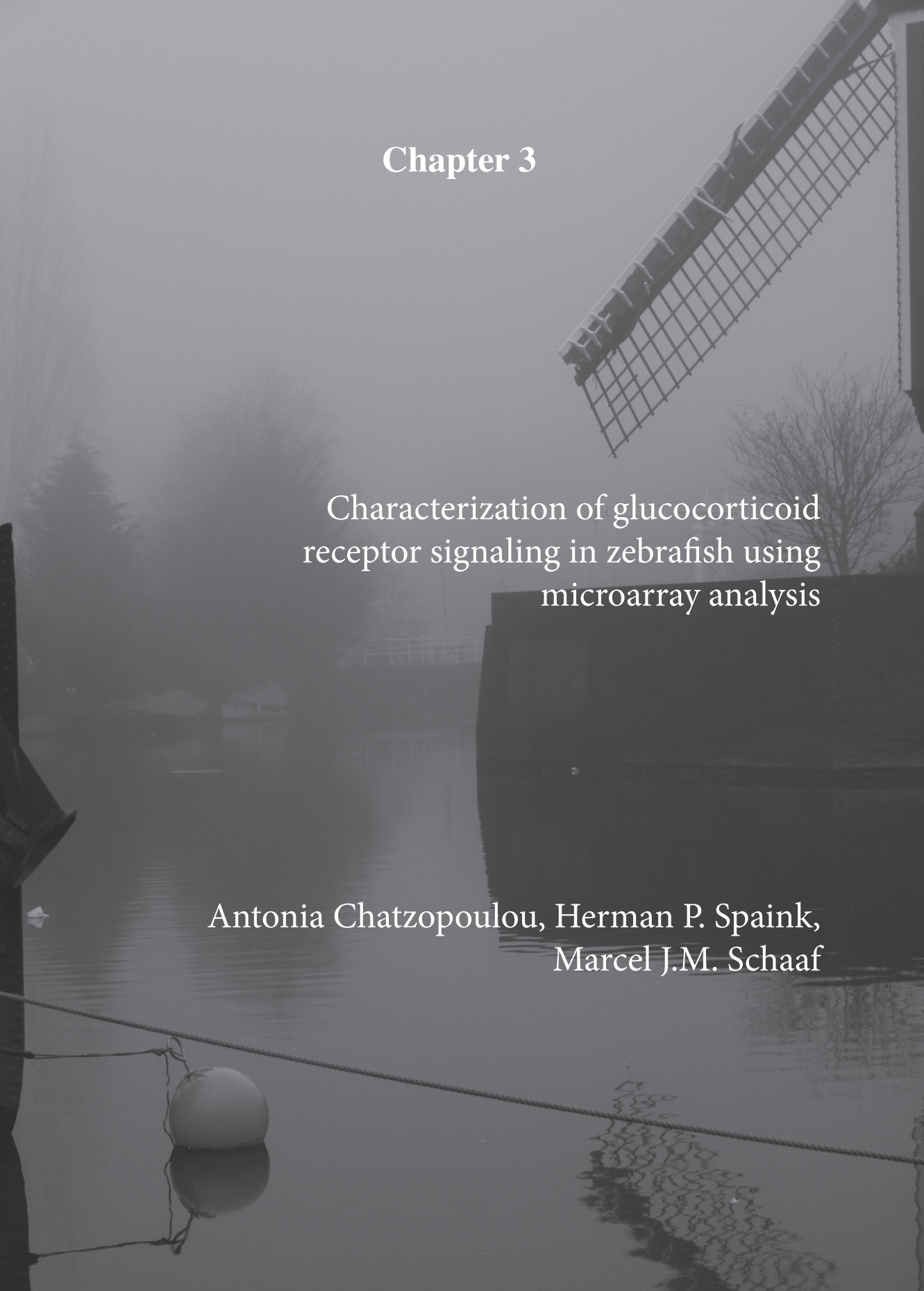


Chapter 3

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).

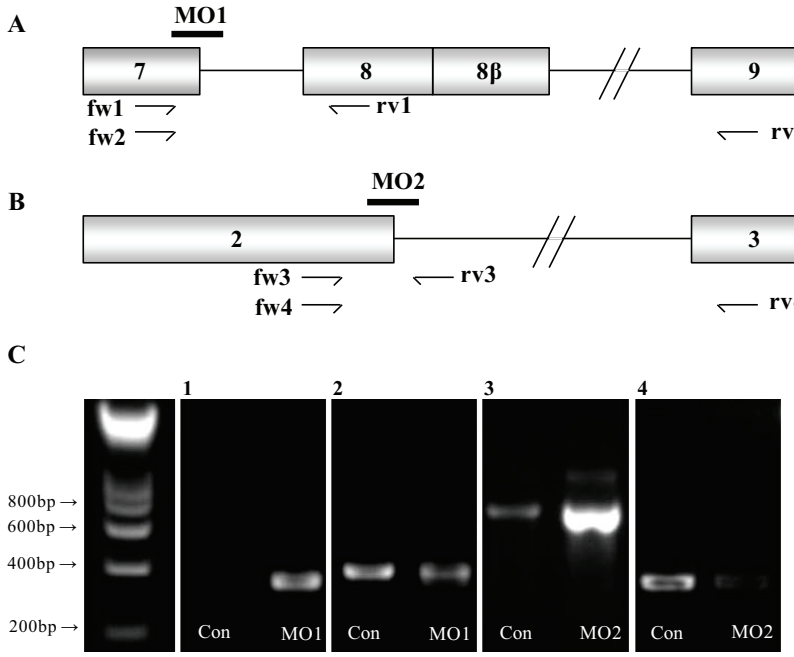


Figure 1. Schematic overview of the location of the target sites of zGR splice-blocking morpholinos MO1 (**A**) and MO2 (**B**). Primers used to analyze the effect of the morpholinos on the splicing pattern are indicated as well. Grey boxes represent the exons (numbers indicated) and black lines connecting 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.

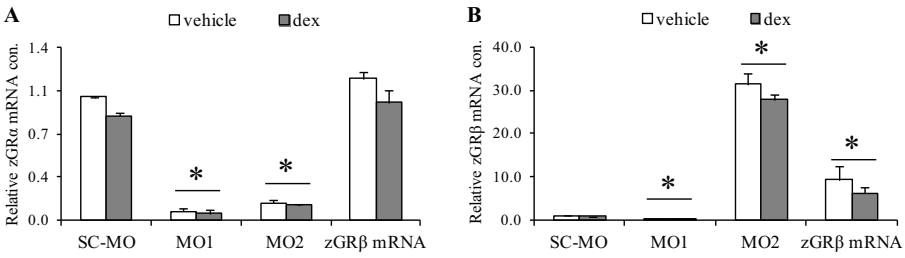


Figure 2. Analysis by qPCR of the effect of injection of morpholinos and zGRβ mRNA on the expression of zGRα and zGRβ transcripts. Zebrafish embryos were injected with either SC-MO, MO1, MO2 or zGRβ mRNA. Injected embryos at 25hpf were treated with dexamethasone (dex) for 6h and total RNA samples were 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.

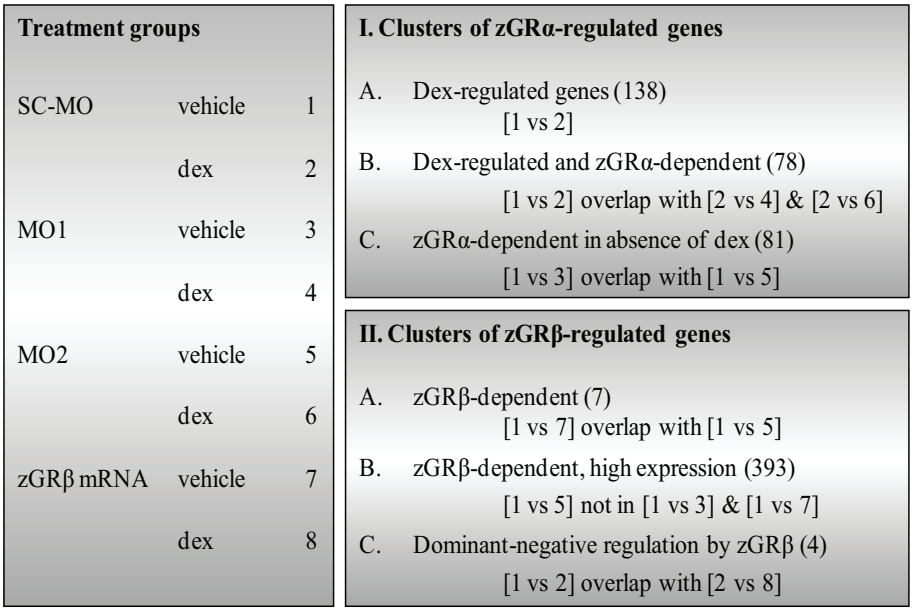


Figure 3. Overview of the microarray results on zGRα- and zGRβ-mediated effects on transcription, analyzed using Rosetta Resolver 7.2 software. The left panel indicates the eight different treatment groups, and the right panels each indicate three clusters of either zGRα- (top panel) or zGRβ-regulated (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.

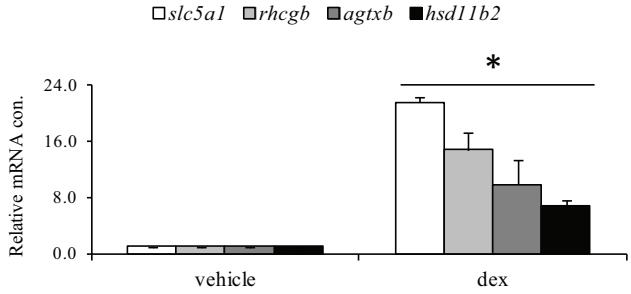


Figure 4. Validation by qPCR of gene regulation by dex identified by microarray analysis. Zebrafish embryos of 1dpf were treated with 100μM dex for 6h and total RNA samples were processed for qPCR analysis. For all genes shown, the mRNA concentration was significantly higher in the dex-treated group 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.

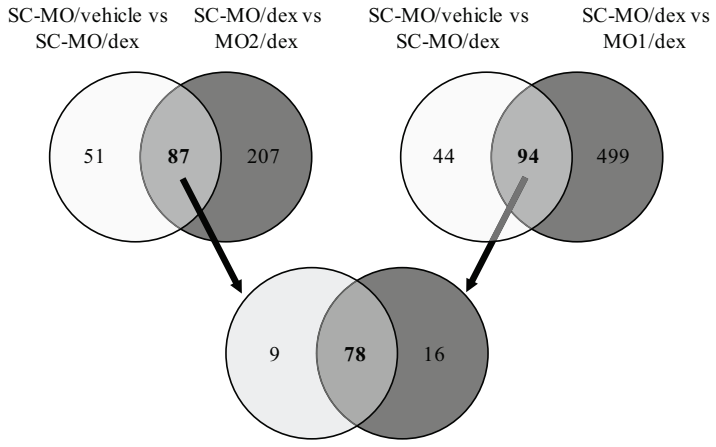


Figure 5. Venn diagrams showing the identification of the cluster of dex-regulated and zGRα-dependent genes. First, the overlap between genes regulated by dex (SC-MO/vehicle vs SC-MO/dex, white circle) and genes regulated by MO2 in the presence of dex (SC-MO/dex vs MO2/dex comparison, grey circle) was 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.

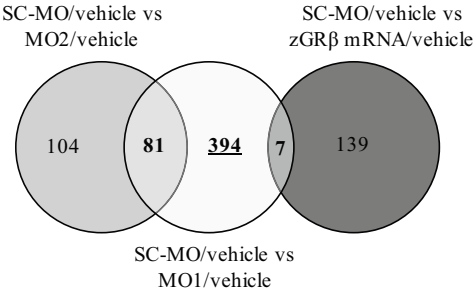


Figure 6. Venn diagram showing the identification of clusters of genes that are zGRα and β-dependent. The left (light grey) circle contains the cluster of genes found to be significantly regulated by MO2 injection in the absence of dex (SC-MO/vehicle vs MO2/vehicle comparison). The middle (white) circle 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.

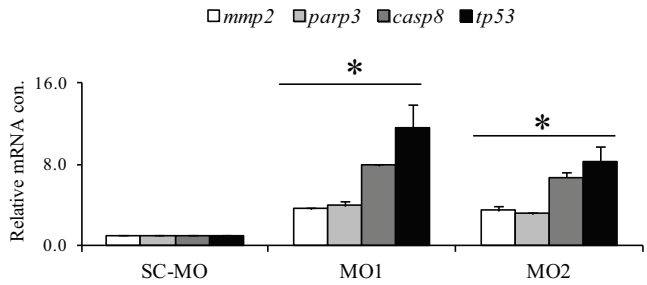


Figure 7. Validation by qPCR of MO-regulated genes identified by microarray analysis. The transcription rates of all genes examined were significantly higher in both MO1- and MO2-injected groups compared to the SC-MO group. These data provide confirmation of the results from our microarray analysis for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

.

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.

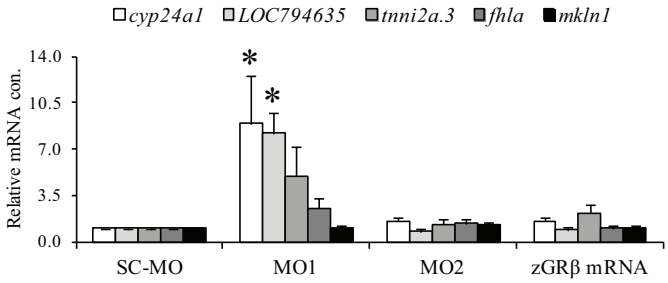


Figure 8. Validation by qPCR of unique MO1-regulated genes identified by microarray analysis. The mRNA concentration levels of *cyp24a1* and *LOC794635* were significantly higher only in MO1 injected group compared to SC one. The rest of the genes tested did not show any differential regulation. Asterisks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

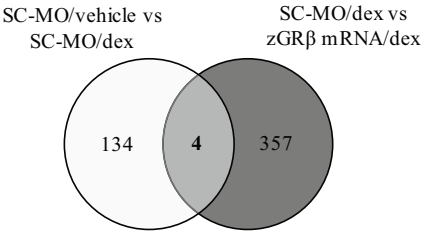


Figure 9. Venn diagram showing the analysis of the possible dominant-negative acivity of zGRβ. The white circle contains dex-regulated genes (SC-MO/vehicle vs SC-MO/dex) and the grey circle encompasses the zGRβ mRNA-regulated genes (SC-MO/dex vs zGRβ mRNA/dex). The overlap contains 4 genes affected by 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.

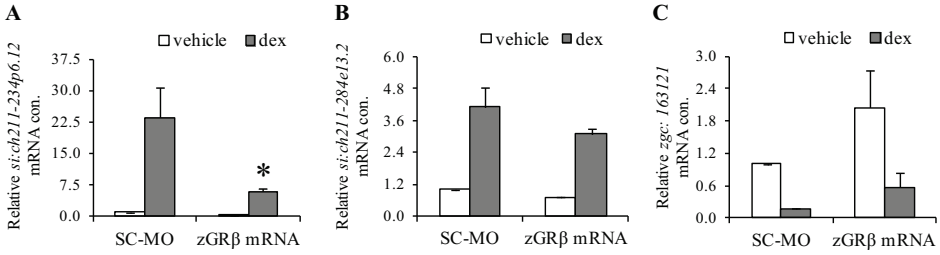


Figure 10. Validation by qPCR of the dominant-negative effect of zGRβ. **A.** The dex-mediated upregulation of *si:ch211-234p6.12* gene was significantly compromised due to zGRβ mRNA injection compared to SC-MO injected group. **B**. The dex-mediated upregulation of *si:ch211-284e13.2* gene was not significantly 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.

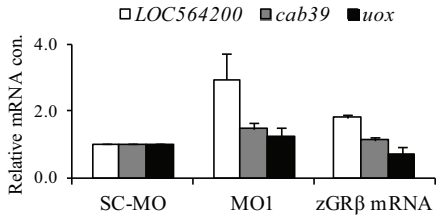
Next, we examined a possible dominant-negative activity of zGRβ and identified only 4 genes for which the dex-induced gene transcription was significantly compromised due to zGRβ mRNA injection, accounting for <3% of the cluster of dex-affected genes. Validation by qPCR of these 4 genes confirmed a dominant-negative 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

The present work was financially supported by the SmartMix program of The Netherlands Ministry of Economic Affairs and the Ministry of Education, Culture and Science.

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.

References

1. Heitzer, M.D., et al., *Glucocorticoid receptor physiology.* Rev Endocr Metab Disord, 2007. **8**(4): p. 321-30.
2. Schoneveld, O.J., I.C. Gaemers, and W.H. Lamers, *Mechanisms of glucocorticoid signalling.* Biochim Biophys Acta, 2004. **1680**(2): p. 114-28.
3. Sapolsky, R.M., L.M. Romero, and A.U. Munck, *How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions.* Endocr Rev, 2000. **21**(1): p. 55-89.
4. Revollo, J.R. and J.A. Cidlowski, *Mechanisms generating diversity in glucocorticoid receptor signaling.* Ann N Y Acad Sci, 2009. **1179**: p. 167-78.
5. de Kloet, E.R., M. Joels, and F. Holsboer, *Stress and the brain: from adaptation to disease.* Nat Rev Neurosci, 2005. **6**(6): p. 463-75.
6. Chrousos, G.P. and T. Kino, *Intracellular glucocorticoid signaling: a formerly simple system turns stochastic.* Sci STKE, 2005. **2005**(304): p. pe48.
7. Nicolaides, N.C., et al., *The human glucocorticoid receptor: molecular basis of biologic function.* Steroids, 2010. **75**(1): p. 1-12.
8. Beato, M. and J. Klug, *Steroid hormone receptors: an update.* Hum Reprod Update, 2000. **6**(3): p. 225-36.
9. Buckingham, J.C., *Glucocorticoids: exemplars of multi-tasking.* Br J Pharmacol, 2006. **147 Suppl 1**: p. S258-68.
10. van der Laan, S. and O.C. Meijer, *Pharmacology of glucocorticoids: beyond receptors.* Eur J Pharmacol, 2008. **585**(2-3): p. 483-91.
11. De Bosscher, K. and G. Haegeman, *Minireview: latest perspectives on antiinflammatory actions of glucocorticoids.* Mol Endocrinol, 2009. **23**(3): p. 281-91.
12. Hollenberg, S.M., et al., *Primary structure and expression of a functional human glucocorticoid receptor cDNA.* Nature, 1985. **318**(6047): p. 635-41.
13. Encio, I.J. and S.D. Detera-Wadleigh, *The genomic structure of the human glucocorticoid receptor.* J Biol Chem, 1991. **266**(11): p. 7182-8.

14. Kino, T., Y.A. Su, and G.P. Chrousos, *Human glucocorticoid receptor isoform β: recent understanding of its potential implications in physiology and pathophysiology.* Cellular and Molecular Life Sciences, 2009. **66**(21): p. 3435-3448.
15. Bamberger, C.M., et al., *Glucocorticoid receptor beta, a potential endogenous inhibitor of glucocorticoid action in humans.* J Clin Invest, 1995. **95**(6): p. 2435-41.
16. Oakley, R.H., et al., *The dominant negative activity of the human glucocorticoid receptor beta isoform. Specificity and mechanisms of action.* J Biol Chem, 1999. **274**(39): p. 27857-66.
17. Goleva, E., et al., *Increased glucocorticoid receptor beta alters steroid response in glucocorticoid-insensitive asthma.* Am J Respir Crit Care Med, 2006. **173**(6): p. 607-16.
18. Zhang, X., *Regulation of Glucocorticoid Responsiveness in Glaucomatous Trabecular Meshwork Cells by Glucocorticoid Receptor-β.* Investigative Ophthalmology & Visual Science, 2005. **46**(12): p. 4607-4616.
19. Lewis-Tuffin, L.J., et al., *Human Glucocorticoid Receptor β Binds RU-486 and Is Transcriptionally Active.* Molecular and Cellular Biology, 2007. **27**(6): p. 2266-2282.
20. Kino, T., et al., *Glucocorticoid receptor (GR) β has intrinsic, GRα-independent transcriptional activity.* Biochemical and Biophysical Research Communications, 2009. **381**(4): p. 671-675.
21. Hecht, K., et al., *Evidence that the beta-isoform of the human glucocorticoid receptor does not act as a physiologically significant repressor.* J Biol Chem, 1997. **272**(42): p. 26659-64.
22. Brogan, I.J., et al., *Interaction of glucocorticoid receptor isoforms with transcription factors AP-1 and NF-kappaB: lack of effect of glucocorticoid receptor beta.* Mol Cell Endocrinol, 1999. **157**(1-2): p. 95-104.
23. Bamberger, C.M., et al., *Regulation of the human interleukin-2 gene by the alpha and beta isoforms of the glucocorticoid receptor.* Mol Cell Endocrinol, 1997. **136**(1): p. 23-8.
24. Carlstedt-Duke, J., *Glucocorticoid Receptor beta: View II.* Trends Endocrinol Metab, 1999. **10**(8): p. 339-342.
25. Gougat, C., et al., *Overexpression of the human glucocorticoid receptor α and β isoforms inhibits AP-1 and NF-κB activities hormone independently.* Journal of Molecular Medicine, 2002. **80**(5): p. 309-318.
26. Kelly, A., et al., *The glucocorticoid receptor β isoform can mediate transcriptional repression by recruiting histone deacetylases.* Journal of Allergy and Clinical Immunology, 2008. **121**(1): p. 203-208.e1.
27. Taniguchi, Y., et al., *Glucocorticoid Receptor-β and Receptor-γ Exert Dominant Negative Effect on Gene Repression But Not on Gene Induction.* Endocrinology, 2010. **151**(7): p. 3204-3213.
28. de Castro, M., et al., *The non-ligand binding beta-isoform of the human glucocorticoid receptor (hGR beta): tissue levels, mechanism of action, and potential physiologic role.* Mol Med, 1996. **2**(5): p. 597-607.
29. Pujols, L., et al., *Expression of glucocorticoid receptor alpha- and beta-isoforms in human cells and tissues.* Am J Physiol Cell Physiol, 2002. **283**(4): p. C1324-31.
30. Schaaf, M.J.M., et al., *Discovery of a Functional Glucocorticoid Receptor β-Isoform in Zebrafish.* Endocrinology, 2008. **149**(4): p. 1591-1599.
31. Hinds, T.D., et al., *Discovery of Glucocorticoid Receptor-β in Mice with a Role in Metabolism.* Molecular Endocrinology, 2010. **24**(9): p. 1715-1727.
32. Brittijn, S.A., et al., *Zebrafish development and regeneration: new tools for biomedical research.* Int J Dev Biol, 2009. **53**(5-6): p. 835-50.
33. Xi, Y., S. Noble, and M. Ekker, *Modeling neurodegeneration in zebrafish.* Curr Neurol Neurosci Rep, 2011. **11**(3): p. 274-82.
34. Lieschke, G.J. and P.D. Currie, *Animal models of human disease: zebrafish swim into view.* Nat Rev Genet, 2007. **8**(5): p. 353-67.
35. Kari, G., U. Rodeck, and A.P. Dicker, *Zebrafish: an emerging model system for human disease and drug discovery.* Clin Pharmacol Ther, 2007. **82**(1): p. 70-80.
36. Kawakami, K., *Transposon tools and methods in zebrafish.* Dev Dyn, 2005. **234**(2): p. 244-54.
37. Schaaf, M.J., A. Chatzopoulou, and H.P. Spaink, *The zebrafish as a model system for glucocorticoid receptor research.* Comp Biochem Physiol A Mol Integr Physiol, 2009. **153**(1): p. 75-82.
38. Alsop, D. and M.M. Vijayan, *Molecular programming of the corticosteroid stress axis during zebrafish development.* Comp Biochem Physiol A Mol Integr Physiol, 2009. **153**(1): p. 49-54.
39. Steenbergen, P.J., M.K. Richardson, and D.L. Champagne, *The use of the zebrafish model in stress research.* Prog Neuropsychopharmacol Biol Psychiatry, 2011. **35**(6): p. 1432-51.
40. Schoonheim, P.J., A. Chatzopoulou, and M.J. Schaaf, *The zebrafish as an in vivo model system for glucocorticoid resistance.* Steroids, 2010. **75**(12): p. 918-25.
41. Wendelaar Bonga, S.E., *The stress response in fish.* Physiol Rev, 1997. **77**(3): p. 591-625.

42. Hillegass, J.M., et al., *Glucocorticoids alter craniofacial development and increase expression and activity of matrix metalloproteinases in developing zebrafish (Danio rerio).* Toxicol Sci, 2008. **102**(2): p. 413-24.
43. Zhang, Y., et al., *In vivo interstitial migration of primitive macrophages mediated by JNK-matrix metalloproteinase 13 signaling in response to acute injury.* J Immunol, 2008. **181**(3): p. 2155-64.
44. Alsop, D. and M. Vijayan, *The zebrafish stress axis: molecular fallout from the teleost-specific genome duplication event.* Gen Comp Endocrinol, 2009. **161**(1): p. 62-6.
45. Bill, B.R., et al., *A primer for morpholino use in zebrafish.* Zebrafish, 2009. **6**(1): p. 69-77.
46. Stockhammer, O.W., et al., *Transcriptome analysis of Traf6 function in the innate immune response of zebrafish embryos.* Mol Immunol, 2010. **48**(1-3): p. 179-90.
47. Rauwerda, H., et al., *Integrating heterogeneous sequence information for transcriptome-wide microarray design; a Zebrafish example.* BMC Res Notes, 2010. **3**: p. 192.
48. van Iersel, M.P., et al., *Presenting and exploring biological pathways with PathVisio.* BMC Bioinformatics, 2008. **9**: p. 399.
49. Bedell, V.M., S.E. Westcot, and S.C. Ekker, *Lessons from morpholino-based screening in zebrafish.* Brief Funct Genomics, 2011. **10**(4): p. 181-8.
50. Mathew, L.K., et al., *Unraveling tissue regeneration pathways using chemical genetics.* J Biol Chem, 2007. **282**(48): p. 35202-10.
51. Pikulkaew, S., et al., *The knockdown of maternal glucocorticoid receptor mRNA alters embryo development in zebrafish.* Dev Dyn, 2011. **240**(4): p. 874-89.
52. Cole, T.J., et al., *Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation.* Genes Dev, 1995. **9**(13): p. 1608-21.
53. Pivonello, R., et al., *Pathophysiology of diabetes mellitus in Cushing's syndrome.* Neuroendocrinology, 2010. **92 Suppl 1**: p. 77-81.
54. D'Acquisto, F., M. Perretti, and R.J. Flower, *Annexin-A1: a pivotal regulator of the innate and adaptive immune systems.* Br J Pharmacol, 2008. **155**(2): p. 152-69.
55. John, S., et al., *Interaction of the glucocorticoid receptor with the chromatin landscape.* Mol Cell, 2008. **29**(5): p. 611-24.
56. Phuc Le, P., et al., *Glucocorticoid receptor-dependent gene regulatory networks.* PLoS Genet, 2005. **1**(2): p. e16.
57. Wang, J.C., et al., *Chromatin immunoprecipitation (ChIP) scanning identifies primary glucocorticoid receptor target genes.* Proc Natl Acad Sci U S A, 2004. **101**(44): p. 15603-8.
58. Rogatsky, I., et al., *Target-specific utilization of transcriptional regulatory surfaces by the glucocorticoid receptor.* Proc Natl Acad Sci U S A, 2003. **100**(24): p. 13845-50.
59. Stojadinovic, O., et al., *Novel genomic effects of glucocorticoids in epidermal keratinocytes: inhibition of apoptosis, interferon-gamma pathway, and wound healing along with promotion of terminal differentiation.* J Biol Chem, 2007. **282**(6): p. 4021-34.
60. James, C.G., et al., *Expression profiling of Dexamethasone-treated primary chondrocytes identifies targets of glucocorticoid signalling in endochondral bone development.* BMC Genomics, 2007. **8**: p. 205.
61. Meijer, O.C., *Understanding stress through the genome.* Stress, 2006. **9**(2): p. 61-7.
62. Lefstin, J.A. and K.R. Yamamoto, *Allosteric effects of DNA on transcriptional regulators.* Nature, 1998. **392**(6679): p. 885-8.
63. So, A.Y., et al., *Determinants of cell- and gene-specific transcriptional regulation by the glucocorticoid receptor.* PLoS Genet, 2007. **3**(6): p. e94.
64. Danilova, N., K.M. Sakamoto, and S. Lin, *Ribosomal protein L11 mutation in zebrafish leads to haematopoietic and metabolic defects.* Br J Haematol, 2011. **152**(2): p. 217-28.
65. Yu, C., et al., *Modulation of hormone-dependent transcriptional activity of the glucocorticoid receptor by the tumor suppressor p53.* Cancer Lett, 1997. **116**(2): p. 191-6.
66. Sengupta, S., et al., *Negative cross-talk between p53 and the glucocorticoid receptor and its role in neuroblastoma cells.* EMBO J, 2000. **19**(22): p. 6051-64.
67. Zhang, L., L. Nie, and C.G. Maki, *P53 and p73 differ in their ability to inhibit glucocorticoid receptor (GR) transcriptional activity.* Mol Cancer, 2006. **5**: p. 68.
68. Crochemore, C., et al., *Enhancement of p53 activity and inhibition of neural cell proliferation by glucocorticoid receptor activation.* FASEB J, 2002. **16**(8): p. 761-70.
69. Urban, G., et al., *Identification of a functional link for the p53 tumor suppressor protein in dexamethasone-induced growth suppression.* J Biol Chem, 2003. **278**(11): p. 9747-53.
70. Murphy, S.H., et al., *Tumor suppressor protein (p)53, is a regulator of NF-kappaB repression by the glucocorticoid receptor.* Proc Natl Acad Sci U S A, 2011. **108**(41): p. 17117-22.

71. Kinyamu, H.K. and T.K. Archer, *Estrogen receptor-dependent proteasomal degradation of the glucocorticoid receptor is coupled to an increase in mdm2 protein expression.* Mol Cell Biol, 2003. **23**(16): p. 5867-81.
72. Nishimura, K., et al., *Altered expression of p53 mRNA in the brain and pituitary during repeated immobilization stress: negative correlation with glucocorticoid receptor mRNA levels.* J Neuroendocrinol, 2004. **16**(1): p. 84-91.
73. Reddy, T.E., et al., *Genomic determination of the glucocorticoid response reveals unexpected mechanisms of gene regulation.* Genome Res, 2009. **19**(12): p. 2163-71.
74. Lewis, D.F., *57 varieties: the human cytochromes P450.* Pharmacogenomics, 2004. **5**(3): p. 305-18.
75. Wang, Z., S. Zhang, and G. Wang, *Response of complement expression to challenge with lipopolysaccharide in embryos/larvae of zebrafish Danio rerio: acquisition of immunocompetent complement.* Fish Shellfish Immunol, 2008. **25**(3): p. 264-70.