Nuclear Receptor Research
vol. 4 (2017), Article ID 101286, 16 pages
doi:10.11131/2017/101286

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Review Article

The First Fifteen Years of Steroid Receptor Research in Zebrafish; Characterization and Functional Analysis of the Receptors

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Abstract. Steroid hormones regulate a wide range of processes in our body, and their effects are mediated by steroid receptors. In addition to their physiological role, these receptors mediate the effects of endocrine disrupting chemicals (EDCs) and are widely used targets for dugs involved in the treatment of numerous diseases, ranging from cancer to inflammatory disorders. Over the last fifteen years, the zebrafish has increasingly been used as an animal model in steroid receptor research. Orthologues of all human steroid receptor genes appear to be present in zebrafish. All zebrafish steroid receptors have been characterized in detail, and their expression patterns have been analyzed. Functional studies have been performed using morpholino knockdown of receptor expression and zebrafish lines carrying mutations in one of their steroid receptor genes. To investigate the activity of the receptors *in vivo*, specific zebrafish reporter lines have been developed, and transcriptomic studies have been carried out to identify biomarkers for steroid receptor action. In this review, an overview of research on steroid receptors in zebrafish is presented, and it is concluded that further exploitation of the possibilities of the zebrafish model system will contribute significantly to the advancement of steroid receptor research in the next decade.

Keywords: steroids; steroid receptors; nuclear receptors; zebrafish; fish.

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Editor
William Baldwin

Dates
Received 9 April 2017
Accepted 13 June 2017

1. Steroids and Steroid Receptors

Steroid hormones are signaling molecules that regulate a wide variety of physiological processes in our body. Two main classes of steroid hormones exist: sex steroids and corticosteroids. The secretion of sex steroids is controlled by the hypothalamus-pituitary-gonadal axis, and these hormones are primarily produced in the ovary in females and in the testis in males. Sex steroids regulate sexual differentiation and reproduction, and they can be subdivided into three groups: estrogens, progestogens and androgens. The hypothalamus-pituitary-adrenal axis regulates the secretion of corticosteroids, which are synthesized in the cortex of the adrenal gland. They can be subdivided into two groups: mineralocorticoids, which regulate our salt and water balance, and glucocorticoids, which are involved in the stress response and regulate processes like glucose metabolism and immune function.

All steroid hormones are synthesized from the common precursor molecule cholesterol. The rate-limiting step in this process is the transport of cholesterol across the mitochondrial membranes, which is controlled by steroidogenic acute regulatory protein (StAR). Next, cholesterol is converted to pregnenolone, which is subsequently converted to progesterone, the main endogenous progestogen in our body. From progesterone, the mineralocorticoid aldosterone is

synthesized in a two-step-process. Pregnenolone can also be converted to 17-OH-progesterone, which serves as a precursor for the biosynthesis of cortisol, our main glucocorticoid hormone. A third steroid biosynthesis pathway generates androgens and estrogens and starts with the production of androstenedione (A2) from pregnenolone. A2 can be converted into the androgens testosterone (T) and dihydrotestosterone (DHT). Finally, T serves as a substrate for the enzyme aromatase which produces estradiol (E2), our main endogenous estrogen.

Upon secretion by the endocrine organs, steroid hormones reach their target cells through the blood. Inside cells, steroid hormones bind and activate steroid receptors. For each of the five subclasses of steroid hormones a specific receptor type exists that mediates their effects: the Estrogen Receptors α and β (ERα and ERβ), the Progesterone Receptor (PR), Androgen Receptor (AR), Glucocorticoid Receptor (GR), and Mineralocorticoid Receptor (MR). These receptors belong to the superfamily of nuclear receptors. Evolutionary analysis has revealed that the members of this receptor family have evolved from one ancestral steroid receptor that was sensitive to estrogens, through a series of gene duplication and diversification events [1]. They show a high level of similarity and share a common modular domain structure. They all contain an N-terminal domain that is variable in length, a small well-conserved DNA-binding domain (DBD), and a larger, moderately conserved, C-terminal ligand-binding domain (LBD).

Steroid receptors are located intracellularly, and in the absence of hormone they form a multiprotein complex with heat shock proteins and immunophilins, which keep them in an inactive state with high affinity for their cognate ligands. Upon ligand binding, the receptors dissociate from the repressor protein complex and a conformational change is induced. In the active conformation, the receptors act as transcription factors. They are able to bind to specific target sites in the DNA, called hormone response elements (HREs), and upon recruitment of different types of transcriptional coregulator proteins the transcriptional rate of a nearby gene is altered. Additionally, steroid receptors are able to alter gene transcription by interacting with other transcription factors and alter the activity of these proteins. Besides these 'genomic' actions of steroid receptors, rapid 'nongenomic' mechanisms of steroid receptor action have been demonstrated, but the intracellular signaling pathways underlying these effects have not been unraveled in full detail yet. Finally, the G protein-coupled estrogen receptor (GPER, previously termed GPR30) has been shown to mediate rapid effects of estradiol and aldosterone [2], and progesterone is known to activate membrane Progestin Receptors (mPRs), which are members of the Progestin and AdipoQ Receptor family [3]. These latter two receptor types are beyond the scope of this review, which will focus on the classical steroid receptors from the nuclear receptor superfamily.

2. Steroid Receptor Research in Zebrafish

In this review, an overview is presented of research on steroid receptors using the zebrafish (*Danio rerio*) as a model system. The zebrafish was originally used as an animal model for studies on embryonic development and morphogenesis, and it is still widely used in research on vertebrate development [4, 5]. However, over the last decade the zebrafish has also emerged as an important model system for eco-toxicological [6] and biomedical research [7, 8]. Several characteristics make it a highly versatile research model. Their easy maintenance, high fecundity and the small size and optical transparency of the embryos and larvae make them highly suitable

for large-scale studies. Since most compounds can easily penetrate the skin of these organisms, drug and toxicological screenings can be performed by simply adding the compounds to the water. The completion of the genomic sequence of the zebrafish greatly facilitated forward and reverse genetic studies in zebrafish. The generation of mutant and transgenic fish lines opened up new possibilities, and novel genome-editing techniques, like the CRISPR/Cas9 technology, will further advance the field.

A large amount of the research that has been performed on steroid receptors in zebrafish has been aimed at studying the effects of endocrine disrupting chemicals (EDCs). EDCs are natural or synthetic compounds that occur in the environment and disrupt the function, levels and distribution of endogenous hormones of exposed organisms, by mimicking or antagonizing the actions of hormones, or by modulating hormone synthesis and metabolism [9]. This may ultimately lead to altered development and/or reproduction in humans and wildlife. EDCs form a heterogeneous group of substances, encompassing natural and synthetic hormones and seemingly unrelated compounds like pesticides, polychlorinated biphenyls (PCBs), Bisphenol A (BPA), phthalates, flavonoids and polycyclic musks [10, 11]. The main mechanism of action for these compounds is generally suggested to be agonistic or antagonistic interaction with steroid receptors. Zebrafish are commonly used for monitoring EDC activity. Phenotypic endpoints like growth, sexual differentiation, sex ratio, egg production and fertilization success are commonly used for monitoring EDC activity, and these biomarkers are used in partial and full life cycle studies [12, 13]. A lot of studies on zebrafish steroid receptor action have aimed to identify novel molecular biomarkers or screening approaches for EDC activity which could replace or complement the phenotypic endpoints. In addition, many investigations were focused on unraveling the mechanism of action of EDCs, especially using approaches for genetic knockdown of steroid receptor function.

Research on steroid receptors in zebrafish started in 2002 with the characterization of the zebrafish ERs, and almost ten years later all zebrafish steroid receptors had been identified and characterized (for an overview see Table 1). All mammalian steroid receptor genes appeared to have one orthologue in zebrafish, apart from the gene encoding Erβ, of which two copies were found in the zebrafish genome. As a first step in the characterization, the zebrafish receptors were used in reporter assays in cultured (mostly mammalian) cells. In general, the ligand specificity did not differ much from the mammalian receptors. Second, expression analysis was performed, mostly by *in situ* hybridization, since the availability of antibodies specific for (zebra)fish receptors is limited. After these initial steps, functional in-vivo studies have been performed. Knockdown of the receptor using morpholinos, which are antisense oligonucleotide analogs often used in zebrafish research, has revealed the function of the receptor during embryonic and larval stages. In recent years, it has become clear that some of the phenotypes caused by this approach may result from unintended nonspecifc morpholino actions [14]. This issue may be overcome by comparing the results from morpholino studies to data obtained using zebrafish lines with mutations in steroid receptor genes, and this has provided further insight into the *in vivo* action of several steroid receptors. For Er and Gr, luciferase and Green Fluorescent Protein (GFP) reporter lines have been generated which allow in-vivo monitoring of the activity of these receptors. Many functional studies have used transcriptomic analysis, initially using microarray and more recently using RNA sequencing, to elucidate molecular pathways regulated by steroid receptor action and to identify biomarkers for steroid receptor activity. In the following sections,

Table 1: Overview of steroid receptor family in zebrafish (*Danio rerio*).

Receptor	Gene name and aliases	ENSEMBL Gene ID[1]	GenBank mRNA Ref. Seq. no.[2]	Reference
Erα	*nr3a1*, *erα*, *era*, *esr1*	ENSDARG00000004111	NM_152959.1	[15–17]
Erβ1	*nr3a2-a*, *erβ1*, *erb1*, *esr2b*	ENSDARG00000034181	NM_174862.3	[15–17]
Erβ2	*nr3a2-b*, *erβ2*, *erb2*, *esr2a*	ENSDARG00000016454	NM_180966.2	[15–17]
Pr	*nr3c3*, *pr*, *pgr*	ENSDARG00000035966	NM_001166335.1	[39, 40]
Ar	*nr3c4*, *ar*	ENSDARG00000067976	NM_001083123.1	[48–50]
Gr	*nr3c1*, *gr*, *utouto*	ENSDARG00000025032	NM_001020711.3	[57]
Mr	*nr3c2*, *mr*	ENSDARG00000102082	NM_001100403.1	[85]

[1]`http://www.ensembl.org/Danio_rerio/Info/Index`

[2]`https://www.ncbi.nlm.nih.gov/genbank/`

for each of the five steroid receptor types the zebrafish research performed over the last fifteen years will be discussed.

3. The Zebrafish Ers

Fifteen years ago, several groups reported on the identification of three different Er encoding genes in zebrafish [15–17]. Phylogenetic analysis showed that one cDNA originated from the zebrafish orthologue of the mammalian *ERα*gene, and that the other two cDNAs were products from two *ERβ*orthologues. It was concluded that the identified *erβ* cDNAs originated from duplicated *erβ* genes in the zebrafish genomes. These duplicate *erβ* genes had been shown previously in other fish species like goldfish [18] and atlantic croaker [19]. Erβ2 had a slightly higher affinity for E2 than the other two zebrafish Ers, which was reflected in a higher potency in in-vitro reporter assays, in which a luciferase gene was driven by a promoter containing an estrogen response element (ERE) [15, 20]. E2, the metabolites estrone (E1) and estriol (E3), and the synthetic estrogen diethylstilbestrol (DES) showed similar agonistic activity on all three receptors [15, 16], whereas the synthetic ligands ICI 164384 (ICI) and 4-hydroxytamoxifen (OHT) acted as antagonists on all zebrafish Ers.

Expression analysis of the three genes encoding Ers showed that during zebrafish development, three phases can be distinguished [16, 20, 21]. During the first phase, the presence of maternal transcripts for Erβ1 [20–22] and Erβ2 [16] has been demonstrated by RT-PCR and RNAse protection assay respectively. Between the mid-blastula stage and 72 hours post fertilization (hpf), the expression levels of all three *er* genes is decreased, although by in-situ hybridization it was shown that some cell types show high levels of specific *er* mRNAs. For example, epidermal cells were demonstrated to contain high *erβ2* mRNA levels at this stage [21]. After 72 hpf, all *er* genes start to be expressed at high levels [16, 20–22], and particularly high levels of *erβ1* and *erβ2* expression were found during the second week of development in neuromasts of the lateral line, a mechanoreceptive system specific to aquatic vertebrates [21]. In adult fish, expression of all three receptor subtypes was demonstrated by RT-PCR in tissues involved in reproduction like brain, pituitary, ovary and testis, but also in nonreproductive tissues like liver, intestine and eyes [15, 22, 23]. In-situ hybridization on brains of adult females showed distinct but partially overlapping patterns of expression in two neuroendocrine regions,

the preoptic area and the mediobasal hypothalamus [15]. Hepatic expression of *erα* mRNA in adult fish was upregulated upon E2 treatment, and this effect appeared to be mediated by Er*α* and Er*β*2, but not Er*β*1 [23, 24].

Knockdown of the expression of the zebrafish *er* genes has been performed in several studies. Temporary knockdown of the *erβ2* expression using a morpholino resulted in the disrupted development of the neuromasts (absence of hair cells) in the morpholino-treated embryos, which was attributed to an aberrant activation of the Notch signaling pathway [25]. Induction of specific target genes of Er*β*2 and Er*α* could also be inhibited by morpholino knockdown of the receptor [26]. Knockdown of *erα* expression during embryonic stages by morpholino-induced blocking of translation of maternal mRNA resulted in severe developmental defects and early mortality [27]. Using a combination of chemical mutagenesis and high-throughput sequencing [28], a mutant zebrafish line was generated, which carried a mutation in the gene encoding Er*β*2 (resulting in an 8 amino acid insertion in the ligand-binding domain [29]). Unfortunately, the readouts used in studies on this mutant line were different from those used in the morpholino studies, so validation of the morpholino data was not possible. This mutant line displayed increased testosterone and 17*β*-estradiol (E2) levels and, probably as a result of the hormonal imbalance, distorted sexual ratios and altered testicular morphology. It was concluded that Er*β*2 plays an important role in the feedback regulation of steroid biosynthesis, in line with its elevated expression in the gonads and neuroendocrine regions of the brain. In addition, a decreased immune response to a viral infection was found in this mutant fish [29].

Several transgenic reporter fish lines are available to study the activity of Er *in vivo*. In these lines, an Er-responsive promoter is genetically fused to a reporter gene encoding either luciferase or GFP. Using a transgenic line with the luciferase gene coupled to a promoter with a single estrogen response element (ERE), Er activity could be measured after 96 hour exposure to estrogens by measuring the luciferase activity in tissue homogenates [17, 30]. This assay has been performed using adult zebrafish and juveniles, in which adults appear to be more responsive. In addition, two transgenic zebrafish lines have been generated in which GFP is used, which makes it possible to locate the reporter activity using fluorescence microscopy. In the first line, a promoter with 5 EREs was utilized, and GFP expression was observed in embryonic and larval zebrafish in the brain, liver and pancreas after estrogen exposure [31, 32]. Estrogen treatment of larvae also induced expression of GFP in cells in the ventral fin adjacent to the cloaca, in the developing olfactory organ, and in the heart. This induction could be inhibited by the ER antagonist ICI182,780 (fulvestrant). Interestingly, GFP induction by estrogens like EE2 and genistein showed marked differences in the tissue specificity of the response [31]. In adult fish, males showed no GFP expression in the liver, whereas females displayed dim expression in this tissue, which could be increased upon estrogen exposure. Ovaries and (male and female) pituitaries were GFP-positive without exposure to exogenous estrogens, so this was considered to reflect Er activation by endogenous estradiol. In another transgenic line GFP was coupled to the promoter of the brain aromatase b gene (*cyp19a1b*), resulting in specific GFP expression in radial glial cells in the area bordering the brain ventricles [33]. Before 9 days post fertilization (dpf), the GFP expression was only observed after induction by estrogens [34].

Various transcriptomic analyses have been carried out in order to identify novel molecular biomarkers for Er activity [35]. The liver and telencephalon transcriptome have been determined using microarray analysis of adult male and female fish after exposure to EE2 [36, 37]. Whole

body transcriptome analysis was performed on male adult fish after exposure to E2 in another microarray study [38]. Microarray analysis has also been performed during early developmental stages (1, 2, 3 and 4 dpf). In addition to well-known Er target genes like *vtg1* (the only gene induced by estrogens at all stages studied [32]), *vtg3*, *vtg4* and *erα*, these studies also revealed Er regulation of many metabolic genes [36, 37] and genes involved in cell cycle and DNA repair [38].

4. The Zebrafish Pr

Just like in most other teleost fish species, a single *pr* gene was found in zebrafish [39, 40], although duplicate *pr* genes (*pr1* and *pr2*) have been demonstrated in the European eel [41]. The two *pr* genes in the eel, like many other duplicated genes, result from a whole genome duplication that happened at the base of teleost evolution around 320–350 million years ago [42]. The orthologue of the eel *pr2* gene was most likely lost early during teleost evolution, soon after the order of Anguilliformes (to which the eels belongs) had branched off the main teleost lineage. The zebrafish Pr binds with high affinity (k_d in the nanomolar range) to the main endogenous progestogen in zebrafish, 17*α*,20*β*-dihydroxy-4-pregnen-3-one (DHP)[39]. DHP is converted from 17-OH-progesterone by 20*β*-hydroxysteroid dehydrogenase (Hsd20b), an enzyme that has only been identified in teleosts [43, 44]. The zebrafish Pr activated the mouse mammary tumor virus (MMTV) promoter in luciferase assays upon binding to DHP, progesterone, 17-OH-progesterone, 17*α*,20*β*,21-trihydroxy-4-pregnen-3-one (20*β*-S, another fish-specific endogenous progestogen), and the synthetic progestin promegestone (R5020) [39, 40]. In testicular explants, the release of 11-KT was induced by DHP and this response could be inhibited by the antagonist RU486 [40].

Pr expression analysis using RT-PCR showed no maternally deposited transcripts, and the first detectable mRNA levels were found at 8 hpf [40]. In adults, the *pr* transcript and protein were detected by RT-PCR and western blot and appeared to be abundant in the ovaries, testis, and brain and scarce or undetectable in the liver, intestine, muscle, heart and gills [39, 40]. In the ovary, high mRNA and protein levels were found in follicular cells and early stage oocytes (stages I and II), with very low levels within maturationally competent late-stage oocytes (IV), which is in line with the nondetectable levels in early-stage fertilized embryos [39, 45]. Similarly, in the testis, mRNA and protein expression was observed in spermatogonia and early spermatocytes and not in spermatids or sperm [39]. In the brain, immunohistochemical analysis showed robust Pr levels in the two main neuroendocrine regions of the brain: the preoptic area and the mediobasal hypothalamus [39]. These expression data indicate that Pr regulates reproductive signaling in the brain, early germ cell proliferation in testis and ovarian follicular functions, but not final oocyte or sperm maturation.

Using transcription activator-like effector nucleases (TALENs), several *pr* mutant fish lines have been made [46, 47]. Three lines were generated with small deletions in exon 1 of the gene encoding Pr, resulting in frameshifts in the gene starting at the sequence encoding amino acid 51, 47 and 205 [46]. Another *pr* mutant line was made by deletion of a large part (13.4 kb) of the *pr* gene between exon 1 and 6, resulting in a frame shift from the codon encoding amino acid 151 [45]. These mutants showed highly similar phenotypes. Homozygous females were infertile, whereas males were fertile [45, 46]. In the mutant females, oocytes appeared to mature normally,

but these mature oocytes were trapped within the follicular cells and failed to ovulate from the ovaries, even after induction by human Chorionic Gonadotropin (hCG) [45, 46]. Apparently, Pr activity is required for ovulation, and it was shown that the Prostaglandin E Receptor 4 was involved, since the expression of this receptor is induced upon Pr activation and antagonists of this receptor inhibited hCG-induced ovulation [45]. Transcriptomic analysis of follicular cells from *pr* mutant fish using RNA sequencing revealed gene networks involved in the induction of ovulation, that are well conserved between zebrafish, mice and humans, and in which genes are enriched that are involved in inflammation, apoptosis, vascularization, cell-matrix adhesion, extracellular matrix remodeling, and the cell cycle [47].

5. The Zebrafish Ar

The cloning and characterization of the zebrafish *ar* gene was reported by several groups [48–50]. Although in several other fish species the occurrence of duplicate *ar* genes had been reported, only one *ar* gene was identified in the zebrafish genome. A single copy of the *ar* gene is present in Cypriniformes (e.g. common carp, goldfish, zebrafish), Characiformes (e.g. black widow tetras) and Siluriformes (e.g. sharptooth catfish), so the duplicate copy must have been lost during the early evolution of the Otophysi lineage, which gave rise to these orders of fish species [51]. The zebrafish *ar* gene appears to be the orthologue of the gene that encodes the Ar-A isoform in fish species containing two *ar* genes.

The zebrafish Ar was demonstrated to bind the endogenous steroids T, DHT, 11-ketotestosterone (11-KT) with high affinity (k_d in nanomolar range), A2 with an approximately ten-fold lower affinity and the synthetic steroids 17α-methyltestosterone (MT) and 17α-dimethyl-19-nortestosterone (mibolerone, MB) with a slightly higher affinity [48, 50]. Transcriptional activation of an MMTV promoter driving a luciferase gene in human and zebrafish cells was found to be similar for T, 11-KT, MT, and DHT (although for DHT the potency was lower in zebrafish cells than in human cells) [49, 50]. Flutamide was shown to act as an antagonist of the zebrafish Ar in these reporter assays [50]. Additionally, It was demonstrated that the main endogenous Ar ligand in zebrafish is 11-KT, which is produced from androstenedione in a two-step process [50]. The biosynthesis of the main mammalian androgens, T and DHT, is limited in zebrafish [50, 52], just like in other fish species [44].

Expression levels of the *ar* gene during development were determined using RT-PCR. Maternal *ar* mRNA was detected and the concentration of *ar* transcripts decreased until around 12 hpf, after which it shows a gradual increase until at least 14 dpf [49]. Using in-situ hybridization, *ar* transcripts were found in the presumptive pronephros and in olfactory placodes in 24 hpf embryos and at 3–5 dpf in the pineal organ anlage and the retina [53]. In adult fish, *ar* mRNA was detected in the gonads, brain, kidney, liver, skin, muscle and eye tissue by RT-PCR [49]. By in-situ hybridization the presence of *ar* mRNA was demonstrated in the testis, in the subpopulation of Sertoli cells contacting early spermatogonia (De Waal 2008) and in brain regions known to be involved in neuroendocrine regulation, like the preoptic area, the periventricular hypothalamus and discrete telencephalic regions [53].

Microarray analysis of the transcriptome of livers from adult female zebrafish exposed to the androgen 17α-methyldihydrotestosterone (MDHT) revealed regulation of genes involved in steroid and retinoic acid metabolism, hormone transport and regulation of cell growth and

proliferation [54]. In another study, the effect of exposure of adult fish to the AR antagonists flutamide and vinclozolin on the gonadal transcriptome in males and females was determined using microarray analysis [55]. Multiple pathways were shown to be regulated, which were involved in steroidogenesis, spermatogenesis and fertilization. These pathways included the integrin, actin, steroidogenic factor-1, Fgf receptor signaling pathway and the polyamine and androgen synthesis pathway [55]. The transcriptional effect of 11-KT exposure of zebrafish larvae between 5 and 6 dpf was investigated by whole body microarray analysis [56]. Four regulated genes involved in hormone metabolism were additionally studied in detail: *cyp2k22*, *slco1f4*, *lipca*, *sult2st3*, and it was demonstrated using the antagonist nilutamide that these genes are specific targets of Ar [56].

6. The Zebrafish Gr

Soon after the Ar, the zebrafish Gr was characterized [57]. Only a single *gr* gene was identified in the zebrafish genome, although many fish species were known to have two *gr* orthologues. Just like the other duplicate steroid receptor gene copies, the two *gr* orthologues were a result of the whole genome duplication that had occurred early in the teleost lineage, and they are called *gr1* and *gr2* [58]. Phylogenetic and syntenic analysis showed that the zebrafish genome contains an orthologue of the *gr2* gene, and that an ancestor of the zebrafish has lost its *gr1* gene due to a genomic rearrangement in chromosome 21 [57]. This loss must have been a relatively recent event, since the common carp, which belongs to the same family (Cyprinidae) as the zebrafish, has both *gr1* and *gr2* orthologue genes [58]. Luciferase assays showed that the zebrafish Gr is able to activate the MMTV promoter upon binding to the synthetic glucocorticoid dexamethasone (EC_{50} in the subnanomolar range) and the endogenous hormone cortisol (with a ~30-fold lower potency)[57]. However, dexamethasone and cortisol were inactive in a larval regeneration-inhibition assay in which other glucocorticoids like beclomethasone dipropionate were shown to be active in a Gr-dependent manner [59, 60]. This activity appeared to correlate with the ability to induce the expression of the *cripto-1* gene. It was demonstrated that specific ligand-induced Gr conformational changes induce these effects, with a particular role for the functional groups at the C17 position of the cortisol backbone [60].

Remarkably, an alternative splice variant of Gr was identified in zebrafish, which shows a high level of similarity with the human GR β-isoform [57]. In humans, this C-terminal splice variant is not able to bind ligand and has been shown to act as a dominant-negative inhibitor of the canonical GR (called GRα), although this function is debated [61, 62]. The zebrafish Grβ diverges from Grα at the same point as its human equivalent, and it showed dominant-negative activity in luciferase reporter assays [57, 63]. The zebrafish Grβ has most likely evolved independently from the human GRβ, since the amino acid sequence of its isoform-specific C-terminus shows no homology with the human sequence, and the exon encoding this Grβ-specific sequence is located at a different position in the *gr* gene [57]. Further analysis of the role of Grβ revealed no dominant-negative activity *in vivo*, and the function of this splice variant remains unclear [63, 64].

The expression levels of *gr* during zebrafish development were measured by RT-PCR and the results showed the presence of maternal transcripts at fertilization and a decrease in mRNA levels until 24 hpf [65]. After this time point, *gr* mRNA levels are increased again at 48 hpf

and remain stable until at least 7dpf [65]. In-situ hybridization showed no spatial restriction for *gr* mRNA during embryonic stages, but high expression levels in the brain, liver and intestinal bulb at 5 dpf [57, 66–68]. This embryonic expression pattern was confirmed using immunohistochemistry [68]. In adults, expression was demonstrated in spleen, liver, intestine, heart, brain, gill and muscle tissue by RT-PCR [57].

The expression of the zebrafish *gr* gene has been knocked down using two types of morpholinos: splice-blocking morpholinos that hybridize to a splice site and interfere with splicing at their target site in the genome and translation-blocking morpholinos, which interfere with translation of a transcript by hybridizing to the translation start site. Interestingly, injection of the first type of morpholinos into early zebrafish embryos did not elicit any visible malformations [59, 64, 67], whereas the latter type resulted in severe developmental defects, like craniofacial and caudal malformations, delayed somitogenesis, and defects in somite and tail morphogenesis [67, 68]. These effects were shown to be specific for the *gr* knockdown, since *gr* mRNA rescued the phenotypes of the morpholino-treated embryos [67, 68]. In addition, morpholino-treated embryos had smaller hearts and reduced cardiac function [69]. The strong effect of the translation-blocking morpholino was explained by its ability to target maternally deposited mRNA, whereas a splice-blocking morpholino only interferes with mRNA which is transcribed *de novo*, so it is only effective after zygotic transcription has started [67]. Microarray analysis of embryos at 5 and 10 hpf demonstrated that the early *gr* expression has an important role in cell survival and inhibits apoptosis [67]. At 24 and 36 hpf, a microarray analysis showed disturbed regulation of genes involved in numerous developmental processes in embryos treated with a translation-blocking morpholino [70].

Interestingly, transcriptomic analysis revealed that knockdown of *gr* expression by a morpholino regulates a different cluster of genes than activation of the receptor by a 6 h treatment with a high dose (100 μM) of the synthetic glucocorticoid dexamethasone [64]. These data suggest that Gr regulates two clusters of genes in zebrafish embryos: one that is regulated under basal conditions and one that is regulated upon increased receptor activation, after stress or upon treatment with a pharmacological dose of a glucocorticoid. The first cluster mainly contains genes involved in cell cycle and cell death, whereas the latter cluster mainly contains genes involved in catabolic processes like proteolysis and glycogen breakdown [64]. In support of this hypothesis, in another microarray study treatment with a low dose of dexamethasone (50 nM) between 0 and 5 dpf was found to mainly regulate genes involved in cell cycle and cell death [71].

Finally, ion regulation was severely compromised in embryos upon morpholino knockdown of *gr* expression. $Ca2^{+}$ uptake was impaired [72], as well as Na+ uptake following 24 h exposure to acidic water [73]. These results are in line with a decreased number of ionocytes in morpholino-treated embryos, in particular Na^{+}-K^{+}-ATPase rich cells (NaRCs) and H^{+}-ATPase rich cells (HRCs) [74]. Furthermore, the secretion of acid was decreased, which could be explained by a suppressed expression of transporters involved in acid secretion [72].

In a forward-genetic screen of chemically mutated zebrafish a mutation was identified in the *gr* gene, which results in a single amino acid substitution in the DBD of the receptor, making the Gr transcriptionally inactive [75]. In humans, a mutation in the equivalent amino acid occurs naturally and also results in a transcriptionally inactive receptor and results in resistance to glucocorticoids [76]. This mutant zebrafish shows no morphological malformations, is

adult-viable and displays increased cortisol levels due to a lack of feedback on the regulation of its secretion. Behavioral studies of adult fish revealed a depression-like profile, including decreased exploratory behavior and impaired habituation to repeated exposure to an anxiogenic environment [75]. Administration of the antidepressant fluoxetine as well as social interactions were shown to restore normal behavior [75, 77]. Using this mutant, it was demonstrated that Gr mediates the glucocorticoid-induced inhibition of leukocyte migration upon wounding [78] and the stimulation of the formation of hematopoietic stem and progenitor cells (HSPCs) by glucocorticoids [79]. In addition, it was found that light adaptation in the retina was disturbed in mutant larvae [80]. The relatively mild phenotype of this mutant zebrafish line has cast doubt on the specificity of some of the morphological malformations observed upon morpholino knockdown. This is supported by very recent data from a Crispr/Cas9 *gr* knockout line, which displays a similarly mild phenotype [81].

Gr activity could be measured using a transgenic line with a luciferase gene driven promoter with four glucocorticoid response elements (GREs). By measuring in-vivo luciferase activity in 5 dpf larvae of this line, the glucocorticoid activity of exogenous compounds could be determined, as well as the action of increased cortisol levels upon exposure to osmotic stress or the administration of the precursor hormone pregnenolone [82]. In two other lines GFP was coupled to a promoter containing six GREs [83] or nine GREs [84]. Ubiquitous fluorescence was detectable from 14 hpf, and at 3 dpf the signal was more specifically expressed in GR target tissues like the brain, liver and pronephros [84]. Due to the high sensitivity Gr activity was also detected in compartments such as fin, eyes, and otic vesicles. In 5 dpf larvae, compounds were screened for glucocorticoid activity, and the specificity of the response was confirmed using morpholino or TALEN knockdown of the receptor or the antagonist RU486 [83, 84]. The endogenous cortisol activity was monitored after osmotic stress and during the diurnal cycle [83, 84]. In adult fish, fluorescence was observed in many tissues, such as the esophageal sacs mucosa, ventricular epicardium, liver, intestinal mucosa, testis and ovary [84].

7. The Zebrafish Mr

Finally, in 2011 the zebrafish *mr* gene was identified, which was shown to be present as a single copy [85]. Using luciferase assays, this receptor was shown to activate the MMTV promoter after binding of aldosterone, cortisol, and 11-deoxycorticosterone (DOC). Fish do not produce aldosterone, but it has been suggested that in fish DOC has a similar function to aldosterone in mammals [86]. DOC is putatively converted from progesterone by steroid 21-monooxygenase (*cyp21a1*), but the presence of this enzyme has not been demonstrated in zebrafish and has been poorly characterized in several other fish species [43, 44]. Interestingly, the human MR antagonist spironolactone showed agonistic activity on the zebrafish Mr, whereas eplerenone and nimodipine acted as antagonists of the zebrafish Mr [85]. A two-hybrid assay showed that aldosterone, cortisol and DOC induced an interaction between the N- and C-terminal ends of the zebrafish Mr, whereas in the human MR this interaction was exclusively induced by aldosterone [85]. Surprisingly, spironolactone did not induce this N/C-interaction, and since this ligand had agonistic activity, the functional significance of the N/C-interaction remains unclear [85].

Little is known about the expression pattern of *mr* in zebrafish. Expression of *mr* mRNA was shown by RT-PCR to be minimal immediately after fertilization and increased 52-fold between

1.5 and 97 hpf, after which it remained stable until 146 hpf. Still, at this time point the *mr* mRNA level was significantly lower than the *gr* mRNA concentration [65]. Morpholino knockdown of *mr* expression was performed in several studies on ion regulation, but no Mr effects on this process were observed [72, 74, 87].

8. Future Perspective

An important aspect of steroid receptor research in zebrafish is the relatively limited number of studies that have been performed in this area. A PubMed (`https://www.ncbi.nlm.nih.gov/pubmedsearch`) search, using 'zebrafish' and the name of the steroid receptors (in as many variations as possible) as search terms, returned a total of 308 published articles, of which more than half involve research on Er (308). For comparison, the same searches with the search term 'mouse' instead of 'zebrafish' retrieved about 50 times more published papers (15,086). This difference is not specific for research on steroid receptors in these animal models and rather reflects the difference in popularity of the models in general, because the terms 'mouse' alone also returned about 50 times more articles than the term 'zebrafish'. Limiting the searches to articles published in 2016 and 2017 showed a 20-fold difference, illustrating an increase in the role of the zebrafish model in steroid receptor research.

In order to further increase the role of the zebrafish model system in steroid receptor research, the advantages of this model system should be better exploited. Morpholino knockdown of the receptor has been performed for all steroid receptor types, but the usage of morpholinos is currently under debate [14]. Fortunately, this technology is rapidly becoming obsolete. Within the Zebrafish Mutation Project (`http://www.sanger.ac.uk/resources/zebrafish/zmp/`), using a combination of chemical mutagenesis and high-throughput sequencing [28], mutant zebrafish lines have been generated for all steroid receptor types. In addition, targeted mutagenesis has become possible with the CRISPR/Cas9 technology [88], which also allows for the generation of tissue-specific [89] and inducible gene knockout strategies [90]. Another challenge that remains is the analysis of steroid receptor expression at the protein level, due to the limited availability of antibodies specific for (zebra)fish receptors.

Approximately half of the zebrafish steroid receptor research involves ecotoxicological studies on EDCs. The zebrafish will become an increasingly popular model for screening for EDCs due to the identification of novel (molecular) biomarkers and the generation of reporter lines for the activity of steroid receptors (currently, only reporter lines for Er and Gr activity are available). In addition, the increased availability of specific receptor mutants will enhance studies on the mechanism of action of EDCs in the zebrafish model. Furthermore, an increasing role for the zebrafish in biomedical research involving steroid receptors can be expected. Current research aiming at the discovery of novel steroid drugs focuses on the identification of selective receptor modulators, which are steroids that induce desired pharmacological effects without eliciting adverse effects. The zebrafish is highly suitable for in-vivo phenotypic screening and can be used to study compounds that dissociate desired from adverse effects. Targeted mutagenesis of receptor domains involved in e.g. DNA binding, dimerization or interaction with transcriptional coregulators will further advance our understanding of the specific molecular actions of steroid drugs, and how they translate into their desired and adverse pharmacological effects. In these ecotoxicological and biomedical studies the evolutionary distance of the zebrafish from humans

compared to mammalian model organisms like mice and rats should be taken into account. Data obtained in zebrafish will often need validation in more closely related animal models before they can be extrapolated to humans. Therefore, the zebrafish model should not be considered as a model organism replacing other animal models, but rather as an additional complementary tool with specific advantages.

In conclusion, the initial characterization and expression analysis of all five steroid receptor types, the generation of mutants and reporter lines and the identification of biomarkers to study their action has made the zebrafish an ideal model system for research on steroid receptors. It is therefore anticipated that in the next decade the role of the zebrafish in steroid receptor research will significantly increase, particularly in research on EDCs and selective steroid receptor modulators.

Competing Interests

The author declares no competing interests.

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