Transcriptional and metabolic effects of glucocorticoid receptor alpha and beta signaling in zebrafish

Chatzopoulou Chatzi, A.; Upasana, R.; Meijer, A.H.; Alia, A.; Spaink, H.P.; Schaaf, M.J.M.

Transcriptional and Metabolic Effects of Glucocorticoid Receptor α and β Signaling in Zebrafish

Antonia Chatzopoulou, Upasana Roy, Annemarie H. Meijer, A. Alia, Herman P. Spaink, and Marcel J. M. Schaaf

Department of Animal Sciences and Health (A.C., A.H.M., H.P.S., M.J.M.S.), Institute of Biology, Leiden University, 2333CC Leiden, The Netherlands; Department of Biophysical Organic Chemistry/Solid State NMR (U.R., A.A.), Leiden Institute of Chemistry, Leiden University, 2333CC Leiden, The Netherlands; and Institute of Medical Physics and Biophysics (A.A.), University of Leipzig, D-04107 Leipzig, Germany

In humans and zebrafish, 2 glucocorticoid (GC) receptor (GR) splice variants exist: the canonical GR α-isoform (GRα), and the GRβ. In the present study, we have used the zebrafish model system in order to reveal genes affected by each of these 2 receptor isoforms. By injecting zebrafish embryos with different splice-blocking morpholinos, we could knock down both GR isoforms or could target the alternative splicing of the GR pre-mRNA in favor of the GRβ. In addition, specific GRβ overexpression was achieved by injecting mRNA. Embryos were treated with the synthetic GC dexamethasone, and transcriptome analysis was performed. Two distinct gene clusters were found that were regulated by GRα: one that was regulated by GRα under basal conditions (presence of endogenous cortisol only), and one that was regulated upon increased activation of GRα (using a pharmacological dose of dexamathasone). GRβ may act as a dominant-negative inhibitor of GRα when GRβ is overexpressed and the GRα expression level is knocked down simultaneously. However, without GRα knockdown, no evidence for this activity was found. In addition, the data indicate regulation of gene transcription through other mechanisms of action by GRβ. We also investigated the concentrations of several metabolites using nuclear magnetic resonance spectroscopy. We found that dexamethasone treatment and knockdown of GRα together with overexpression of GRβ had opposite effects on glucose, amino acid, and fatty acid levels. Thus, we have shed new light on the molecular mechanisms of GC-induced effects on metabolism, which are known to increase the risk of obesity, hyperglycemia, and diabetes. (***Endocrinology*** **156: 1757–1769, 2015)**

The glucocorticoid (GC) 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 (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 GC response elements, 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 (eg, Nuclear Factor-κB [NF-κB] and Activating Protein-1 [AP-1]), via which it can either synergistically induce gene expression or repress activation of

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

Received November 24, 2014. Accepted March 4, 2015.
First Published Online March 10, 2015

Abbreviations: 2D, 2 dimensional; DAVID, The Database for Annotation, Visualization and Integrated Discovery; dex, dexamethasone; GC, glucocorticoid; GR, GC receptor; hGRα, human GR α-isoform; hpf, hours post fertilization; HR-MAS, high resolution magic angle spinning; LBD, ligand-binding domain; MO, morpholino; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; qPCR, quantitative PCR; SC-MO, standard control MO; veh, vehicle.

genes. The latter mode comprises one of the classical ways by which GCs exert their antiinflammatory 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). An issue limiting the clinical use of GCs is the severity of the side effects, which are reflected in among others a reduction in bone and muscle mass, and endocrine and metabolic effects (12). These metabolic effects include dyslipidemia, hyperglycemia, and insulin resistance, thereby promoting the susceptibility to type 2 diabetes (13, 14).

When the human GR was cloned, 2 splice variants were discovered, named as human GR α-isoform (hGRα) and hGRβ, which derive from alternative splicing within the most 3′ exon, encoding the C-terminal end of the ligand-binding domain (LBD) (15, 16). Hence, the hGRα (777 amino acids, representing the canonical receptor) is able to interact with GCs, whereas the hGRβ (742 amino acids) has a shorter LBD with a unique C-terminal 15-amino acid sequence, which renders it unable to bind GCs (15–17). Experimental data have provided evidence for a dominant-negative effect of hGRβ on hGRα's transcriptional properties (18–21) as well as intrinsic transcriptional activity (independent of hGRα) (22, 23). However, the biological significance of hGRβ is still debated, mainly due to inconsistent results regarding its transcriptional role, (24–30) its low expression levels (18, 31, 32), and low level of evolutionary conservation (33, 34).

Over the last decade, the zebrafish has emerged in biomedical research as an important model system for a variety of human diseases (35–37), and it has been considered as a potent model organism for GC research (38–41). As humans, zebrafish have a single GR gene, and cortisol is the main endogenous GC. Its secretion is regulated by the hypothalamus-pituitary-interrenal axis, which is the equivalent to the mammalian hypothalamus-pituitary-adrenal axis (39, 40, 42). Upon secretion of cortisol or treatment with synthetic GCs, the activated zebrafish GRα (zGRα) can mediate gene transcription in a similar way as the hGRα (38, 41, 43, 44). Interestingly, zebrafish express both zGR splice variants, because the zGR gene gives rise to zGRα and zGRβ as a result of alternative splicing (33, 45). The zGRβ is highly similar to its human equivalent in structure, subcellular localization and expression level (33). 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 (33).

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 zGRα and zGRβ. Transcriptional effects were investigated using a microarray analysis, and metabolic effects were studied by nuclear magnetic resonance (NMR) spectroscopy. Our results show that zGRα is able to regulate 2 clusters of genes: 1 under basal conditions (in the presence of endogenous cortisol only), and 1 upon increased activation of the receptor (by a high dose of an exogenous GC). In addition, zGRβ may act as a dominant-negative inhibitor of zGRα, depending on the ratio between the zGRα and zGRβ expression levels. The observed transcriptional effects were reflected in the levels of several metabolites analyzed using NMR spectroscopy.

Materials and Methods

Zebrafish strain, husbandry, and 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 egg water containing 60 μg/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.

Morpholino (MO) preparations, zGRβ mRNA synthesis, and injections

Three different MOs were used (purchased from GeneTools, primer sequences presented in Supplemental Table 1), all of which prepared and stored according to the manufacturer's instructions. Before injections, MOs were diluted at a final concentration of 0.05mM in 1× Danieau's buffer (58mM NaCl, 0.7mM KCl, 0.4mM $MgSO_4$, 0.6mM $Ca(NO_3)_2$, and 5mM HEPES at pH 7.6) containing 0.05% phenol red (Sigma).

An expression vector for zGRβ, PCS2+zGRβ (33), was linearized with *Nsi*I (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). Before injections, zGRβ mRNA was denatured at 60°C for 5 minutes and diluted at a final concentration of 5 ng/μL in 1× 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 1 nL of each MO or zGRβ mRNA solution was injected into the yolk of 1–2 cell stage fertilized eggs, and 0.05 pmol standard control MO (SC-MO) was coinjected in the zGRβ mRNA group for proper comparisons with the other groups.

GC treatment, collection of embryos, and RNA isolation

Twenty-four hours after fertilization embryos were placed in 2% agarose-coated Petri dishes and incubated with 100μM

dexamethasone (dex) (Sigma) in egg water for 6 hours at 28°C. Per treatment group, 20 embryos were collected in TRIzol reagent (Invitrogen). Three individual experiments were performed, yielding triplicate samples per treatment group. Total RNA was extracted using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA was dissolved in water and denatured for 5 minutes at 60°C. Samples were treated with desoxyribonuclease (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).

PCR analysis of modified splicing of the GR mRNA

One hundred nanograms of desoxyribonuclease (DNase)-treated total RNA samples were used as a template for One-Step RT-PCR analysis of zebrafish GR RNA sequences using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase kit (Invitrogen), according to the manufacturer's manual. Primer sequences are presented in Supplemental Table 2. Additional qPCR analysis was performed using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad). A detailed description of the qPCR analysis is provided in Supplemental Methods. Primer sequences used for qPCR are presented in Supplemental Table 3.

Microarray analysis

A 4x180k microarray chip platform (design ID:028233; customized by Agilent Technologies) was used in this study (46). 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. Further details on the microarray amplification, labeling hybridization, scanning, and data processing are found in Supplemental Methods. The raw data from the microarray experiment were submitted to the Gene Expression Omnibus database under accession number GSE63360. Data analysis was performed setting cutoff for the P value of less than 10^{-10} and for fold change of either more than 2 or less than -2.

Gene ontology analysis

As a starting point for the gene ontology analysis of the microarray results, clusters of genes were analyzed using the online functional classification tool DAVID (http://david.abcc.ncifcrf.gov/summary.jsp). In addition, for genes not classified by DAVID, information was gathered on their function (using the websites GeneCards (http://www.genecards.org/), National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/gene), Genetics Home Reference (http://www.ncbi.nlm.nih.gov/gene), and Wikipedia (http://en.wikipedia.org/wiki/). Using this information, all genes were classified in one of the categories assigned by DAVID, or in a new category.

^{1}H HR-MAS NMR spectroscopy

For high resolution magic angle spinning (^{1}H HR-MAS) NMR spectroscopy measurement, intact embryos at 30- or 48-hpf stage were placed in a 4 mm Bruker zirconium rotor, and subsequently, 10 μL of 100mM deuterated phosphate buffer (pH 7.4) containing 3-trimetylsilyl-2,2,3,3-tetradeuteropropionic acid (1mM) were added as NMR reference. The rotor was immediately placed in a Bruker DMX 400-MHz NMR spectrometer, operating with a proton resonance frequency of 399.427 Hz and equipped with a 4 mm HR-MAS dual inverse ^{1}H/^{13}C probe. The whole HR-MAS study was performed at 4°C to minimize tissue degradation. The spectra were acquired at a spinning rate of 2500 Hz using a Carr-Purcell- Meiboom-Gill pulse sequence with the repetition time and echo time of 3500 and 0.4 ms, respectively. Total number of averages was 256 with 8 dummy scans. All spectra were processed using a 0 filling of 32 k and line broadening for exponential window function of 1 Hz before Fourier transformation. Spectra are shown in Supplemental Figures 2 and 3. Further details on the NMR spectroscopy analysis are found in Supplemental Methods.

Statistical analysis

Statistical analyses (t tests and ANOVAs with Bonferroni post hoc tests) on qPCR data were performed using the GraphPad Prism version 4.00 (GraphPad Software). All statistical analyses of the NMR results were performed using OriginPro version 8.5.

Results

MO treatment, GRβ mRNA injection, and dex administration

Two splice-blocking MOs (chemically modified antisense oligonucleotides) were used to manipulate the expression levels of zGRα and zGRβ in zebrafish embryos. MO1 targets the splice donor site of exon 2 of the zGR gene, causing retention of intron 2 in both zGRα and zGRβ mRNA (Figure 1B). The resulting transcript encodes a GR protein, which is truncated at amino acid 367, lacks the DNA binding domain and LBD, and is therefore considered inactive. Total zGRα and zGRβ mRNA levels were significantly decreased upon MO1 treatment (Figure 1, C and D). MO2 targets the splice donor site of exon 8, which directs the alternative splicing of the zGR pre-mRNA towards increased production of the zGRβ and a decreased production of the zGRα (Figure 1, B–D). In addition, injection of zGRβ mRNA was performed to specifically increase the zGRβ expression (Figure 1D). A SC-MO was used to inject the control group. At 24 hpf, the embryos injected with SC-MO, MO1, MO2, and zGRβ mRNA were treated with dex (100μM) or vehicle treated for 6 hours. Thus, 8 experimental groups were generated: SC-MO/vehicle, SC-MO/dex, MO1/vehicle, MO1/dex, MO2/vehicle, MO2/dex, zGRβ mRNA/vehicle, and zGRβ mRNA/dex (Table 1).

Neither of the MO treatments caused any obvious morphological abnormalities during embryonic development. This observation is consistent with other studies, in which different splice-blocking MOs were used for a functional knockdown of zGR (47, 48), whereas translation-block-

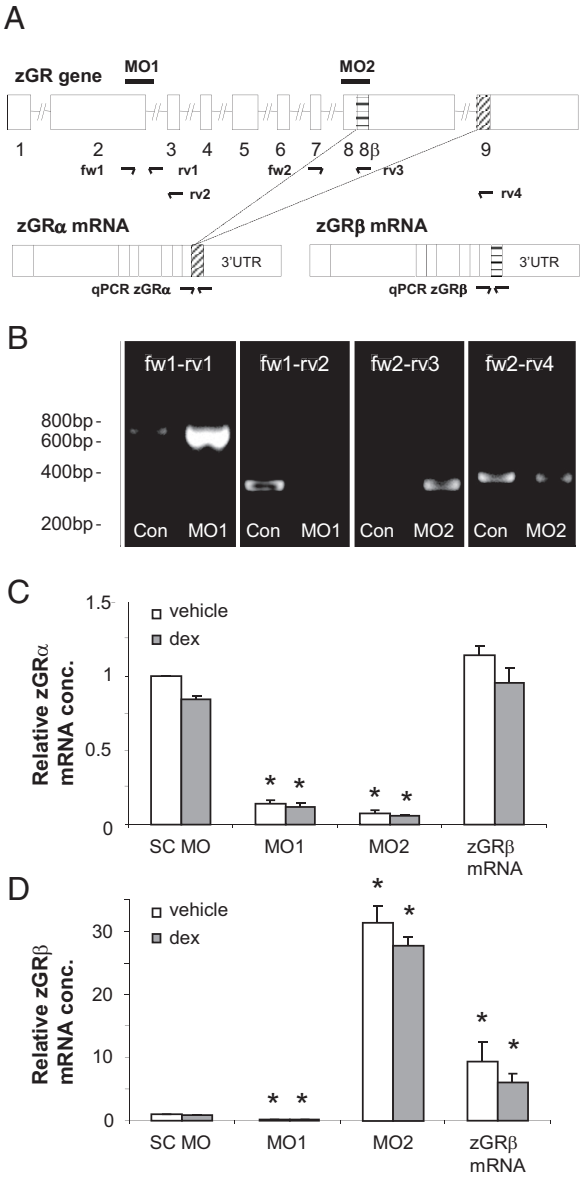

Figure 1. A, Schematic overview of the zebrafish GR gene and its possible transcripts, zGRα and zGRβ mRNA. Boxes represent exons, and exon numbers are indicated. Alternative splicing occurs between exon 8 and 8β. When the splice site between these exons is utilized, zGRα mRNA is produced (containing exon 1–9), and when this splice site is skipped, zGRβ mRNA is produced (containing exon 1–8 and 8β). The location of the target sites of zGR splice-blocking MO1 and MO2 is shown and primers used to analyze the effect of the MOs on the splicing pattern are indicated. B, Agarose gel showing products of RT-PCR analysis of RNA samples from 30-hpf embryos injected with either MO1 (panels 1 and 2) or MO2 (panels 3 and 4). Panel 1 shows a 789-bp product after amplification using primers fw1 and rev1. The MO1-injected sample shows an increased production of this amplicon. Panel 2 shows a 361-bp product using primers fw1 and rev2. The MO1-injected sample shows a decreased generation of this PCR product compared with control. Panel 3 shows a 360-bp product after using primers fw2 and rev3. Increased production of this band was shown in MO2-injected samples. Panel 4 shows a 400-bp product after using primers fw2 and rev4. The MO2-injected sample shows a decreased production of this amplicon. C, Analysis by qPCR of the effect of injection of MOs and zGRβ mRNA on the expression of zGRα mRNA. Both MO treatments resulted in a significant down-regulation of the zGRα mRNA concentration compared with SC-MO injections, whereas no significant difference between SC-MO and zGRβ mRNA-injected samples was detected. Dexamethasone did not affect the expression of the zGRα mRNA. *, significantly different from vehicle control ($P < .001$). D, Analysis by qPCR of the effect of injection of MOs and zGRβ mRNA on the expression of zGRβ mRNA. MO2 caused a down-regulation of the zGRβ mRNA concentration, whereas MO1 injections resulted in an up-regulation of the zGRβ mRNA concentration compared with SC-MO injections. Similarly, zGRβ mRNA injections resulted in an increase in the zGRβ mRNA concentration compared with SC samples. No effect on the expression of the zGRβ due to dex treatment was detected. *, significantly different from vehicle control ($P < .001$).

Table 1. Overview of Experimental Groups in Present Study

Injection	Treatment	Group Number
SC-MO	Vehicle	1
	Dexamethasone	2
MO1 (zGRα ↓ , zGRβ ↓)	Vehicle	3
	Dexamethasone	4
MO2 (zGRα ↓ , zGRβ ↑)	Vehicle	5
	Dexamethasone	6
zGRβ mRNA (zGRα−, zGRβ ↑)	Vehicle	7
	Dexamethasone	8

ing MOs have been shown to cause malformations during development (48, 49). This paradox could be due to the fact that splice-blocking MO treatment does not affect maternally deposited mRNAs (so MOs start to act after the onset of zygotic transcription; ~4–5 hpf). Dexamethasone treatment did not cause any abnormalities either, probably because of the short duration of the treatment (6 h), because dex treatment for longer periods (>24 h) during embryonic development has been shown to alter craniofacial morphogenesis (43). 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.

The transcriptional role of zGRα

Microarray analysis of dex effects on gene transcription

As a first step in the analysis of the microarray experiment, the regulation of gene transcription due to dex treatment was studied. For this purpose, the SC-MO/veh and SC-MO/dex groups were compared. We identified 138 genes to be significantly dex regulated. The vast majority of these genes (129) was up-regulated in response to dex, which suggests that this cluster mainly contains genes that are transactivated by the dex-activated GR. Gene ontology analysis showed that 28 genes in this cluster were involved in metabolic processes. From these 28 genes, 8

genes were involved in glucose metabolism (4 in gluconeogenesis and 3 in glycogenolysis), 11 in protein metabolism (5 proteases), and 3 in lipid metabolism. Other gene ontology groups that were highly represented in this cluster were genes encoding transcription factors (12 genes), those involved in the immune system (11 in total, 5 anti-inflammatory and 6 proinflammatory), and those involved in nuclear receptor signaling (11 in total, 3 involved in GR signaling). An overview of the gene ontology analysis is presented in Figure 2A, and detailed information is presented in Supplemental Table 4. By means of qPCR, the induction of 4 genes from this gene cluster was verified (Supplemental Figure 1A).

In order to study whether gene regulation by dex was affected upon zGRα knockdown, we compared the cluster of dex-regulated genes with clusters of genes 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 77 genes was identified to be present in all 3 clusters (Figure 2B and Supplemental Table 4), which means that the observed regulation of these genes by dex is dependent on the presence of zGRα. These 77 genes account for 56% of the genes in the dex-regulated cluster, indicating that most dex regulation observed in our microarray is abolished upon MO1 and MO2 knockdown of zGRα, and that the MOs effectively knocked down the expression of zGRα. The cluster of genes affected by MO knockdown especially contained genes with higher levels of up-regulation by dex and those of which the up-regulation is supported by multiple probes on the microarray, suggesting that this approach filters out false positives from the dex-regulated cluster. Therefore, a corroborated set of 77 marker genes for studying GR signaling in zebrafish embryos is presented here (Supplemental Table 4).

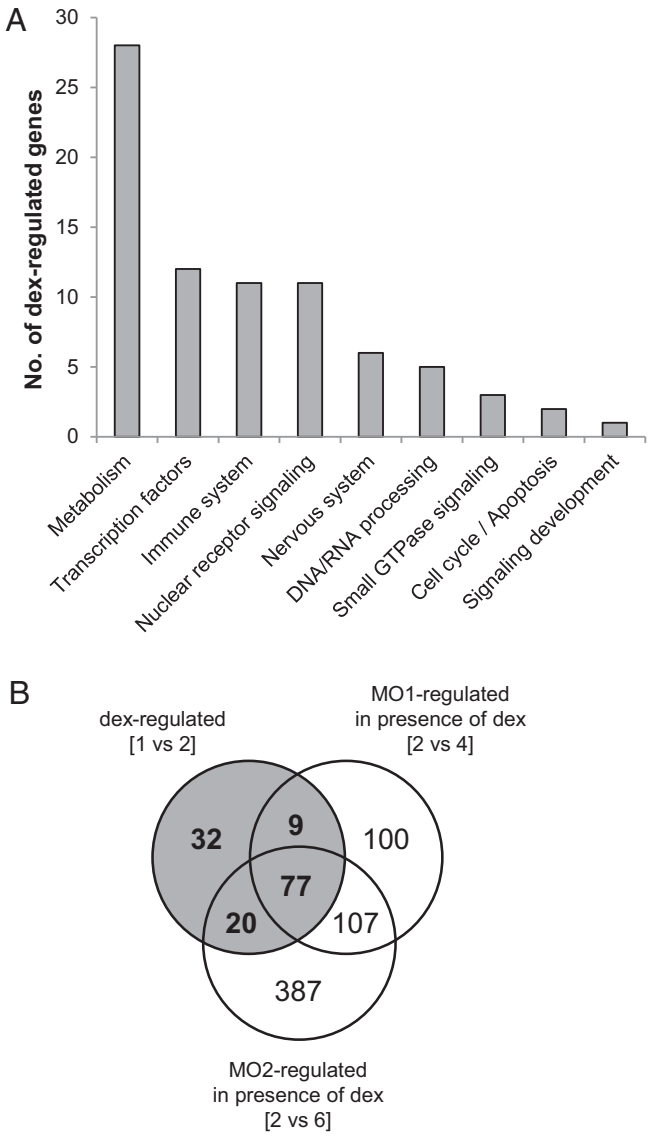

Figure 2. Microarray analysis of dex regulation. A, Gene ontology groups represented in the cluster of 138 dex-regulated genes. The results show that dex mainly regulated genes involved in metabolism. Details on individual genes are presented in Supplemental Table 1. B, Venn diagram showing the identification of the cluster of dex-regulated and zGRα-dependent genes. The overlap between genes regulated by dex (SC-MO/vehicle vs SC-MO/dex, gray circle) and genes regulated by MO1 and MO2 I the presence of dex (SC-MO/dex vs MO1/dex and SC-MO/dex vs MO2/dex comparison, white circles) was determined (77 genes). This cluster represents genes regulated by dex in a GRα-dependent way.

Microarray analysis of MO1 effects on gene transcription

The effect of zGRα knockdown was further studied by comparing the SC-MO/veh and MO1/veh groups. This analysis showed that injection of MO1 (in the absence of dex) significantly affected the expression of 185 genes (Figure 3A). Surprisingly, this MO1-regulated gene cluster was very different from the cluster regulated upon dex treatment. Only 8 genes were present in both the cluster of MO1-regulated genes and the cluster of 138 dex-regulated genes, of which only 2 genes showed regulation in opposite direction by the MO and dex. In addition, gene ontology analysis showed that MO1 and dex affected different gene ontology groups (Figures 2A and 3B). MO1 affected relatively more genes involved in cell cycle and apoptosis, whereas more genes involved in the immune system and nuclear receptor signaling were affected by dex. Even though the gene ontology group of genes involved in metabolism was highly represented in both the cluster regulated by dex (28) and MO1 (30), only 3 were present in both clusters (of which 1 regulated in opposite direction). These differences could not be explained by off-target effects of MO1, because a large overlap was observed between the clusters of MO1- and MO2-regulated genes (Figure 2A).

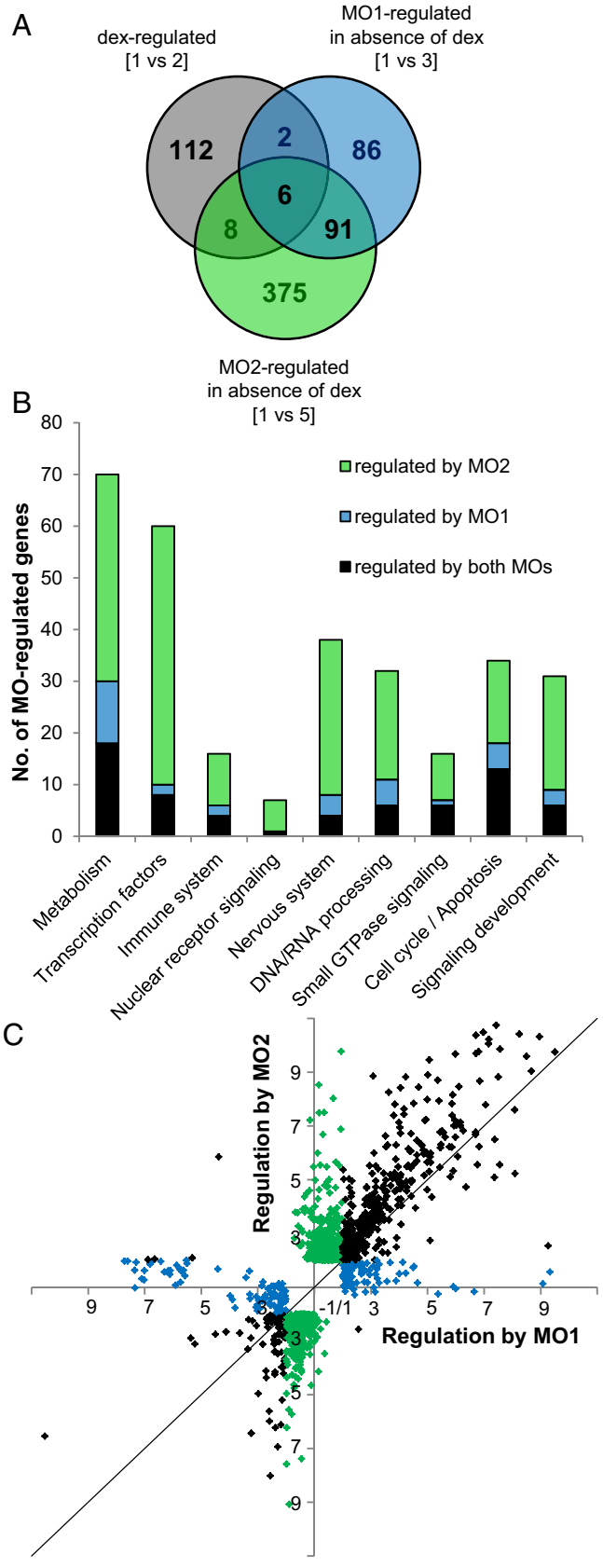

Figure 3. Microarray analysis of effects of MO treatment. A, Venn diagram showing the overlap between the cluster of genes by dex (SC-MO/vehicle vs SC-MO/dex, gray circle) and genes regulated by MO1 and MO2 (SC-MO/veh vs MO1/veh and SC-MO/veh vs MO2/veh blue and green circle, respectively). The diagram shows very little overlap, indicating that dex and MO treatment regulate highly different clusters of genes. B, Gene ontology groups represented in the clusters of genes by MO1 and/or MO2 treatment in absence of dex. The results show that MO treatment mainly regulated genes involved in metabolism and genes encoding transcription factors. Details on individual genes are presented in Supplemental Table 2. C, Scatter plot showing the level of gene regulation by MO2 treatment plotted against regulation of genes by MO1 treatment. Data points indicate levels for individual probes. Data are shown for probes that were significantly regulated at least 2-fold by MO1 and/or MO2. Black diamonds indicate data for probes significantly regulated by both MOs, blue diamonds for probes significantly regulated by MO1, and green diamonds for probes significantly regulated by MO2.

Apparently, upon dex treatment, a different set of genes was regulated by zGRα than in the absence of dex. It can therefore be suggested that zGRα acts upon different genes in the presence of endogenous cortisol only than upon activation by a pharmacological dose of an exogenous GC like dex. Moreover, both dex and MO1 treatment mainly induced gene up-regulation (respectively, 94% and 72% of the regulated genes was up-regulated). It can therefore further be suggested that zGRα mainly suppresses gene transcription upon activation by endogenous cortisol and that it enhances transcription (of a different set of genes) upon increased activation by an exogenous GC.

The transcriptional role of zGRβ

Microarray analysis of MO2 effects on gene transcription

Subsequently, genes were investigated that were regulated by MO2 (comparison SC-MO/veh vs MO2/veh). Like MO1, this MO decreases the expression of zGRα, but it simultaneously increases the expression of zGRβ (whereas MO1 causes decreased expression of zGRβ) (Figure 1). We identified a larger number of genes (480) to be regulated upon MO2 injection, of which 67% was up-regulated. A large overlap between MO1- and MO2-regulated genes was observed: 97 genes appeared to be regulated by both MO1 and MO2 (Figure 3A). This was expected because both MOs decrease the expression of zGRα. Validation by qPCR was performed for 4 of these genes confirming the observed regulation (Supplemental Figure 1B).

However, when we further compared gene regulation by MO1 and MO2, we found some interesting differences. First, MO2 showed stronger gene regulation. More genes were regulated by MO2 (480 vs 185) (Figure 3A), and most genes regulated by both MOs showed stronger regulation by MO2 than by MO1 (Figure 3C). Most likely, this is a result of the increased expression of zGRβ by MO2, compared with the decreased zGRβ expression by

MO1. Based on these results, we suggest that increased expression of zGRβ inhibits zGRα-induced suppression of gene transcription. Alternatively, the differences in gene regulation by MO1 and MO2 may be explained by a more efficient knockdown of zGRα expression by MO2. However, this seems unlikely because the observed difference in zGRα mRNA is small (Figure 1C), and upon MO1 treatment, most of the available zGRα mRNA encodes a truncated nonfunctional protein (Figure 1B).

Second, of the 159 genes down-regulated by MO2, only 14 were regulated by MO1 as well, indicating that MO2 regulates a cluster of genes that is not regulated by MO1. MO2 regulated relatively more genes encoding transcription factors and genes involved in the nervous system, processing of DNA and RNA, and signaling pathways important during development (Figure 3B and Supplemental Table 5). Thus, the increased zGRβ expression results in regulation (mainly suppression) of a cluster of genes not affected by zGRα. Alternatively, this difference may be explained by off-target effects of MO2.

Microarray analysis of zGRβ mRNA effects on gene transcription

In order to further investigate genes specifically affected due to zGRβ overexpression, we studied genes regulated upon injection of zGRβ mRNA (comparison SC-MO/veh vs GRβ mRNA/veh). A cluster of 146 genes was found to be regulated by zGRβ mRNA injection in the absence of dex, of which the majority (87 genes) was down-regulated (Figure 4A). Of these 146 genes, 67 genes were also found to be present in the cluster of 161 genes regulated by GRβ mRNA injection in the presence of dex (Figure 4A). This indicates that the observed gene regulation upon GRβ mRNA injection is mainly independent of zGRα activation. Gene ontology analysis of the cluster of genes regulated by GRβ mRNA injection either in absence or presence of dex (Figure 4B and Supplemental Table 6) revealed that 21 of these genes were involved in regulation of the immune system, of which 3 were encoding interleukins, and 4 novel immune-type receptors. Other gene ontology groups enriched in this cluster were genes involved in metabolism (14 genes, of which 10 involved in protein metabolism), the nervous system (14), and cell cycle/apoptosis (15, of which 13 involved in the cell cycle).

To determine whether this zGRβ mRNA-regulated cluster of genes is also regulated upon MO2 treatment (which results in an increased expression of zGRβ in addition to a decreased expression of zGRα), we compared it with the MO2-regulated cluster (comparison GRβ mRNA/veh vs MO2/veh). This analysis showed that only 13 genes were affected by both injection of MO2 and zGRβ mRNA (Figure 4A). This may be due to differences

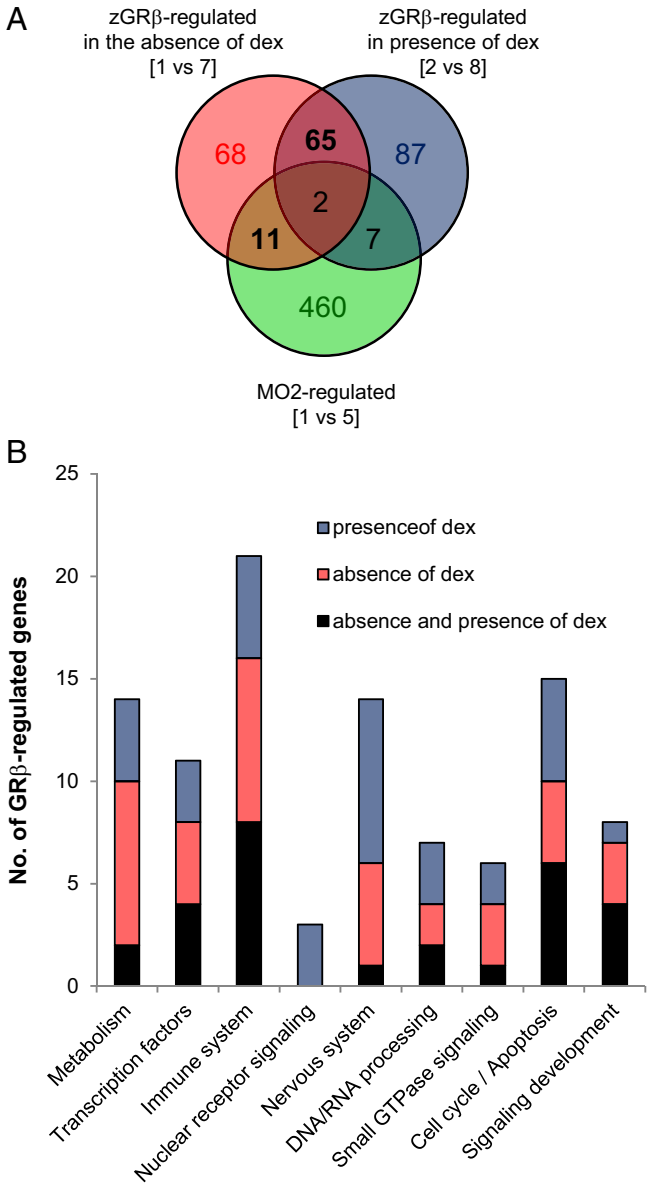

Figure 4. Microarray analysis of effects of zGRβ mRNA injection. A, Venn diagram showing the overlap between the cluster of genes regulated by zGRβ mRNA injection in the absence (SC-MO/veh vs zGRβ mRNA/veh, red circle) and presence of dex (SC-MO/dex vs zGRβ mRNA/dex, blue circle). The diagram shows large overlap between these 2 clusters (67 genes), indicating that gene regulation upon zGRβ mRNA injection is mainly independent of zGRα activation. The overlap between these 2 clusters and the cluster of genes regulated by MO2 (SC-MO/veh vs MO2/veh, green circle) is very small, although MO2 treatment also increases zGRβ expression. B, Gene ontology groups represented in the clusters of genes by zGRβ mRNA injection in absence and presence of dex. The results show that zGRβ mRNA injection mainly regulated genes involved in regulation of the immune system. Details on individual genes are presented in Supplemental Table 3.

in the context (ie, expression level of zGRα), in which zGRβ is overexpressed between the 2 methods of zGRβ overexpression.

To analyze a possible dominant-negative activity of zGRβ on the transcriptional activity of zGRα, we studied

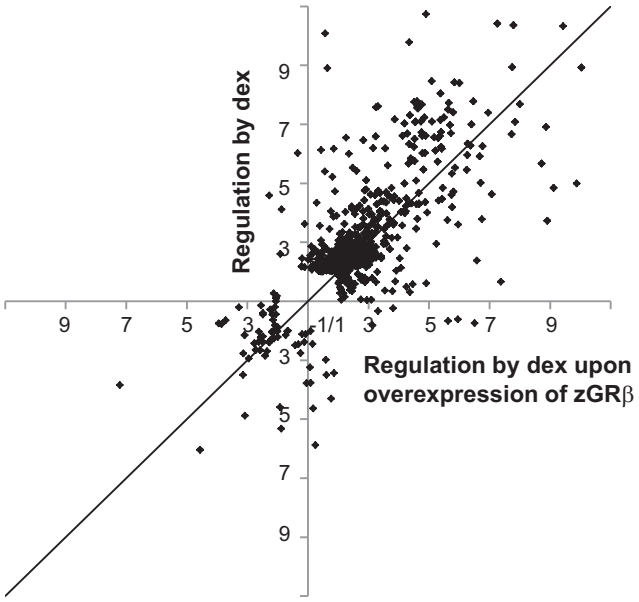

Figure 5. Scatter plot showing the level of gene regulation upon dex treatment (SC-MO/veh vs SC-MO/dex) plotted against regulation of genes by dex treatment after zGRβ mRNA injection (GRβ mRNA/veh vs GRβ mRNA/dex). Data points indicate levels for individual probes. Data are shown for probes that were significantly regulated at least 2-fold. The plotted line indicates points where dex regulation without GRβ mRNA injection equals dex regulation after GRβ mRNA injection. The scatter plot shows that generally dex regulates gene expression after zGRβ mRNA injection similarly to regulation without zGRβ mRNA injection, indicating that zGRβ displays very little dominant-negative activity under these conditions.

the effect of zGRβ overexpression on gene regulation upon dex administration. For this purpose, the level of regulation by dex after zGRβ mRNA injection (comparison zGRβ mRNA/veh vs zGRβ mRNA/dex) was plotted against the regulation by dex without zGRβ mRNA injection (comparison SC-MO/veh vs SC-MO/dex), for all probes significantly regulated in at least one of these comparisons (Figure 5). The resulting scatter plot shows that generally gene regulation by dex is not different between these 2 conditions. Thus, this analysis shows no evidence for a dominant-negative activity of zGRβ on zGRα-mediated gene transcription induced by dex.

Linking transcriptional effects to metabolic effects

NMR analysis of metabolic effects of dex and MO treatment

Because the observed transcriptional effects of dex and MO treatment mainly concerned the metabolism of the embryos, we decided to study the metabolic effects of these treatments by NMR spectroscopy. In order to study the effect of dex, embryos were treated with vehicle or dex at 24 hpf for 24 hours, and ^{1}H HR-MAS NMR spectroscopic analysis was performed on 48-hpf embryos. Metabolic profiles of vehicle- and dex-treated embryos were obtained by both 1 dimensional and 2 dimensional (2D) NMR (Supplemental Figure 2), and quantitative data on metabolite levels were acquired from the 2D profiles (Figure 6A). The NMR data showed that the glucose levels were 1.7-fold increased due to the dex treatment and that the lactate concentrations were similarly increased (1.8-fold). In addition, increased levels of the amino acids alanine, glycine, and glutamine/glutamic acid were observed (1.4-, 1.9-, and 1.7-fold increased, respectively). No effects were observed on cholesterol levels, but increased levels of fatty acids were detected (1.7-fold).

In order to study the effects of MO treatment on the

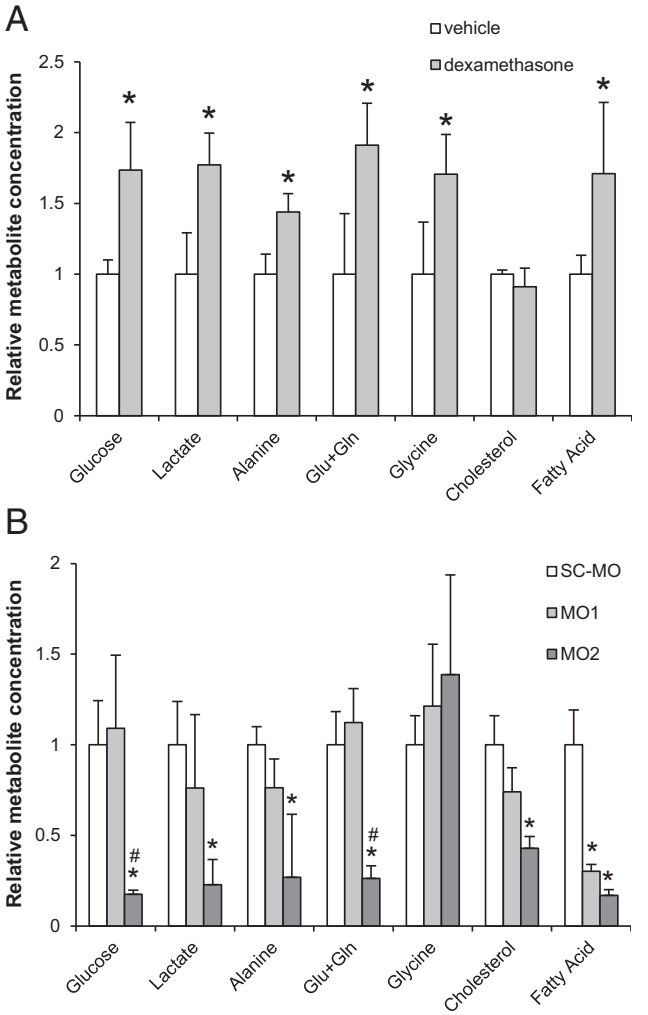

Figure 6. NMR analysis of metabolic effects of dex and MO treatment. Relative metabolite concentrations determined by 2D ^{1}H HR-MAS NMR spectroscopic analysis. A, Relative metabolite concentrations in 48-hpf embryos after 24 hours of dex treatment. All metabolites measured, except cholesterol, show a significant increase after dex treatment. *, significantly different from vehicle control ($P < .05$). B, Relative metabolite concentrations in 30-hpf embryos injected with MO1 or MO2 at the 1–2 cell stage. MO1 treatment decreased the concentration of fatty acids, whereas MO2 decreased the concentration of all metabolites, except glycine. *, significantly different from vehicle control ($P < .05$); #, significantly different from MO1-treated group ($P < .05$).

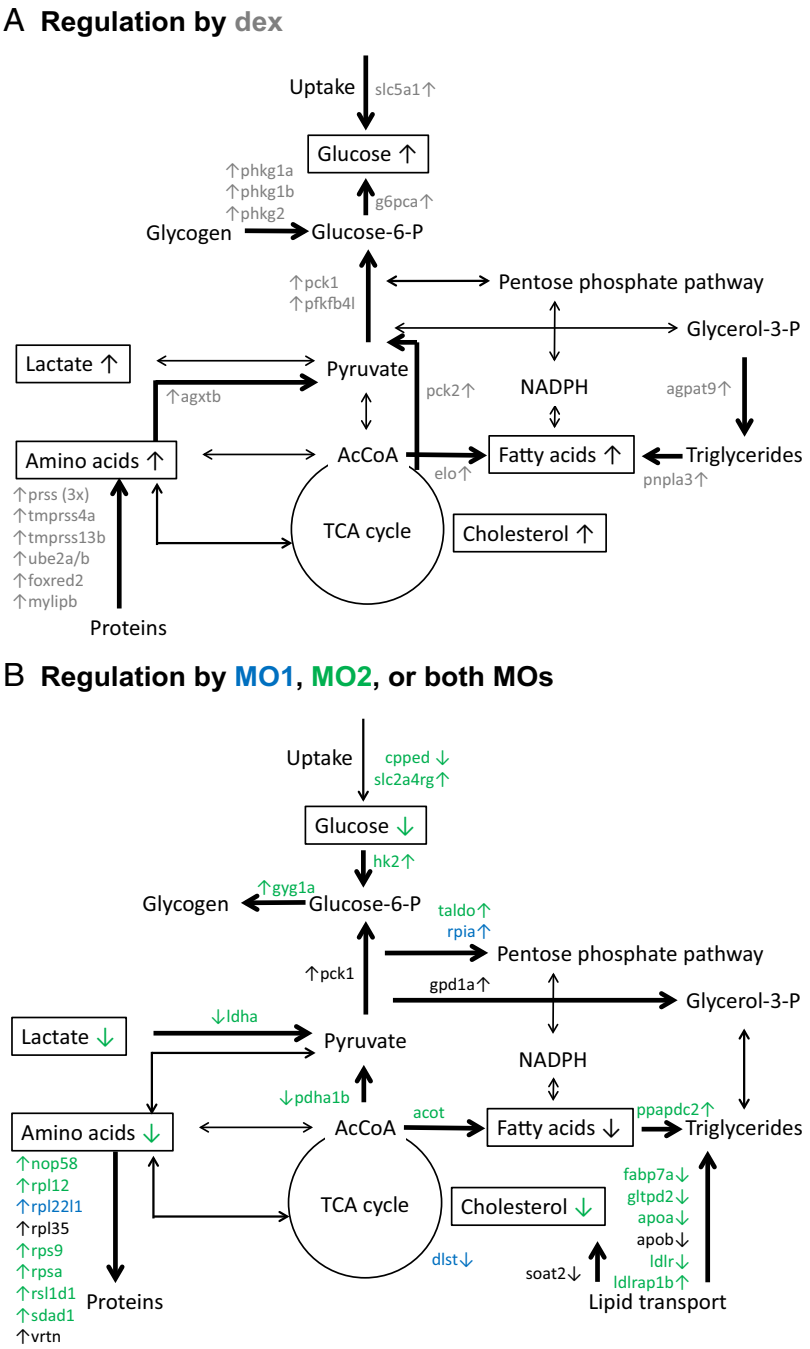

Figure 7. Schematic representation of metabolic pathways showing the link between transcriptomic and metabolomic data. Names of regulated genes involved in the regulation of a specific metabolic process are indicated (direction of regulation indicated by arrows). This regulation may explain the changes in the concentrations of specific metabolites, which are shown in boxes. A, Transcriptomic and metabolomic changes upon dex treatment. The increased glucose concentration may result from increased expression of the *pck1*, *pck2*, *pfkfb4l*, and *g6pca* genes, encoding key enzymes in the control of gluconeogenesis. The *phkg1a*, *phkg1b*, and *phkg2* genes were up-regulated, which are involved in glycogenolysis. The uptake of glucose is stimulated as well through increased expression of the *slc5a1* gene. The increased levels of amino acids may result from increased expression of 5 different serine proteases, and 3 genes involved in the ubiquitin/proteasome pathway (*ube2a/b*, *mylipb*, and *foxred2*). The expression of the *agtxb* gene was increased, which links the proteolysis to the gluconeogenesis pathway. Increased concentrations of fatty acids and cholesterol upon dex treatment may result from the increased expression of *pnpla3*, and from the increased expression of *elo*. Another regulated enzyme involved in lipid metabolism is encoded by the *agpat9* gene. B, Transcriptomic and metabolomic changes upon MO treatment. Black arrows and gene names indicate regulation by

embryonic metabolism, embryos were injected with the SC-MO, MO1, or MO2 at the 1–2 cell stage. At 30 hpf, the metabolic effects of these treatments were determined by NMR spectroscopy (Figure 6B). In general, MO1 showed little metabolic effects and MO2 showed effects opposite to those observed upon dex treatment. MO1 treatment only decreased the fatty acid concentration. MO2 treatment decreased the glucose levels as well as lactate levels (both by ~5-fold). The alanine and glutamine/glutamic acid levels were lowered after MO2 treatment (~4-fold), whereas the glycine level remained unchanged. Finally, the NMR data showed a MO2-induced decrease in free cholesterol and fatty acid levels (approximately 2- and 6-fold, respectively).

Linking metabolomic data to transcriptomic data

By combining the metabolomic data obtained using NMR spectroscopy with the transcriptomic data obtained using microarray analysis, we could determine the transcriptional GR targets underlying the observed metabolic effects. An overview of this analysis is presented in Figure 7, and it demonstrates that the metabolic changes observed in the NMR study can be explained by the transcriptional changes that were found in the microarray analysis. This way, the transcriptional targets of GCs that underlie their metabolic effect were identified, and a detailed description and discussion of these results is presented in Supplemental Results and Discussion.

Discussion

In the present study, we have revealed specific target genes of the zGRα and zGRβ. Zebrafish embryos were used as an in vivo model sys-

tem, and this whole-body approach resulted in a broad view of systems regulated by GCs. Our results show that zGRα regulates 2 distinct gene clusters. Furthermore, we show evidence for a dominant-negative role of zGRβ on zGRα's activity, and for a role of this splice variant as a regulator of transcription independent of zGRα.

It was expected that most genes regulated by dex would show an inverse regulation upon GRα knockdown by the MO treatment. However, we found a surprisingly small overlap between the cluster of dex-regulated genes and the clusters of MO-regulated genes, which indicates that zGRα regulates a different set of target genes upon activation by endogenous cortisol than upon activation by a high dose of dex. At the stage of development investigated in this study (24–30 hpf), endogenous cortisol levels are at their minimum, because the maternally deposited cortisol is depleted, and the embryonic hypothalamus-pituitary-interrenal axis is not functional yet (39). It can therefore be argued that in our study zGRα is minimally activated by endogenous cortisol. Moreover, it might be suggested that the unliganded zGRα is a regulator of gene expression under these conditions, as previously observed in mammary epithelial cells (50). In contrast, the dose of dex used in our study (100μM) is considered to result in maximal activation of the receptor, based on pilot studies in which a maximal induction of the *fkbp5* gene was observed at this dose (A.C. and M.J.M.S., unpublished data).

The lack of overlap between MO- and dex-regulated genes is in line with the observation of Pikulkaew et al (49), who noted that genes known to be up-regulated in response to dex, like *fkbp5* and *gilz* (*tsc22d3*), were not regulated by MO knockdown of GR. In our study, upon dex treatment mainly genes involved in metabolic pathways were regulated, whereas MO knockdown of the receptor regulated in addition to metabolic genes many genes involved in the cell cycle and apoptosis. Because both receptor knockdown and dex treatment generally increased gene transcription rates in our study, we further suggest that upon activation by endogenous cortisol zGRα mainly suppresses gene transcription, and that it mainly enhances transcription upon increased activation by a high dose of dex.

These findings put forward the idea of 2 distinct gene sets: one set to be regulated under basal conditions for the maintenance of homeostasis and the other to be regulated upon a stressful stimulus in order to restore homeostasis (51). It can be hypothesized that the potency of zGRα to regulate transcription differs between target genes. Hence, some genes could already be activated upon zGRα activation by low cortisol levels, whereas other genes may need increased levels of activated zGRα (51–53). In support of this notion, a recent study by Reddy et al (54) showed variation in the sensitivity of genes to small interfering RNA (siRNA) treatment against the GR. This may be due to different binding affinities for ligand-bound receptors to the GC-response elements. In line with this idea, Polman et al (55) have recently found 2 populations of DNA target sites with different receptor binding affinities in the genome of rat hippocampal cells. In addition, it was previously demonstrated in rat neuronal cells that 1 hour after GR activation, only down-regulation of genes was induced, whereas after 3 hours, most regulated genes were up-regulated. This suggests that up-regulation of genes requires increased activation of GR (56), which is in line with our finding that dex treatment mainly increases gene expression, whereas zGRα activation by endogenous cortisol levels seems to mainly down-regulate gene expression. Finally, Frijters et al (57) found differences in the dependence on GR dimerization between different gene clusters regulated in mouse liver by prednisolone. The cluster of genes of which the prednisolone induction was abolished in mice expressing a dimerization-deficient receptor mainly contained genes involved in cell cycle and apoptosis, whereas the cluster of genes least affected by the lack of dimerization mainly contained genes involved in glucose metabolism. Interestingly, our data show that upon increased activation mainly metabolic genes are regulated by zGRα, and under basal conditions, many genes involved in cell cycle and apoptosis.

Our dex-responsive gene cluster could complement data derived from previous studies in which researchers also used microarray technology in order to identify mammalian GR target genes (58–61). In some of these studies, gene ontology analysis also revealed GR activation to affect metabolic pathways as we found in our dataset (62, 63). Moreover, further examination showed that for most

Figure. 7 (Continued). both MOs, blue arrows and gene names regulation by MO1, and green arrows and gene names regulation by MO2. Glucose and lactate levels were decreased by MO2 treatment, which may result from increased expression of the *hk2*, *pdha1b*, and *ldha* genes, suggesting that both aerobic and anaerobic catabolism of glucose are decreased. Conversion to glycogen appears to be up-regulated, indicated by an increased expression of the *gyg1a* gene. An increased incorporation of cholesterol and fatty acids in triglycerides may explain the decreased free cholesterol and fatty acid levels observed by NMR, and the increased expression of the *ppapdc2*, *taldo1*, and *gpd1a* genes may be involved in increased triglyceride synthesis. Six genes involved in lipid transport (*apoa*, *apob*, *ldlr*, *gltpd2*, *fabp7a*, and *soat2*) were down-regulated by MO2 treatment (whereas the *ldlrap1b* was up-regulated). Furthermore, the expression of 8 ribosomal proteins (*nop58*, *rpl12*, *rpl35*, *rpsa*, *rps9*, *rsl1d1*, *sdad1*, and *vrtn*) was up-regulated upon MO2 administration, as well as 4 genes involved in mRNA processing (*ddx19*, *eif4e1b*, *ppan*, and *zc3h14*) and 7 genes involved in rNRA processing (*dkc1*, *ebna1bp2*, *isg20l2*, *mki67ip*, *mphosph10*, *npm1a*, and *rrp8*), indicating an increased rate of de novo protein synthesis, which may explain the decreased alanine and glutamine/glutamic acid levels.

of these dex-regulated genes the induction upon dex treatment was significantly reduced due to suppression of zGRα expression upon MO1 and MO2 injections. Thus, this analysis provided us with a corroborated set of genes that are regulated upon dex treatment and of which this regulation is zGRα dependent.

We have been able to link the observed transcriptional changes to physiological effects by measuring metabolic effects using NMR spectroscopy. Using this approach, it was observed that dex increases the levels of glucose, amino acids, and fatty acids. This could be explained by the increased expression of genes involved in gluconeogenesis and glycogenolysis, proteolysis and peptidase activity, and triglyceride breakdown. Thus, by combining the effects on transcription to metabolic effects, we identified the transcriptional targets underlying the GC regulation of metabolism. A detailed description of this analysis is presented in Supplemental Results and Discussion. This description shows that the described transcriptional and metabolic effects are very similar to well-established GC effects in (adult) mammalian systems, which in humans are known to increase the risk of obesity, hyperglycemia, and diabetes in humans. Apparently, the observed transcriptional and metabolic GC effects reflect GC-induced changes that occur generally in vertebrate organisms. The zebrafish embryo model, although it is a developing organism, can therefore be considered an appropriate model system for studies on GC effects on metabolism and related human pathology.

In addition to the analysis of zGRα signaling, the transcriptional role of zGRβ was investigated. We first examined a possible dominant-negative activity of zGRβ. When zGRβ was overexpressed using a splice-blocking MO (MO2), the results suggested that zGRβ inhibits the transcriptional activity of zGRα. However, when zGRβ was overexpressed by injection of zGRβ mRNA, the resulting data indicated the absence of a significant inhibitory role of zGRβ on the transcriptional properties of zGRα. This apparent discrepancy may be explained by a higher zGRβ and a lower zGRα expression level after MO2 injection compared with GRβ mRNA injection, resulting in an increased zGRβ to zGRα ratio. Conflicting data on the dominant-negative role of GRβ have been obtained from overexpression studies of the human GRβ as well, which revealed inconsistencies concerning its inhibitory effects among different experimental setups (19–21, 24, 25, 29, 30). In addition, zGRβ's inhibitory role may be more specific than previously thought, because it was recently shown that hGRβ only inhibits the transrepression activity of hGRα (30) and that GRβ's dominant-negative activity was specific for a subset of mainly metabolic genes in mice (34).

In addition, the human GRβ has been suggested to have intrinsic transcriptional activity, which was shown in studies in cell lines overexpressing hGRβ (22, 23). In the present study, overexpression of zGRβ, either by injection of zGRβ mRNA or MO2, resulted in the regulation of a large number of genes, suggesting intrinsic transcriptional activity of zGRβ. Both methods of overexpression mainly showed a suppressive activity independent of zGRα, but surprisingly little overlap was found between the clusters of genes regulated by the 2 approaches. This discrepancy may be due to differences in the zGRβ to zGRα ratio, but also the temporal pattern of zGRβ overexpression. A novel possible mode of action has recently been suggested for the human GRβ in which it acts as a coactivator of a transcription factor of the T-cell factor/Lymphoid enhancer-binding factor (TCF/LEF) family (in particular TCF-4), thereby enhancing downstream effects of the Wingless/Integrated (Wnt) signaling pathway (64, 65). In our study, genes involved in signaling pathways important for development (especially Wnt signaling) were preferentially regulated by MO2, suggesting that zGRβ may play a role as a transcriptional coactivator in these signaling pathways.

In conclusion, using the zebrafish as an in vivo model system, we have identified the transcriptional targets of zGRα and zGRβ action. Two distinct clusters of zGRα-regulated genes were observed, and evidence for a functional role of zGRβ was found.

Acknowledgments

Address all correspondence and requests for reprints to: Marcel J.M. Schaaf, Einsteinweg 55, 2333CC Leiden, The Netherlands. E-mail: m.j.m.schaaf@biology.leidenuniv.nl.

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

Disclosure Summary: The authors have nothing to disclose.

References

1. **Heitzer MD, Wolf IM, Sanchez ER, Witchel SF, DeFranco DB.** Glucocorticoid receptor physiology. *Rev Endocr Metab Disord*. 2007; 8:321–330.
2. **Schoneveld OJ, Gaemers IC, Lamers WH.** Mechanisms of glucocorticoid signalling. *Biochim Biophys Acta*. 2004;1680:114–128.
3. **Sapolsky RM, Romero LM, Munck AU.** How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr Rev*. 2000;21:55–89.
4. **Revollo JR, Cidlowski JA.** Mechanisms generating diversity in glucocorticoid receptor signaling. *Ann NY Acad Sci*. 2009;1179:167–178.
5. **de Kloet ER, Joëls M, Holsboer F.** Stress and the brain: from adaptation to disease. *Nat Rev Neurosci*. 2005;6:463–475.

6. **Chrousos GP, Kino T.** Intracellular glucocorticoid signaling: a formerly simple system turns stochastic. *Sci STKE*. 2005:pe48.
7. **Nicolaides NC, Galata Z, Kino T, Chrousos GP, Charmandari E.** The human glucocorticoid receptor: molecular basis of biologic function. *Steroids*. 2010;75:1–12.
8. **Beato M, Klug J.** Steroid hormone receptors: an update. *Hum Reprod Update*. 2000;6:225–236.
9. **Buckingham JC.** Glucocorticoids: exemplars of multi-tasking. *Br J Pharmacol*. 2006;147(suppl 1):S258–S268.
10. **van der Laan S, Meijer OC.** Pharmacology of glucocorticoids: beyond receptors. *Eur J Pharmacol*. 2008;585:483–491.
11. **De Bosscher K, Haegeman G.** Minireview: latest perspectives on antiinflammatory actions of glucocorticoids. *Mol Endocrinol*. 2009;23:281–291.
12. **Schäcke H, Döcke WD, Asadullah K.** Mechanisms involved in the side effects of glucocorticoids. *Pharmacol Ther*. 2002;96:23–43.
13. **Vegiopoulos A, Herzig S.** Glucocorticoids, metabolism and metabolic diseases. *Mol Cell Endocrinol*. 2007;275:43–61.
14. **Rose AJ, Herzig S.** Metabolic control through glucocorticoid hormones: an update. *Mol Cell Endocrinol*. 2013;380:65–78.
15. **Hollenberg SM, Weinberger C, Ong ES, et al.** Primary structure and expression of a functional human glucocorticoid receptor cDNA. *Nature*. 1985;318:635–641.
16. **Encío IJ, Detera-Wadleigh SD.** The genomic structure of the human glucocorticoid receptor. *J Biol Chem*. 1991;266:7182–7188.
17. **Kino T, Su YA, Chrousos GP.** Human glucocorticoid receptor isoform β: recent understanding of its potential implications in physiology and pathophysiology. *Cell Mol Life Sci*. 2009;66:3435–3448.
18. **Bamberger CM, Bamberger AM, de Castro M, Chrousos GP.** Glucocorticoid receptor β, a potential endogenous inhibitor of glucocorticoid action in humans. *J Clin Invest*. 1995;95:2435–2441.
19. **Oakley RH, Jewell CM, Yudt MR, Bofetiado DM, Cidlowski JA.** The dominant negative activity of the human glucocorticoid receptor β isoform. Specificity and mechanisms of action. *J Biol Chem*. 1999;274:27857–27866.
20. **Goleva E, Li LB, Eves PT, Strand MJ, Martin RJ, Leung DY.** Increased glucocorticoid receptor β alters steroid response in glucocorticoid-insensitive asthma. *Am J Respir Crit Care Med*. 2006;173:607–616.
21. **Zhang X, Clark AF, Yorio T.** Regulation of glucocorticoid responsiveness in glaucomatous trabecular meshwork cells by glucocorticoid receptor. *Invest Ophthalmol Vis Sci*. 2005;46:4607–4616.
22. **Lewis-Tuffin LJ, Jewell CM, Bienstock RJ, Collins JB, Cidlowski JA.** Human glucocorticoid receptor binds RU-486 and is transcriptionally active. *Mol Cell Biol*. 2007;27:2266–2282.
23. **Kino T, Manoli I, Kelkar S, Wang Y, Su YA, Chrousos GP.** Glucocorticoid receptor (GR) β has intrinsic, GRα-independent transcriptional activity. *Biochem Biophys Res Commun*. 2009;381:671–675.
24. **Hecht K, Carlstedt-Duke J, Stierna P, Gustafsson J, Brönnegård M, Wikström AC.** Evidence that the β-isoform of the human glucocorticoid receptor does not act as a physiologically significant repressor. *J Biol Chem*. 1997;272:26659–26664.
25. **Brogan IJ, Murray IA, Cerillo G, Needham M, White A, Davis JR.** Interaction of glucocorticoid receptor isoforms with transcription factors AP-1 and NF-κB: lack of effect of glucocorticoid receptor β. *Mol Cell Endocrinol*. 1999;157:95–104.
26. **Bamberger CM, Else T, Bamberger AM, Beil FU, Schulte HM.** Regulation of the human interleukin-2 gene by the α and β isoforms of the glucocorticoid receptor. *Mol Cell Endocrinol*. 1997;136:23–28.
27. **Carlstedt-Duke J.** Glucocorticoid Receptor β: view II. *Trends Endocrinol Metab*. 1999;10:339–342.
28. **Gougat C, Jaffuel D, Gagliardo R, et al.** Overexpression of the human glucocorticoid receptor α and β isoforms inhibits AP-1 and NF-κB activities hormone independently. *J Mol Med (Berl)*. 2002;80:309–318.
29. **Kelly A, Bowen H, Jee Y, et al.** The glucocorticoid receptor β isoform can mediate transcriptional repression by recruiting histone deacetylases. *J Allergy Clin Immunol*. 2008;121:203–208.e201.
30. **Taniguchi Y, Iwasaki Y, Tsugita M, et al.** Glucocorticoid receptor- and receptor- exert dominant negative effect on gene repression but not on gene induction. *Endocrinology*. 2010;151:3204–3213.
31. **de Castro M, Elliot S, Kino T, et al.** The non-ligand binding β-isoform of the human glucocorticoid receptor (hGR β): tissue levels, mechanism of action, and potential physiologic role. *Mol Med*. 1996;2:597–607.
32. **Pujols L, Mullol J, Roca-Ferrer J, et al.** Expression of glucocorticoid receptor alpha- and β-isoforms in human cells and tissues. *Am J Physiol Cell Physiol*. 2002;283:C1324–C1331.
33. **Schaaf MJ, Champagne D, van Laanen IH, et al.** Discovery of a functional glucocorticoid receptor β-isoform in zebrafish. *Endocrinology*. 2008;149:1591–1599.
34. **Hinds TD Jr, Ramakrishnan S, Cash HA, et al.** Discovery of glucocorticoid receptor- in mice with a role in metabolism. *Mol Endocrinol*. 2010;24:1715–1727.
35. **Brittijn SA, Duivesteijn SJ, Belmamoune M, et al.** Zebrafish development and regeneration: new tools for biomedical research. *Int J Dev Biol*. 2009;53:835–850.
36. **Xi Y, Noble S, Ekker M.** Modeling neurodegeneration in zebrafish. *Curr Neurol Neurosci Rep*. 2011;11:274–282.
37. **Lieschke GJ, Currie PD.** Animal models of human disease: zebrafish swim into view. *Nat Rev Genet*. 2007;8:353–367.
38. **Schaaf MJ, Chatzopoulou A, Spaink HP.** The zebrafish as a model system for glucocorticoid receptor research. *Comp Biochem Physiol A Mol Integr Physiol*. 2009;153:75–82.
39. **Alsop D, Vijayan MM.** Molecular programming of the corticosteroid stress axis during zebrafish development. *Comp Biochem Physiol A Mol Integr Physiol*. 2009;153:49–54.
40. **Steenbergen PJ, Richardson MK, Champagne DL.** The use of the zebrafish model in stress research. *Prog Neuropsychopharmacol Biol Psychiatry*. 2011;35:1432–1451.
41. **Schoonheim PJ, Chatzopoulou A, Schaaf MJ.** The zebrafish as an in vivo model system for glucocorticoid resistance. *Steroids*. 2010;75:918–925.
42. **Wendelaar Bonga SE.** The stress response in fish. *Physiol Rev*. 1997;77:591–625.
43. **Hillegass JM, Villano CM, Cooper KR, White LA.** Glucocorticoids alter craniofacial development and increase expression and activity of matrix metalloproteinases in developing zebrafish (*Danio rerio*). *Toxicol Sci*. 2008;102:413–424.
44. **Zhang Y, Bai XT, Zhu KY, et al.** In vivo interstitial migration of primitive macrophages mediated by JNK-matrix metalloproteinase 13 signaling in response to acute injury. *J Immunol*. 2008;181:2155–2164.
45. **Alsop D, Vijayan M.** The zebrafish stress axis: molecular fallout from the teleost-specific genome duplication event. *Gen Comp Endocrinol*. 2009;161:62–66.
46. **Rauwerda H, de Jong M, de Leeuw WC, Spaink HP, Breit TM.** Integrating heterogeneous sequence information for transcriptome-wide microarray design; a zebrafish example. *BMC Res Notes*. 2010;3:192.
47. **Mathew LK, Sengupta S, Kawakami A, et al.** Unraveling tissue regeneration pathways using chemical genetics. *J Biol Chem*. 2007;282:35202–35210.
48. **Pikulkaew S, Benato F, Celeghin A, et al.** The knockdown of maternal glucocorticoid receptor mRNA alters embryo development in zebrafish. *Dev Dyn*. 2011;240:874–889.
49. **Nesan D, Kamkar M, Burrows J, Scott IC, Marsden M, Vijayan MM.** Glucocorticoid receptor signaling is essential for mesoderm formation and muscle development in zebrafish. *Endocrinology*. 2012;53:1288–1300.
50. **Ritter HD, Mueller CR.** Expression microarray identifies the unli-

ganded glucocorticoid receptor as a regulator of gene expression in mammary epithelial cells. *BMC Cancer*. 2014;14:275.
51. **Meijer OC.** Understanding stress through the genome. *Stress*. 2006; 9:61–67.
52. **So AY, Chaivorapol C, Bolton EC, Li H, Yamamoto KR.** Determinants of cell- and gene-specific transcriptional regulation by the glucocorticoid receptor. *PLoS Genet*. 2007;3:e94.
53. **Lefstin JA, Yamamoto KR.** Allosteric effects of DNA on transcriptional regulators. *Nature*. 1998;392:885–888.
54. **Reddy TE, Pauli F, Sprouse RO, et al.** Genomic determination of the glucocorticoid response reveals unexpected mechanisms of gene regulation. *Genome Res*. 2009;19:2163–2171.
55. **Polman JA, de Kloet ER, Datson NA.** Two populations of glucocorticoid receptor-binding sites in the male rat hippocampal genome. *Endocrinology*. 2013;154:1832–1844.
56. **Morsink MC, Joëls M, Sarabdjitsingh RA, Meijer OC, De Kloet ER, Datson NA.** The dynamic pattern of glucocorticoid receptor-mediated transcriptional responses in neuronal PC12 cells. *J Neurochem*. 2006;99:1282–1298.
57. **Frijters R, Fleuren W, Toonen EJ, et al.** Prednisolone-induced differential gene expression in mouse liver carrying wild type or a dimerization-defective glucocorticoid receptor. *BMC Genomics*. 2010;11:359.
58. **John S, Sabo PJ, Johnson TA, et al.** Interaction of the glucocorticoid receptor with the chromatin landscape. *Mol Cell*. 2008;29:611–624.
59. **Phuc Le P, Friedman JR, Schug J, et al.** Glucocorticoid receptor-dependent gene regulatory networks. *PLoS Genet*. 2005;1:e16.
60. **Wang JC, Derynck MK, Nonaka DF, Khodabakhsh DB, Haqq C, Yamamoto KR.** Chromatin immunoprecipitation (ChIP) scanning identifies primary glucocorticoid receptor target genes. *Proc Natl Acad Sci USA*. 2004;101:15603–15608.
61. **Rogatsky I, Wang JC, Derynck MK, et al.** Target-specific utilization of transcriptional regulatory surfaces by the glucocorticoid receptor. *Proc Natl Acad Sci USA*. 2003;100:13845–13850.
62. **Stojadinovic O, Lee B, Vouthounis C, et al.** Novel genomic effects of glucocorticoids in epidermal keratinocytes: inhibition of apoptosis, interferon-γ pathway, and wound healing along with promotion of terminal differentiation. *J Biol Chem*. 2007;282:4021–4034.
63. **James CG, Ulici V, Tuckermann J, Underhill TM, Beier F.** Expression profiling of Dexamethasone-treated primary chondrocytes identifies targets of glucocorticoid signalling in endochondral bone development. *BMC Genomics*. 2007;8:205.
64. **Yin Y, Zhang X, Li Z, et al.** Glucocorticoid receptor β regulates injury-mediated astrocyte activation and contributes to glioma pathogenesis via modulation of β-catenin/TCF transcriptional activity. *Neurobiol Dis*. 2013;59:165–176.
65. **Wang Q, Lu PH, Shi ZF, et al.** Glucocorticoid receptor β acts as a co-activator of T-cell factor 4 and enhances glioma cell proliferation. *Mol Neurobiol*. 2014; Published online ahead of print October 10, 2014. 10.1007/s12035-014-8900-9.