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Review

The zebrafish as a model system for glucocorticoid receptor research☆

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ABSTRACT

Glucocorticoids regulate a plethora of physiological processes, and are widely used clinically as anti-inflammatory drugs. Their effects are mediated by the glucocorticoid receptor (GR), a ligand-activated transcription factor. Currently, zebrafish embryos are being developed into a model system for GR research, since they are easy to manipulate genetically and their phenotype can easily be visualized because of their transparent bodies. In addition, the zebrafish GR gene shows a relatively high level of similarity with its human equivalent. First, both the zebrafish and the human genome contain only a single gene encoding the GR. In all other fish species studied thus far, two GR genes have been found. Second, the zebrafish contains a C-terminal GR splice variant with high similarity to the human GRβ, which has been shown to be a dominant-negative inhibitor of the canonical GRα and may be involved in glucocorticoid resistance. Thus, zebrafish embryos are potentially a useful model system for glucocorticoid receptor research, but currently only a limited number of tools is available. In this review, we discuss which tools are available and which need to be developed, in order to exploit the full potential of the zebrafish as a model system for GR research.

Contents

1. Introduction

In the present review we will discuss how research on the glucocorticoid receptor (GR) may benefit from using the zebrafish (*Danio rerio*) as an animal model system. The zebrafish could be a valuable tool, both in fundamental studies on the molecular mechanisms of GR action and in applied research like screening of glucocorticoid drugs. We will present the advantages of this model system for GR research. However, since the zebrafish has mostly been used as an animal model in the field of developmental biology, several specific tools required for research on the GR in zebrafish are lacking. We will give an overview of which tools are already available and which tools need to be developed in order to exploit the full potential of the zebrafish as a model system for GR research.

1.1. Glucocorticoids and the glucocorticoid receptor

Glucocorticoids are steroid hormones that are secreted by the adrenal gland after stress and in a circadian rhythm. In humans and

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fish, the main endogenous glucocorticoid is cortisol, whereas corticosterone is the main glucocorticoid in rodents. These hormones regulate a wide range of processes, like the immune response (Barnes, 2006), neural activity and behavior (de Kloet et al., 2005), metabolism (Wang, 2005) and bone formation (Migliaccio et al., 2007). They are well known for their anti-inflammatory effects, and are widely used clinically to treat immune-related diseases like asthma and rheumatoid arthritis. Synthetic analogs of glucocorticoids are among the most prescribed drugs in the world.

The effects of glucocorticoids are mediated by an intracellular receptor, the glucocorticoid receptor (GR). This receptor is a member of the family of steroid receptors, which in turn belong to the superfamily of nuclear receptors (Zhang et al., 2004). Like all nuclear receptors, the GR acts as a ligand-activated transcription factor, and it is well conserved among vertebrate animal species (Bridgham et al., 2006). It consists of a large N-terminal domain, involved in transcriptional activation, a small DNA binding domain which contains two zinc-fingers and a C-terminal ligand-binding domain (Giguere et al., 1986). In the absence of hormone, the GR resides in the cytoplasm where it forms a complex with heat shock proteins and immunophilins (Pratt and Toft, 2003). Upon ligand binding, the receptor dissociates from the complex and translocates to the nucleus. There the activated GR can bind to glucocorticoid response elements (GREs) in the promoter region of target genes and interact with transcriptional cofactors (Beato and Klug, 2000). In this way, gene transcription of the downstream gene is activated and this process is called transactivation. Alternatively, the GR can inhibit gene expression induced by other transcription factors like nuclear factor(NF)-κB and activator protein(AP)-1 (De Bosscher et al., 2008). This process is called transrepression and forms the basis of the anti-inflammatory action of glucocorticoids, since these transcription factors are involved in the transcription of many pro-inflammatory genes. The exact mechanism of transrepression has not been elucidated yet, but physical interaction between GR and the other transcription factor and recruitment of specific transcriptional cofactors appears to be involved.

1.2. The zebrafish as a model organism

The zebrafish has many advantages over other vertebrate animal model systems (Trede et al., 2004; Lieschke and Currie, 2007; Hsu et al., 2007; Levraud et al., 2008). It is small, easily maintained and breeds well under laboratory conditions. Each female can produce hundreds of eggs per day, that are fertilized externally. Upon fertilization, the embryos develop rapidly and most organ systems have been formed 5 days later. The *ex utero* development makes the zebrafish embryos easily accessible for transient genetic manipulation by microinjection of DNA, mRNA or morpholinos, which are antisense DNA oligonucleotides that can alter protein synthesis in the developing embryo by blocking a specific translation start site or a splice donor or acceptor site. The embryos are transparent, which allows for microscopic imaging at the subcellular level, especially when performed in combination with fluorescent labeling of specific cells or proteins. Furthermore, an increasing number of transgenic and mutant zebrafish lines are available, as well as several zebrafish cell lines derived from embryos and adult tissues, that can be used as a complementary tool allowing more refined biochemical characterizations (Driever and Rangini, 1993; Chen et al., 2002). The zebrafish genome, as available in the zv7 assembly on the Ensembl website (http://www.ensembl.org/index.html), is virtually complete. Seventy percent of the genome has been sequenced with >99.999% accuracy. For the rest of the genome, a so-called whole genome shotgun approach has been used, which has a coverage of 5.5 times. The sequence database has been compared to the data obtained from a double haploid zebrafish line.

2. The zebrafish glucocorticoid receptor

2.1. A single GR gene in zebrafish

Most teleostean fish species contain two glucocorticoid receptor genes, as a result of a genome duplication that occurred during fish evolution between 350 and 400 million years ago, soon after the fish and tetrapod lineages diverged (Volff, 2005). The resulting receptor proteins are called GR1 and GR2 (Stolte et al., 2006). These isoforms have been established for rainbow trout (Bury et al., 2003), Burton's mouthbrooder (Greenwood et al., 2003), green spotted puffer fugu (Stolte et al., 2006), common carp (Stolte et al., 2008a), and sea bass (Terova et al., 2005; Vizzini et al., 2007). In some fish species like the Japanese flounder and brown trout (Stolte et al., 2006), only one GR gene has been found thus far, but it is yet unclear if they contain a second GR gene, since most of these fish species are poorly studied.

The organization of these fish GR1 and GR2 genes is highly similar to the organization of the human GR gene (Stolte et al., 2006). They consist of 9 exons, of which the first is entirely noncoding and the ninth contains the 3'UTR. Alternative splicing has been demonstrated to occur in the GR1 gene between exon 3 and 4, resulting in a 9 amino acid insert between the two zinc fingers of the DNA binding domain that decrease the DNA binding affinity of the receptor (this longer GR1 isoform is called GR1a, whereas the shorter form is GR1b). At the protein level, fish GRs display a high level of similarity to the human GR as well. In the ligand-binding domain, between 85% and 95% of the amino acids of fish GRs are similar to those in the human GR and in the DNA binding domain this number exceeds 98% for most fish GRs studied (Fig. 1A).

GR1 and GR2 both appear to induce transcription on GRE-containing promoters, but the concentrations at which transactivation is induced differs greatly. The EC50 for cortisol in *in vitro* reporter assays was approximately 65 times higher for rainbow trout GR1 compared to GR2 (Bury et al., 2003), and similar results have been found for the Burton's mouthbrooder and common carp GR1 and GR2 (Greenwood et al., 2003; Stolte et al., 2008c). It is therefore hypothesized that GR2 is active at low basal cortisol levels, whereas GR1 is the 'stress receptor' that becomes active at higher circulating cortisol concentrations. Differential regulation of the expression of GR1 and GR2 has been observed after stress and immune challenges, again implying different roles for the two GRs (Stolte et al., 2008b,c).

Surprisingly, the zebrafish genome only contains one GR gene (Stolte et al., 2006; Schaaf et al., 2008; Alsop and Vijayan, 2008). This lack of a second GR gene has been reported in several studies, and three lines of evidence support this finding (Schaaf et al., 2008). First, BLAST searches in the most recently released version of the zebrafish genome (the zv7 assembly on the Ensembl website) using other fish GRs as queries returned all other zebrafish steroid receptors, and many other nuclear receptors, but not a second GR gene. Second, searches in GenBank for transcripts derived from a zebrafish GR gene revealed fourteen putative zebrafish GR cDNA and EST sequences, but further analysis demonstrated that all these sequences were transcripts from the single GR gene that had been identified already. Third, analysis of the syntenic regions of the fish GR genes shows that the genomic region surrounding the zebrafish GR gene is well conserved and is highly similar to the region surrounding the GR2 gene of fugu, green spotted puffer medaka and stickleback. The region surrounding the GR1 gene in these fishes has undergone major rearrangement, which has resulted in the loss of the GR1 gene in zebrafish. This is in line with our finding that the zebrafish GR clusters within the GR2 clade of fish GRs in a phylogenetic tree (Fig. 2). The loss of the GR1 gene has happened relatively late in the evolution of the zebrafish, since the common carp (which is a member of the family of cyprinids, like the zebrafish) has been shown to contain two GR genes (Stolte et al., 2008a).

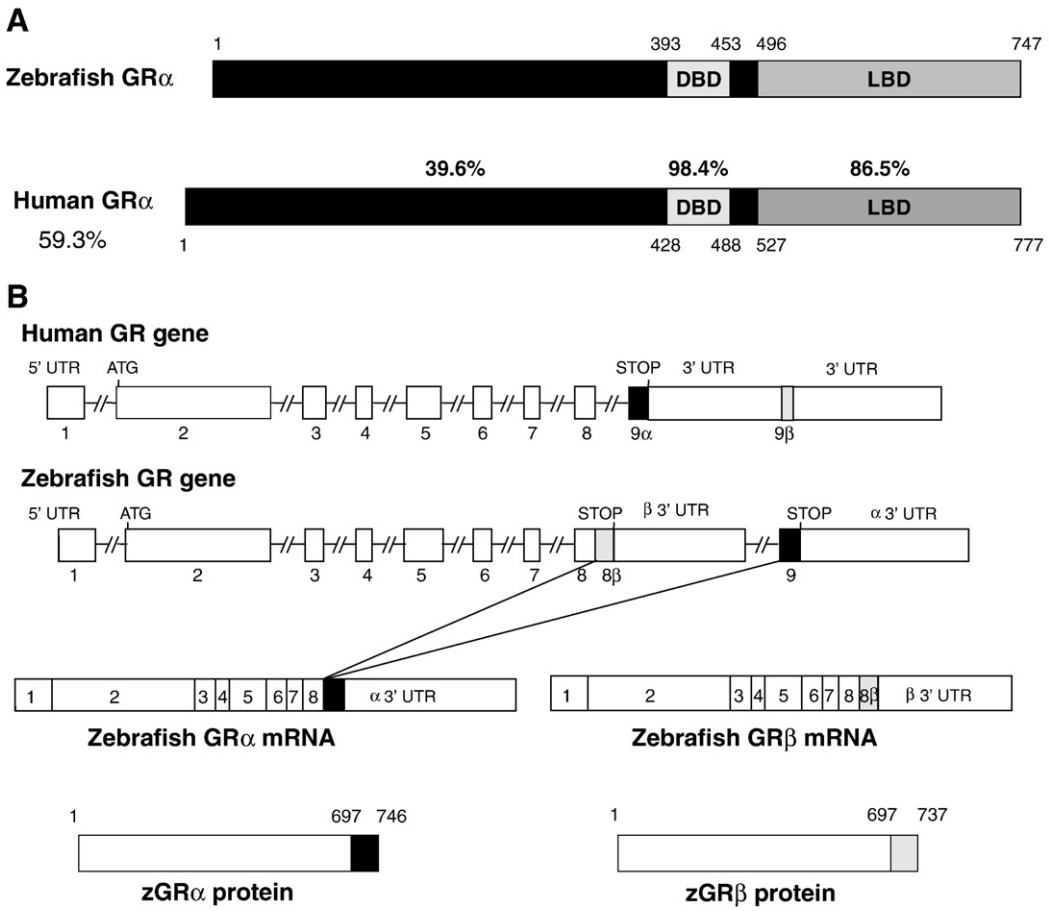

Fig. 1. Comparison between the human and zebrafish GRs. A. Similarity between the human and zebrafish GR α-isoforms. The α-isoform represents the classical, canonical GR. It contains a large N-terminal domain, a DNA-binding domain (DBD) and a ligand-binding domain (LBD).Percentages indicate the fraction of amino acids similar between human and zebrafish per domain. The overall level of similarity is 59.3%. B. The human and zebrafish GR genes. Both genes contain 9 exons, of which exon 1 is non-coding. A remarkable difference is the location of the sequence encoding β-isoform-specific amino acids. In the human gene, this sequence is located in exon 9, whereas in the zebrafish gene it is found in exon 8. In zebrafish, the use of the most 5′ splice donor site in this exon results in a shorter version of exon 8 and an open reading frame that includes exon 9, resulting in mRNA encoding zGRα (GenBank Acc No. EF436284). The use of the most 3′ site results in an extended version of exon 8, introducing a stop codon in exon 8, which results in zGRβ mRNA (EF436285). The zebrafish GRα and GRβ protein are identical between amino acids 1 and 696. An additional 40 specific amino acids form the C-terminus of zGRβ.

2.2. The zebrafish GR β-isoform

Another remarkable characteristic of the zebrafish GR gene is the possibility of alternative splicing, which results in a GR isoform that is identical to the canonical GR in the N-terminal domain, the DNA binding domain and most of the ligand binding domain, but contains a different amino acid sequence at its C-terminus (Fig. 1B) (Schaaf et al., 2008). This isoform is called the zebrafish GRβ, since it highly resembles the human GR β-isoform. The human GRβ (hGRβ) is a result of alternative splicing in exon 9 (Hollenberg et al., 1985; Oakley et al., 1996). This isoform is identical to the canonical GR (hGRα) between amino acid 1 and 727, after which it diverges. The human GR β-isoform contains an additional 15 C-terminal amino acids, which show no homology to the 50 additional amino acids in hGRα's C-terminus.

The human GR β-isoform does not bind glucocorticoid agonists and is predominantly localized in the nucleus. It has been shown in *in vitro* reporter assays that hGRβ does not induce transcription on GRE-containing promoters, but acts as a dominant-negative inhibitor of hGRα's transactivational properties (Bamberger et al., 1995; Oakley et al., 1996, 1997, 1999). In line with this dominant-negative activity, a correlation has been found between resistance to glucocorticoid treatment in patients suffering from several immune-related diseases and increased expression levels of hGRβ (Leung et al., 1997; Hamid et al., 1999; Shahidi et al., 1999; Honda et al., 2000; Goleva et al., 2006). In addition, the occurrence of diseases like ulcerative colitis (Honda et al., 2000), leukemia (Shahidi et al., 1999) and severe asthma (Bergeron et al., 2006) has been demonstrated to correlate with an increased expression of this GR isoform in various immune cells.

However, some issues still remain unresolved. Several researchers could not reproduce the dominant-negative activity of hGRβ *in vitro* (Hecht et al., 1997; de Lange et al., 1999). In addition, the high hGRβ expression levels at which the dominant-negative activity *in vitro* is observed are in sharp contrast with its low expression levels *in vivo* (Oakley et al., 1996), which makes the relevance of the *in vitro* results questionable. A recent study suggests that hGRβ may regulate gene transcription independent of hGRα, and that this activity can be altered by the synthetic GRα antagonist RU486 which has been shown to bind hGRβ (Lewis-Tuffin et al., 2007). It has also been suggested that hGRβ acts as a constitutive transrepressor of genes that are transrepressed in a ligand-dependent way by hGRα (Kelly et al., 2008).

Until recently, a GR β-isoform had only been found in humans, and its absence has been demonstrated in rodents (Otto et al., 1997). Therefore, an animal model that may help resolving some of the issues mentioned here has been lacking, until the recent discovery of a GR β-isoform in zebrafish. The zebrafish GR β-isoform is similar to its human equivalent in structure, function and expression level (Schaaf et al., 2008). The zebrafish GR α- and β-isoform are identical between amino acids 1 and 696. An additional 40 specific amino acids form the

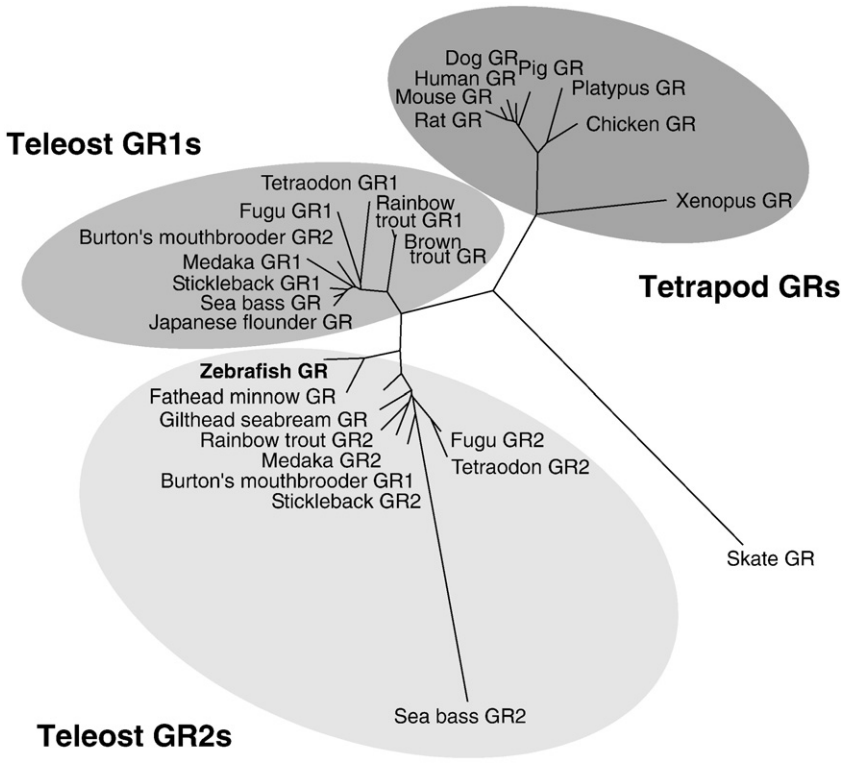

Fig. 2. Phylogenetic tree of the teleost fish and tetrapod GRs. Protein sequences were generated by translating cDNA sequences or predicted mRNA sequences. Sequences were only used if complete coding sequence was available (see also (Schaaf et al., 2008)). ClustalW software (version 1.83, available at http://clustalw.ddbj.nig.ac.jp/top-e.html) was used with default parameters. The zebrafish GR clusters within the GR2 clade of teleostean GRs.

C-terminus of zGRβ, and these amino acids show no homology to the 57 specific amino acids in the C-terminus of zGRα. Sequence alignment of the human and zebrafish GRs show that the divergence point between the α- and β-isoform is identical in humans and zebrafish. In reporter assays, zGRβ has been shown to act as a dominant-negative inhibitor of zGRα's transactivational activity, similar to the effect of hGRβ on hGRα-induced transcription. The expression levels of zGRβ at the mRNA level in adult zebrafish and in embryos are significantly lower (between 10- and 100-fold) than the zGRβ mRNA levels, which resembles the lower expression level of hGRβ mRNA relative to the hGRα mRNA level that is found in several human tissues and cells (Oakley et al., 1996; Dahia et al., 1997; Mu et al., 1998; Honda et al., 2000).

3. The zebrafish as a model system for GR research

The zebrafish could be a valuable tool for at least two types of GR research. First, the zebrafish can be used to advance our knowledge on the molecular mechanisms underlying the effects of GR activation *in vivo*. Using techniques for transient or stable genetic manipulation in combination with imaging-based phenotypic readouts, the zebrafish can be used for analysis of how specific molecular mechanisms alter the phenotype of a living vertebrate organism. Most of these phenotype-based assays are based on the imaging of fluorescent cells in zebrafish embryos, that could be used on a relatively large number of individuals.

Second, its potential could be used in studies towards the discovery of novel drugs and drug targets (Zon and Peterson, 2005; Mathew et al., 2007). Because of its small size and suitability for imaging studies, the zebrafish could be an ideal tool for the screening of novel glucocorticoid drugs. These screening assays could be implemented as an extra step between high-throughput drug screening assays (often performed in cell cultures) and subsequent studies in mammalian animal models like rodents. This way, compounds which appear to be ineffective in *in vivo* studies are filtered out at an early stage, limiting the number of compounds to be tested in mammalian models. In addition, using forward genetic screens using glucocorticoid responsiveness as a readout, novel drug targets may be discovered that may be exploited as a target for drugs that could increase the effectiveness of glucocorticoid treatment.

4. Tools for GR research in zebrafish

Since only a few studies on the GR in zebrafish have been performed, a limited number of tools is currently available to study GR function in zebrafish. In Table 1 these tools are listed and they will be briefly discussed below.

4.1. Molecular genetic tools

Several mutant zebrafish lines possibly interesting for GR research are available. A mutant zebrafish line is available that carries a mutation in the retinal homeobox gene 3 (*rx3*), resulting in a loss of corticotrope cells in the pituitary and severely reduced cortisol levels (Loosli et al., 2003; Dickmeis et al., 2007). In addition, other cortisol-deficient mutants are available that lack the entire pituitary, like the fibroblast growth factor 3 mutant (*lia*/*fgf3*, (Herzog et al., 2004)) and the achaete scute-complex like 1a mutant (*pia*/*ascl1a*, (Pogoda et al., 2006)). Another mutant, eyes absent 1 (*aal*/*eya1*, (Kozlowski et al., 2005)), only contains the lactotrope cells of the pituitary.

In addition, a few relevant morpholino studies have been performed. Transient knockdown of steroid biosynthesis using a morpholino reducing the cyp11a1 gene expression (the enzyme which converts cholesterol into pregnenolone, the first step in the steroid biosynthesis pathway) results in severe developmental defects, but which class of steroids is responsible for this effect is yet unclear (Hsu et al., 2006). In another study a morpholino approach is used to knock down GR function by blocking the splice acceptor site at the 5′end of

Table 1
Tools currently available for GR research in zebrafish.

A. Molecular genetic tools		
Manipulation of GR activity		
Cortisol-deficient mutants	rx3	Loosli et al., 2003; Dickmeis et al., 2007
	lia/fgf3	Herzog et al., 2004
	pia/ascl1a	Pogoda et al., 2006
	aal/eya1	Kozlowski et al., 2005
Morpholinos	cyp11a1	Hsu et al., 2006
	GR	Mathew et al., 2007
Detection of GR mRNA and protein level		
qRT–PCR	GRα	Dickmeis et al., 2007; Mathew et al., 2007; Alsop and Vijayan, 2008
	GRα and GRβ	Schaaf et al., 2008
In situ hybridization	GRα and GRβ	Schaaf et al., 2008
Western blots	GRα	Dickmeis et al., 2007
Immunohistochemistry	GRα	Present paper (Fig. 3)
Detection GR target gene mRNA level		
qRT–PCR	FKBP5, GILZ, sox9b	Mathew et al., 2007
	MMP-2, -9, -13	Hillegass et al., 2007, 2008
	FKBP5, IκBα, PEPCK	Present paper (Fig. 4)
	IL-8, IL-1β, TNFα	Present paper (Fig. 4)
B. Phenotype-based assays		
Assays for immunosuppressive effects of GR		
Inflammation models	Leukocyte migration assay	
	mpo:GFP	Renshaw et al., 2006; Mathias et al., 2006; Mathew et al., 2007
	Enhancer trap line	Meijer et al., 2008
	lysC:GFP	Hall et al., 2007
	Chronic inflammation model	
	hai1 mutant	Mathias et al., 2007
T-cells in thymus	lck:GFP	Langenau et al., 2004
	rag2:GFP	Langenau et al., 2004
Infection model	Fluorescently labeled bacteria	
	M. marinum	Davis et al., 2002
	S. typhimurium	Van der Sar, 2003
Assays for other effects of GR		
Bone formation	Visualization of skeletal structures	
	Calcein/alizarin red	Du et al., 2001; Fleming et al., 2005
Cortisol levels	Immuno-assay	Dickmeis et al., 2007; Alsop and Vijayan, 2008

exon 6, resulting in a GR transcript that lacks this exon (Mathew et al., 2007). The altered splicing results in a mRNA that encodes a GR protein that lacks the LBD (in more detail: it is identical to the wild type zebrafish GR until amino acid 552 and contains an additional 3 amino acids). Injection of this morpholino did not result in any obvious early developmental defects, suggesting that GR is not essential for early embryonic development (Mathew et al., 2007). This does not mean that alterations in GR function do not affect embryonic development, since glucocorticoid treatment during the first days of development has been reported to result in craniofacial abnormalities, altered somitogenesis, blood pooling and pericardial and yolk sac edema (Hillegass et al., 2007, 2008).

Expression levels of zGRα and zGRβ mRNA can be determined by qRT–PCR (Mathew et al., 2007; Dickmeis et al., 2007; Schaaf et al., 2008; Alsop and Vijayan, 2008) and the expression pattern has been studied by in situ hybridization (Schaaf et al., 2008). For detection at the protein level, western blotting has been performed using an anti-hGR antibody (p-20, available from Santa Cruz (Dickmeis et al., 2007)), which is directed against the receptor C-terminus, and is therefore specific for the GR α-isoform. In our laboratory, we have used this antibody to perform immunohistochemistry on embryos 24 hours post fertilization (Fig. 3).

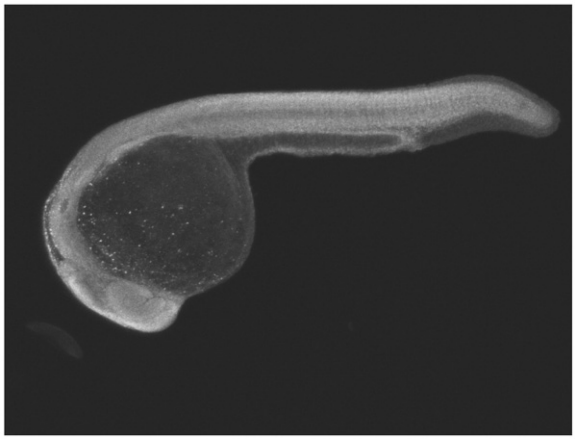

Fig. 3. Whole mount immunohistochemistry on 24 hpf embryo. An antibody was used against the C-terminus of human GRα (p-20, Santa Cruz Biotechnology Inc.). No spatial restriction was observed in the immunostaining, which is in line with previously described in situ hybridization data (Schaaf et al., 2008).

Alterations in the expression of specific GR target genes can be used as a readout of GR activity. In zebrafish embryos, using qRT–PCR the upregulation of the well-known GR target genes FK506 binding protein 5 (FKBP5), glucocorticoid-induced leucine zipper (GILZ) and sox9b after glucocorticoid treatment has been shown (Mathew et al., 2007), and the induction of the matrix metalloproteinase-2, -9, and -13 has been demonstrated (Hillegass et al., 2007; Hillegass et al., 2008). In our laboratory, we have assembled a small panel of six GR target genes, of which three (FKBP5, IκBα, and phosphoenolpyruvate carboxykinase (PEPCK)) are upregulated and three (Interleukin(IL)-8, IL-1β and tumor necrosis factor (TNF)α) are downregulated upon dexamethasone treatment in 1 day old embryos, so we have *in vivo* readouts for both transactivation and transrepression (Fig. 4).

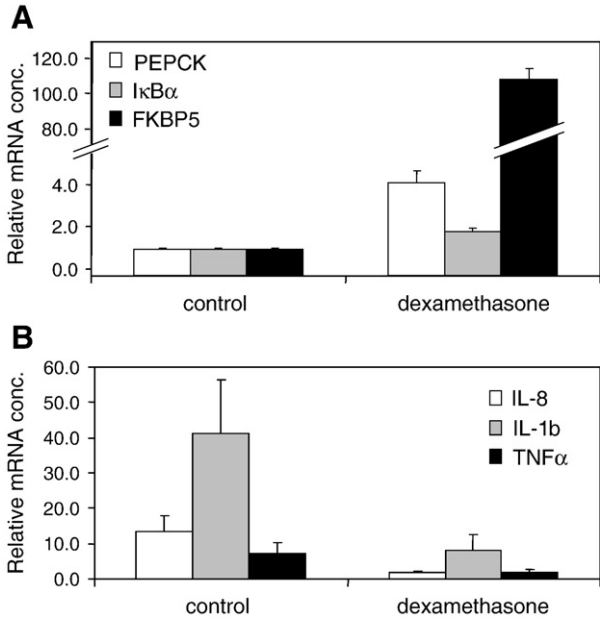

Fig. 4. Analysis of glucocorticoid-induced alterations in gene expression in zebrafish embryos. At 28 h post fertilization, embryos were incubated in 100 μM dexamethasone for 6 h. Total RNA was isolated and qRT–PCR was performed using specific primer sets for the indicated genes. Three genes were upregulated by dexamethasone treatment (A), and three genes were downregulated (B). In the experiment in panel B embryos were incubated in PMA and ionomycin during the dexamethasone treatment to induce the expression of the indicated genes.

4.2. Phenotype-based assays

In assays screening for the action of glucocorticoids, their immunosuppressive action could be evaluated *in vivo*. It should be noted that zebrafish embryos only contain an innate immune system, and that the adaptive immune system does not arise until four weeks after fertilization (Trede et al., 2004). First, the action of glucocorticoids in zebrafish inflammation models can be screened. A transgenic fish line can be utilized containing the green fluorescent protein (GFP) gene driven by the myeloperoxidase (MPO) promoter, expressing GFP in the neutrophil granulocytes (Renshaw et al., 2006; Mathias et al., 2006). These cells migrate to the site of injury after a wound has been made, and this experimental paradigm is considered as a model for acute inflammation (Renshaw et al., 2006). Treatment of embryos with the synthetic glucocorticoid beclomethasone results in a significant decrease in the number of neutrophils migrating to the trauma site upon amputation of a part of the tail (Mathew et al., 2007). A transgenic line (generated by enhancer trapping) which expresses yellow fluorescent protein (YFP) in a subset of neutrophils (Meijer et al., 2008), and a line expressing GFP in a subset of macrophages (with the GFP expression driven by the lysozyme C promoter (Hall et al., 2007)), have been used in similar assays of immune cell migration. In addition, several other transgenic lines are available in which a subpopulation of immune cells express GFP (Ward et al., 2003; Hsu et al., 2004). Recently a transgenic zebrafish line that can be used as a model for chronic inflammation has been generated, caused by a mutation of the hepatocyte growth factor activator inhibitor 1 (hai1) gene, that shows accumulation of (GFP-labeled) neutrophils in the fin (Mathias et al., 2007). The effect of glucocorticoids on the behavior of the labeled immune cells has not been tested in any of these latter lines yet.

Second, the presence of T-cells in the thymus can be monitored. A transgenic zebrafish line can be used that expresses GFP under control of the T-cell specific tyrosine kinase (lck) promoter, resulting in GFP-labeled T cells. Treatment of embryos from this line with the glucocorticoid receptor agonist dexamethasone results in the ablation of GFP-labeled T-cells in the thymus of these embryos (Langenau et al., 2004). Another line in which GFP expression is controlled by the recombinant activating gene 2 (rag2) promoter (resulting in GFP labeled immature T and B cells) showed similar results (Langenau et al., 2004).

Third, several zebrafish infection models exist in which the status of the infection can be monitored. An increase in the proliferation of the infectious agent could be used as a measure for the immunosuppressive activity of a GR agonist. Infecting zebrafish embryos with fluorescently labeled bacteria enables the analysis of the infection in real time and *in situ*. This approach has been successfully used for *Mycobacterium marinum* and *Salmonella typhimurium* infections (Davis et al., 2002; van der Sar et al., 2003).

In addition to their use in screening assays for the anti-inflammatory activity of glucocorticoids, zebrafish embryos can also be used for screening of other effects of glucocorticoid treatment, like decreased bone formation which is a common side effect of glucocorticoid treatment. Recently, a zebrafish model system for glucocorticoid-induced osteoporosis has been developed, based on the visualization of skeletal structures of zebrafish larvae using calcium-binding dyes like calcein or alizarin red (Du et al., 2001; Fleming et al., 2005). As a proof of principle, treatment of 5-day-old zebrafish larvae with prednisolone, a glucocorticoid that is widely used clinically, significantly reduced bone formation in this assay (Barrett et al., 2006). Using these assays in embryos restricts the screening to the osteoblast activity, since the first osteoclasts appear in twenty-day old individuals (Witten et al., 2001).

Another common side effect of glucocorticoid treatment is a decrease in circulating cortisol levels, and this effect can be studied in zebrafish as well. Total cortisol levels can be measured in homogenates from pools of zebrafish embryos of any age using an immunoassay (Dickmeis et al., 2007; Alsop and Vijayan, 2008). Increased cortisol levels in response to a stressor can be detected from 97 hours post fertilization (Alsop and Vijayan, 2008), and a circadian rhythm in cortisol level has been observed at 6 days post fertilization (Dickmeis et al., 2007). This indicates that the hypothalamus-pituitary-interrenal gland (HPI) axis is functional in zebrafish larvae, and it can be expected that glucocorticoid treatment results in a decrease in circulating cortisol levels.

5. Conclusions and perspective

In conclusion, the zebrafish system could be a valuable model system for research on the GR, in which it can be used for investigating the molecular mechanism of glucocorticoid receptor action and in drug discovery studies. Two characteristics make it a very favorable system for this type of research. First, the zebrafish GR displays a high level of similarity to the human GR. The genome of both species contains a single (well conserved) GR gene from which two receptor isoforms, GRα and GRβ can be produced through alternative splicing. Second, the zebrafish embryo system has many practical advantages, among which the relatively easy stable or transient genetic manipulation of vertebrate organisms in combination with opportunities to screen the phenotype of a large number of individuals using imaging-based technology.

Although some molecular genetic tools and screening assays are available already, new tools need to be developed in order to fully exploit the opportunities of this model. The generation of a GR knockout zebrafish line, for example by TILLING (targeting induced local lesions in genomes (Wienholds et al., 2003)), would be a top priority in this respect. Mice with a disruption in the GR gene die soon after birth because of respiratory failure (Cole et al., 1995), but a deficiency in GR signaling may not be lethal in fish. Transgenic fish lines overexpressing the GR β-isoform would be a useful tool to study the effects of this isoform *in vivo*, especially if the expression would be inducible (e.g. by using the heat shock protein (hsp) 70 promoter (Shoji and Sato-Maeda, 2008)), or spatially restricted (e.g. by using the Gal4/UAS system (Asakawa and Kawakami, 2008). Transgenic reporter fish lines can be made using the bacterial artificial chromosomes (BAC) modification strategy, in which a large genomic region can be cloned and the GFP coding sequence can be inserted at the translation start site of a specific gene. After inserting these sequences into the zebrafish genome, GFP is expressed driven by all the promoter/enhancer elements regulating the expression of the original protein (Jessen et al., 1998; Yang et al., 2006). By using GR responsive genes like FKBP5 or IL-8 in this approach, reporter fish lines for the activity of GR can be generated and used as readouts in screening assays. A more general view on glucocorticoid-induced alterations on gene expression will be offered by using custom-made zebrafish-specific microarrays (Meijer et al., 2005; Krens et al., 2008) and serial analysis of gene expression (SAGE) experiments using megasequencing. Finally, specific antibodies against the zebrafish GR α- and β-isoform will be required for studying (alterations in) the localization and expression level of these proteins.

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