAAGC
interleukin 26	*il26*	TAAACCCTTCCATCGGATATTGG	TCATCATAGATTTCCAGGACAcG
serine/threonine kinase 35	*stk35*	CCACAGGGACCTCAAACCAGA	CCTTGATGACAGGATTGCcG
uncoupling protein 2	*ucp2*	CGTTCCTCCTACTGCCACcG	GGTGTCCAGTGGAAAGGTGAAG

hydroxysteroid 11-beta dehydrogenase 2	*hsd11b2*	AACCCCAGGTGCGATACTAcG	AACCTGTCGCTGATGCTGAGTG
rhesus blood group, C glycoprotein b	*rhcgb*	GTGCTTCGTCTGGCAGGTGT	GTGTCTGCCTCCTCGTTATACcG
solute carrier family 2 (facilitated glucose transporter), member 12	*Slc2a12*	ACTATGCAGAGCCGCACACAC	CGTGAAGTTTGCTGTATTACcG
hydroxysteroid (17-beta) dehydrogenase 3	*hsd17b3*	CGCAATGACAGGACATCAGAAG	CTTCTCACAAACTCCTCAGCcG
alanine-glyoxylate aminotransferase b	*agxtb*	TGAAGCATCACCAGATCGAGTTT	GTATCCCATGAGCCCAATCcG

Supplemental Table 4: Sequences of all primers used for qPCR analysis.

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Chapter 5

General discussion & summaries

General discussion

In this thesis, we embarked on a series of experimental settings in order to explore the functional role of the glucocorticoid receptor (GR) pathway. We chose the zebrafish model system that in recent years has emerged as a versatile system for conducting biological research, due to the relative ease of genetic manipulation and handling, its transparency and resemblance of many physiological systems to those of mammals, such as the stress axis and the immune response. Moreover, we opted to use microarray technology to analyze gene expression, in order to explore GR target genes at the whole transcriptome level. This way, we investigated the specificity and dynamics of transcriptional regulation by both the GR α- and β-isoform under different physiological conditions.

The validity of the zebrafish as a model organism for research on glucocorticoid (GC) action has previously been shown. Our work also supports this notion, since zGRα, upon stimulation with synthetic glucocorticoids, affects the transcriptional rate of a large number of genes. Among them, many are known to be GR targets in mammals as well (e.g. *fkbp5, pepck, il8, il1b, mmp9*). Additionally, activation of zGRα leads to immunosuppression, thereby recapitulating the well known anti-inflammatory effects of GC drug treatment.

Our microarray analysis identified many molecular pathways to be affected by zGRα activation such as metabolic, immune, and cell cycle-related signaling routes that could be used as a platform for further investigation of how exactly GCs control physiological systems in a whole organism. In our studies we also determined sets of genes to be upregulated (e.g. *hsd11b2, fkbp5, agtxb, rhcgb*) and downregulated by GCs (e.g. *il8, cyp2aa3, mmp9, mmp13a*), which could constitute marker genes in more elaborate further studies, either in order to elucidate the transcriptional mechanisms of GR action or to perform screenings for novel GC drugs using the zebrafish as a model organism.

In order to model local inflammation in zebrafish larvae at 3dpf (when only the innate immune system is active), we used a tail fin amputation assay. In this assay, it has been shown that within a few hours upon amputation a localized inflammatory response is generated, illustrated by migration of neutrophils and macrophages to the wound site. In our experiments, activation of zGRα led to a significant attenuation of this wound-induced migration of neutrophils, but not macrophages. In addition, large alterations in gene expression were found. Interestingly, it appeared that GC treatment suppressed virtually all amputation-induced gene regulation. Of all genes significantly upregulated by amputation, 84% showed an attenuated upregulation in the presence of GCs. Furthermore, 92% of the genes significantly downregulated by amputation displayed an attenuation of this downregulation after GC treatment. Interestingly, GC treatment did not entirely block the transcriptional response to amputation for the vast majority of genes, but rather resulted in a decreased response. Thus, GC treatment appears to have a very general dampening effect on alterations in gene expression induced by tail fin amputation.

Gene ontology analysis showed that among the pathways that were induced upon amputation we found mainly immune-related signaling routes. GCs, apparently, attenuate all these inflammatory signaling cascades, ruling out specificity of GCs for specific pathways or immune-enhancing effects in this model. It could therefore be argued that the GR inhibits the activity of a ‘master switch’ of immune activation. An important role in this process could be for the AP-1 transcription factor complex, which is known to be an important transcription factor in the induction of the inflammatory response. The amputation-induced induction of 3 subunits of this transcription factor complex (*fos, junb, atf3*) was attenuated by GC treatment, and that finding can be added to the already known

physical interaction of GR and specific AP-1 subunits at the protein level, which also results in inhibition of AP-1 activity.

The overall dampening effect of GC treatment on amputation-induced changes in immune-related gene expression is in contrast with specific effects observed on leukocyte migration, which show a significant suppression in neutrophils, however no effect in macrophages. It could therefore be argued that the observed effects on gene expression do not necessarily reflect the alterations at the protein level or that the amputation-induced migration of leukocytes is mediated at a non-genomic level as well.

In further studies, the modulatory effects of GCs could be further explored using more physiological and cellular readouts (e.g. tissue regeneration, cell proliferation, apoptosis, phagocytosis), also in combination with specific GR knockdown or overexpression in particular tissues or cell types. Additionally, other types of immune challenges could be employed such as systemic bacterial infections as well as assessments at later stages of development when the adaptive immune system is present as well, so that GR action could be studied in more detail.

Using morpholino knockdown of zGRα, it was observed that this receptor regulates the transcription of two distinct subsets of genes. We found that knockdown of zGRα under basal conditions resulted in the alteration of the transcription rate of a different cluster of genes than when zGRα was activated by treatment with the synthetic GC dexamethasone. In general, the first cluster of genes contains mainly genes involved in metabolism, whereas the second cluster of genes mainly involves cell-cycle-related genes. For example, we identified the p53 pathway to be regulated by decreased levels of zGRα under no exogenous stimulation with GCs. A very interesting interplay between GR and p53 has been reported before, and the zebrafish could become a powerful *in vivo* model for studying this interaction at the whole organism level, for instance via looking at alterations in the level of apoptotic cell death during development. The finding of two distinct clusters of GR-regulated genes could open up new research lines concerning the biological significance of this receptor and its function under specific physiological conditions. It could be hypothesized that under resting conditions, the GR controls basal functions like the cell cycle to maintain homeostasis, whereas upon activation by increased GC levels (e.g. those resulting from the response to a stressful stimulus), the GR starts to regulate metabolic pathways in order to restore homeostasis. Further examination of the architecture of the gene promoters (e.g. the GRE structure) in these two clusters could explain their differential response to zGRα signaling and therefore contribute to a better understanding of the molecular mechanism of GRα-mediated transcription under various physiological conditions.

Finally, we were also interested in the biological significance of the GR β-isoform. In humans this splice variant has been shown to act as a dominant-negative inhibitor of GRα and it has been suggested to be involved in GC resistance in asthma patients. However, there is a lot of controversy regarding its low expression level and its effects on transcription. The zebrafish also expresses a GR β-isoform and hence, it could be an interesting model to elucidate the role of this receptor isoform *in vivo*.

In the present work, using overexpression of zGRβ through injection of zGRβ mRNA, and through the generation of a transgenic fish, we aimed to unravel its function with respect to the regulation of gene expression. First we studied whether zGRβ acts as a dominant-negative inhibitor of zGRα. Our data do not support such a role, since only 1 gene (*si:ch211-234p6.12*, encoding an unknown protein) could be determined as a target for zGRβ's dominant negative activity, which most likely represents a false-positive result. Second, we studied whether zGRβ has intrinsic transcriptional activity, i.e. activity

independent of zGRα activation. Both the results obtained using zGRβ mRNA injection and those obtained using the zGRβ overexpressing transgenic fish did not show any evidence for an intrinsic transcriptional activity of zGRβ. This apparent lack of transcriptional activity of zGRβ suggests that the function of splicing of the zGR pre-mRNA into zGRβ mRNA could merely be a physiological way to lower the expression of the canonical GR α-isoform in order to render a cell less responsive to GCs.

Surprisingly however, upon injection of a morpholino altering the splice pattern of zGR pre-mRNA towards increased zGRβ production, we found more genes of which the expression was altered than after treatment with a morpholino that only resulted in decreased zGRα expression. Although a large overlap existed between the gene sets regulated by the two morpholinos there was a large cluster of genes that was exclusively regulated by the morpholino that increased zGRβ expression, and this cluster contained genes involved in pathways not regulated by the other morpholino. This gene cluster could be explained as evidence for an intrinsic transcriptional activity of zGRβ, but might also be a result of a more efficient knockdown of zGRα by the morpholino.

In conclusion, the zebrafish model system with its opportunities for genetic manipulation constitutes a versatile *in vivo* system, in order to further explore GR gene function and signaling. Our exploratory studies provided us a set of GR regulated genes to be used for future investigation of the mechanisms of GR action, interaction and biological significance, regarding GC regulation of various biological processes (e.g. development, inflammation, cancer, stress response).

Summary

The glucocorticoid receptor (GR) signaling pathway is essential for the survival and wellbeing of most vertebrate organisms and in addition, it significantly contributes in combating inflammation (reviewed in **Chapter 1)**. Hence, the scope of the present thesis was to elucidate the biological significance and action of the glucocorticoid receptor by means of genetic manipulation and stimulation with synthetic glucocorticoids (GCs). As a model organism, we employed the zebrafish, that allows fine genetic, molecular and cellular experimental approaches and as our main readout we used transcriptome analysis, since the GR is a transcription factor. 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.

In **chapter 2**, we explored the role of the zebrafish GR α-isoform (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 towards the wound site 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 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. Our results show that beclomethasone treatment of amputated larvae attenuated the migratory behavior of neutrophils, but not of macrophages. Additionally, GC treatment had a very general dampening effect on the induction of gene expression upon amputation, without any apparent specificity for particular pathways. These results show that the zebrafish larva model of tail fin amputation and beclomethasone treatment recapitulates the anti-inflammatory GC effects, thus providing a reliable model system to further elucidate the molecular mechanisms of GC signaling.

In **chapter 3**, we investigated the specificity and function of both zebrafish GR α- and β-isoforms (zGRα and zGRβ, respectively). Zebrafish embryos were injected with two splice-blocking morpholinos (one leading to knockdown of both zGRα and zGRβ, 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 allowed us to answer 3 questions. First, which specific genes are affected by zGRα under different physiological conditions. Second, whether zGRβ exhibits a dominant-negative activity on zGRα's transcriptional properties. Third, which genes are specifically altered due to a possible intrinsic zGRβ transcriptional activity (independent of zGRα). Our transcriptome analysis showed two distinct gene clusters regulated by zGRα. One cluster that was affected by zGRα under basal conditions, and one that was regulated upon activation of zGRα by dexamethasone treatment. In general, the first cluster mainly contains cell-cycle related genes whereas the second cluster mainly involves genes related to metabolism. Furthermore, our data did not support a significant role of zGRβ as a dominant-negative inhibitor of zGRα. Nevertheless, zGRβ could have an intrinsic transcriptional activity that would require high expression levels of this splice variant.

In **chapter 4**, we embarked on a series of experimental approaches allowing us 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. We overexpressed zGRβ transiently by cell transfections using a zGRβ expression plasmid. Luciferase reporter assays in transiently transfected COS-1 cells demonstrated a dominant-negative effect of zGRβ on the transactivation properties of zGRα. However, no such effect was observed in zebrafish PAC2 cells using the induction of the *fkbp5* gene as readout. In addition, upon zGRβ mRNA injections in zebrafish embryos, no dominant-negative effect was observed on the zGRα-induced transactivation of the *fkbp5*, *pepck* and *nfkbiaα* genes. Subsequently, we generated a transgenic fish line with inducible zGRβ overexpression. Transcriptome analysis by microarray and subsequent qPCR validation using this line did not reveal any regulatory effect of zGRβ (either as a dominant-negative inhibitor of zGRα or as a transcription factor independent of zGRα). Based on these results, we suggest that the zebrafish GR β-isoform does not have a regulatory role in transcription and that splicing of the GR pre-mRNA into a messenger encoding an alternative splice variant could instead represent a physiological mechanism to downregulate the levels of the canonical receptor variant.

In **chapter 5**, results from all three experimental chapters were discussed collectively in order to draw conclusions about the validity of zebrafish as a model system for GC research and, most importantly, the role and function of both zGR splice variants. Our work showed that the zebrafish is a reliable system to study the GR signaling pathway since this model organism recapitulates the classical effects of GRα activation such as transactivation of genes and immunosuppressive activity. Furthermore, our whole organism transcriptome analysis provided us with a wealth of information on GRα-dependent gene expression regulation under different conditions. These data can further be investigated in more detail, in order to study the biological significance and molecular properties and interactions of this receptor with respect to specific physiological settings. For instance, different inflammatory responses could easily be modeled in zebrafish, and comparative analysis of zGRα-mediated regulation of the immune response could be undertaken. As for the biological role of zGRβ, our data do not support any zGRβ dominant-negative activity on zGRα's transcriptional properties, whereas some specific zGRβ intrinsic transcriptional activity (meaning independent of zGRα) cannot be ruled out.

Samenvatting

Signaling gemedieerd door de glucocorticoid receptor is essentieel voor het overleven en welzijn van de meeste gewervelde dieren, en het draagt bij aan het bestrijden van ontstekingen (voor een review **zie hoofdstuk 1**). De focus van dit proefschrift is dan ook om de biologische significantie en de werking van de GR op te helderen door middel van genetische manipulatie en stimulatie met synthetische glucocorticoïden (GCs). Als modelsysteem hebben we de zebravis gebruikt, die het mogelijk maakt om nauwgezette genetische, moleculair- en celbiologische experimenten uit te voeren. Als belangrijkste uitleessysteem hebben we transcriptoomanalyse gebruikt, omdat de GR een transcriptiefactor is. Het doel van het onderzoek was verder om de functie van deze veelzijdige signaalcascade te karakteriseren in zebravis, om dit diermodel op te zetten als systeem om zowel zeer gedetailleerd als high-throughput GR onderzoek te doen.

In **hoofdstuk 2** hebben we de rol van de zebravis GR α-isoform (zGRα) onderzocht in de modulatie van de immuunrespons na een verwonding. Hiervoor hebben we het staartvinamputatie-model gebruikt in drie dagen oude zebravis larven die na de amputatie met het synthetische GC beclomethason werden behandeld. Amputatie veroorzaakte migratiegedrag naar de plaats van verwonding voor neutrofielen en macrofagen, en veroorzaakte daarnaast de activatie van een groot aantal immuun-gerelateerde signaalroutes. Met behulp van microscopische en transcriptoomanalyse hebben we het GC effect op leukocytmigratie onderzocht en de transcriptionele activiteit van genen bestudeerd die een rol spelen in de door amputatie veranderde moleculaire netwerken. Onze resultaten laten zien dat behandeling van geamputeerde larven met beclomethason de migratie van neutrofielen remt, maar niet die van macrofagen. Daarnaast heeft GC behandeling een zeer algemeen dempend effect op de inductie van genexpressie na amputatie, zonder enige zichtbare specificiteit voor bepaalde signaalroutes. Deze resultaten laten zien dat het model van staartvin amputatie in zebravis larven gecombineerd met beclomethasonbehandeling goed de ontstekingsremmende werking van GCs nabootst, en dus een betrouwbaar modelsysteem is voor verder onderzoek naar de moleculaire mechanismen van GC signaling.

In **hoofdstuk 3** hebben we de specificiteit en functie van de zebravis GR α- en β-isoform (zGRα en zGRβ) onderzocht. Zebravisembryo's werden geïnjecteerd met twee morfolino's die splice-sites blokkeerden (één die zowel zGRα als zGRβ expressie omlaag reguleerde en één die de splice-site die to zGRβ expressie leidt blokkeerde) en met zGRβ mRNA (wat tot zGRβ overexpressie leidt). Embryo's werden behandeld met het synthetische GC dexamethason en vervolgens werd transcriptoomanalyse uitgevoerd met behulp van microarray technologie. Dit experiment maakte het mogelijk om drie vragen te beantwoorden. Ten eerste, welke specifieke genen worden door zGRα gereguleerd onder specifieke fysiologische condities? Ten tweede, heeft zGRβ een dominant-negatieve werking heeft op de transcriptionele activiteit van zGRα? Ten derde, welke genen worden specifiek gereguleerd door een mogelijk intrinsieke transcriptionele activiteit van zGRβ? Onze transcriptoomanalyse liet twee verschillende clusters van genen zien die werden gereguleerd door zGRα. Eén cluster, dat werd gereguleerd onder basale condities, en een dat werd gereguleerd na zGRα activatie door beclomethasonbehandeling. In het algemeen bevat het eerste cluster vooral genen die betrokken zijn bij de celcyclus terwijl het tweede cluster vooral genen bevat die een spelen in het metabolisme. Verder vonden we geen bewijs voor een dominant-negatieve werking van zGRβ op de transcriptionele activiteit

van zGRα. Een intrinsieke transcriptionele activiteit van zGRβ kunnen we op basis van deze resultaten niet uitsluiten, al zou hiervoor wel een hoog expressieniveau van deze splice variant nodig zijn.

In **hoofdstuk 4** hebben we een serie van experimentele benaderingen gekozen die het ons mogelijk maakte om de biologische significantie van de zGR β-isoform te bestuderen. In het bijzonder waren we geïnteresseerd in twee vragen. Ten eerste, veroorzaakt zGRβ dominant-negatieve inhibitie op de transcriptionele activiteit van zGRα? Ten tweede, laat zGRβ intrinsieke transcriptionele activiteit zien, dus onafhankelijk van zGRα? We hebben zGRβ transiënt tot overexpressie gebracht door cellen te transfecteren met een zGRβ expressievector. Luciferase reporter assays op getransfecteerde COS-1 cellen lieten een dominant-negatief effect zien van zGRβ op de trasncriptionele activiteit van zGRα. Echter, dit effect was niet te zien in zebravis PAC2 cellen, wanneer we gebruik maakten van de inductie van het *fkbp5* gen als uitleessysteem. Daarnaast zagen we geen dominant-negatief effect van zGRβ op de zGRα-geïnduceerde transactivatie van het *fkbp5*, *pepck* en *nfkbiaa* gen. Vervolgens hebben we een transgene zebravislijn vervaardigd met induceerbare overexpressie van zGRβ. Gebruik makend van deze lijn werd transcriptoomanalyse met behulp van microarrays en qPCR validatie gedaan, en geen effect van zGRβ overexpressie kon worden gedetecteerd (noch de dominant-negatieve activiteit, noch de intrinsieke transcriptionele activiteit). Door deze resultaten kunnen we stellen dat de zebravis GR β-isoform geen regulerende rol heeft op transcriptie en dat splicing van het GR pre-mRNA tot een mRNA dat codeert voor zGRβ een fysiologisch mechanisme zou kunnen zijn dat er enkel op gericht is de expressie van de klassieke GR α-isoform omlaag te reguleren.

In **hoofdstuk 5** worden de resultaten van de drie voorgaande hoofdstukken bediscussieerd om vervolgens conclusies te trekken over de validiteit van het zebravis modelsysteem voor GC onderzoek en, nog belangrijker, de rol en activiteit van beide zGR splice varianten. Ons werk laat zien dat de zebravis een betrouwbaar systeem is om de GR signaalroute te bestuderen, omdat in dit modelorganisme klassieke effecten van GRα activatie nagebootst kunnen worden als transactivatie van specifieke genen en de immuunsuppressieve werking. Verder heeft de transcriptoomanalyse, uitgevoerd op een compleet, intact organisme, een schat aan informatie opgeleverd over GRα-afhankelijke genexpressie onder verschillende fysiologische condities. Deze gegevens kunnen verder en in meer detail worden bestudeerd, om de biologische significantie, moleculaire eigenschappen en interacties van deze receptor te bestuderen in relatie tot specifieke fysiologische omstandigheden. Zo kunnen bijvoorbeeld verschillende vormen van ontstekingsreacties makkelijk worden gemodelleerd in zebravis, om vervolgens de zGRα-gemedieerde regulatie van deze reactie geanalyseerd. Wat betreft de biologische functie van zGRβ vinden we in onze data geen bewijs voor een dominant-negatieve rol van zGRβ op de transcriptionele activiteit van zGRα, terwijl een intrinsieke transcriptionele activiteit niet kan worden uitgesloten.

Curriculum vitae

Antonia Chatzopoulou Chatzi was born in 1980 in Athens, Greece. In 1998, she graduated from the 3rd Lyceum of Alimos, Athens and in 1999 she was admitted to the School of Biology at the University of Athens. During her BSc internship she studied the effect of insulin and oxidative stress on the signaling of MAP kinases and the transcription factor NF-κB on mammalian skeletal myoblasts under the supervision of Prof. Dr. C. Gaitanaki at the Human and Animal Physiology department at the University of Athens. In 2004, she obtained her BSc degree in Biology and in 2005 she was admitted to the MSc Neurosciences programme at the Free University of Amsterdam. Her first internship during her MSc study was done under supervision of Dr. S. Spijker at the Molecular and Cellular Neurobiology department at Free University, examining the effects of the antidepressant drug fluoxetine on specific brain regions of socially defeated rats, by means of gene expression profiling. Her second internship was conducted at the Medical Pharmacology department of Leiden University, under the supervision of Dr. O.C. Meijer and Dr. C. Fitzsimons, investigating how the specific composition of Glucocorticoid Response Elements of different Glucocorticoid Receptor-responsive genes account for different potency and efficacy upon steroid treatment. Her subsequent literature study focused on the synaptic inputs into the paraventricular nucleus that, during the stress response, regulate the Corticotrophin-Releasing Factor gene transcription, and was supervised by Dr. O.C. Meijer. In 2007 she obtained her MSc degree and in the same year she started her PhD training at the Molecular and Cell Biology department of Leiden University, under the supervision of Dr. M.J.M. Schaaf. In her project she explored the action and biological role of the Glucocorticoid Receptor α and β-isoforms, employing the zebrafish model and applying transcriptome analysis.

List of publications

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