Nuclear receptor research in zebrafish

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Abstract

Nuclear receptors (NRs) form a superfamily of transcription factors that can be activated by ligands and are involved in a wide range of physiological processes. NRs are well conserved between vertebrate species. The zebrafish, an increasingly popular animal model system, contains a total of 73 NR genes, and orthologues of almost all human NRs are present. In this review article, an overview is presented of NR research in which the zebrafish has been used as a model. Research is described on the three most studied zebrafish NRs: the estrogen receptors (ERs), retinoic acid receptors (RARs) and peroxisome proliferator-activated receptors (PPARs). The studies on these receptors illustrate the versatility of the zebrafish as a model for ecotoxicological, developmental and biomedical research. Although the use of the zebrafish in NR research is still relatively limited, it is expected that in the next decade the full potential of this animal model will be exploited.

Key Words

- ▶ zebrafish
- ▶ nuclear receptor
- ▶ estrogen receptor
- ▶ retinoic acid receptor
- ▶ peroxisome proliferator-activated receptor

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Introduction

The nuclear receptor (NR) superfamily forms the largest family of transcription factors in metazoan organisms, in which they regulate reproduction, development, metabolism and the immune response (Bookout *et al.* 2006, Evans & Mangelsdorf 2014). Many NRs become active upon binding of small lipophilic compounds, like steroid and thyroid hormones, vitamins and dietary lipids. However, about half of the NRs are designated as orphan receptors, since an activating ligand has not been identified for them. In these orphan NRs, the ligand-binding pocket is either absent or continuously occupied by a small lipid or heme molecule.

Ligand activation of NRs induces an active conformation, enabling transcriptional regulation (Moore *et al.* 2006). Within the NR superfamily, two modes of action exist. The first group of ligand-activated NRs already interact with their DNA recognition site before ligand binding, but since they recruit transcriptional corepressors, they are kept in an inactive state. In contrast, the members of the steroid receptor family are

not bound to their DNA target sites in the absence of a ligand. Upon activation by a ligand, both groups of NRs recruit coregulator molecules, which modulate the local chromatin structure whereby the transcriptional rate of a nearby gene is either enhanced or repressed. Since their activity is not induced by a ligand, orphan receptors display constitutive transcriptional activity.

NRs 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). These common structural domains have greatly facilitated the identification of NR genes in genomic sequences and the generation of phylogenetic trees, enabling a thorough analysis of the evolution of the NR superfamily (Bertrand *et al.* 2004, Escriva *et al.* 2004, Markov *et al.* 2008, Bridgham *et al.* 2010). Evolutionary studies have demonstrated that the NR superfamily has evolved from a single ligand-activated ancestral receptor. Through a process of gene duplication

and subsequent mutation, the capacity to bind different ligands and the ligand-independent activation were acquired. Phylogenetic analysis has led to the classification of the NR superfamily into seven subfamilies (NR0–6) on which the current nomenclature of the NR superfamily is based.

The zebrafish was initially used as a model system for studies on embryonic development and morphogenesis. Its easy maintenance, high fecundity and optical transparency made it a highly versatile research model. Forward and reverse genetic studies in zebrafish were facilitated when the complete genomic sequence of the zebrafish became available. Since embryos and larvae were small in size and could easily be obtained in large numbers, large-scale studies could be performed. 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 generation of mutant and transgenic fish lines opened up new possibilities for zebrafish research, and novel genome-editing techniques like CRISPR/Cas9 will further advance the field. As a result, over the last decade, the zebrafish has emerged as an important model system not only for research on vertebrate development (Beis & Stainier 2006, Collins & Stainier 2016) but also for ecotoxicological (Dai *et al.* 2014) and biomedical research (Lieschke & Currie 2007, MacRae & Peterson 2015). In this review, an overview is presented of the research on NRs using the zebrafish as a model system.

Ten years ago, Bertrand *et al.* (2007) found 70 NR genes in the zebrafish genome. When they compared the zebrafish NR family to its mammalian equivalent (48 NR genes in the human genome, 49 in the mouse genome), they could not find zebrafish orthologues of RAR β , LXR β and CAR. Actually, LXR β and CAR have not been identified in any fish species. Compared to mammals, 19 NR genes were found to be duplicated in zebrafish, as a result of a genome duplication that occurred in the teleost lineage soon after the divergence from the tetrapods (Glasauer & Neuhauss 2014): TR α , RAR α , RAR γ , PPAR α , PPAR β , Rev-ErbA β , Rev-ErbA γ , ROR α , ROR γ , VDR, RXR α , RXR β , COUP-TFI, EAR2, ER β , ERR β or γ , SF-1, GCNF and SHP. Although the official nomenclature for duplicate NRs in (zebra) fish is the addition of ‘-A’ or ‘-B’ suffixes (e.g. PPAR β -A and PPAR β -B, see <http://zf.in.org>), for several receptor duplicates, the suffixes ‘1’ and ‘2’ are commonly used (e.g. ER β 1 and ER β 2). In a recent study, Zhao and coworkers (2015) found three more NR genes in the zebrafish genome: paralogues of the RXR γ and NURR1 gene and a third paralogue of the ROR γ gene. Thus, a total of 73 NR

genes have been identified in the zebrafish genome, and an overview of the zebrafish NR superfamily is presented in Fig. 1.

The vast majority of the duplicate NR genes found in the zebrafish genome were also found in other fish species like pufferfish, medaka, tilapia and stickleback. However, differences exist as a result of lineage-specific loss of duplicate NR genes during evolution. Analysis of the relative evolution rate of the zebrafish NRs, by comparison with their human orthologue, showed that for eight duplicated NRs (PPAR α , RevErbA β , ROR α , ROR γ , COUP-TFI, ERR β , GCNF and SHP), the evolution rate was highly different between the two proteins (Bertrand *et al.* 2007). This suggests neofunctionalization of the rapidly evolved copy (i.e. it has acquired a novel function), and this was confirmed by divergent expression patterns of the two NR genes (Bertrand *et al.* 2007). The alternative fate of duplicate genes, subfunctionalization (i.e. the two copies share the function of their nonduplicated ancestor (Force *et al.* 1999)), was suggested for the other duplicated NR genes.

The expression pattern of all NR genes has been analyzed using whole-mount *in situ* hybridizations on zebrafish embryos (Bertrand *et al.* 2007). The results demonstrated that most NR genes are not expressed during early embryogenesis, but start to be expressed after 24 h post fertilization (hpf), during organogenesis. Many of these genes are expressed in the developing central nervous system and retina, and very few in the lens, blood somites and heart. Based on the expression patterns, the zebrafish NRs could be divided in 7 clusters, and this division appeared to be very different from a similar clustering that was done based on expression of NR genes in adult mice (Bookout *et al.* 2006). This may indicate species-specific roles of NRs or different roles of NRs during embryonic development.

A PubMed (<https://www.ncbi.nlm.nih.gov/pubmedsearch>) search, using ‘zebrafish’ and the name of the nuclear receptor (in as many variations as possible) as search terms, showed which NRs are being studied in zebrafish. The results of these searches are shown in Fig. 2. The searches returned a total of 678 published articles. This may be considered a small number compared to the 28,062 articles that the search term ‘zebrafish’ alone returns. Apparently, the zebrafish is not (yet) a very popular model system for NR research, even though among vertebrates the richest abundance of NRs is found in teleosts like the zebrafish (Zhao *et al.* 2015). Almost half of the published articles on zebrafish NRs involves research on steroid receptors (298), and approximately

nr1a1	TR α	TR α	nr2a1	HNF4 α		nr3a1	ER α	
nr1a2	TR β		nr2a2	HNF4 γ		nr3a2	ER β	ER β
nr1b1	RAR α	RAR α	nr2a3	HNF4 δ		nr3b1	ERR α	
nr1b3	RAR γ	RAR γ	nr2b1	RXR α	RXR α	nr3b2	ERR β	
nr1c1	PPAR α	PPAR α	nr2b2	RXR β	RXR β	nr3b3	ERR γ	ERR γ
nr1c2	PPAR β	PPAR β	nr2b3	RXR γ	RXR γ	nr3b4	ERR δ	
nr1c3	PPAR γ		nr2c1	TR2		nr3c1	GR	
nr1d1	Rev-ErbA α		nr2c2	TR4		nr3c2	MR	
nr1d2	Rev-ErbA β	Rev-ErbA β	nr2e1	TLL		nr3c3	PR	
nr1d4	Rev-ErbA γ	Rev-ErbA γ	nr2e3	PNR		nr3c4	AR	
nr1f1	ROR α	ROR α	nr2f1	COUP-TFI	COUP-TFI	nr4a1	NGFIB	
nr1f2	ROR β		nr2f2	COUP-TFII		nr4a2	NURR1	NURR1
nr1f3	ROR γ	ROR γ	nr2f5	COUP-TFg		nr4a3	NOR1	
nr1h3	LXR α		nr2f6	EAR-2	EAR-2	nr5a1	SF-1	SF-1
nr1h4	FXR α					nr5a2	LRH-1	
nr1h5	FXR β					nr5a5	FF1C	
nr1i1	VDR	VDR				nr6a1	GCNF	GCNF
nr1i2	PXR					nr0b1	DAX1	
						nr0b2	SHP	SHP

Figure 1

Overview of the nuclear receptor superfamily in zebrafish. The zebrafish genome contains 73 genes encoding nuclear receptors. No orthologues of the mammalian RAR β , LXR β and CAR have been found, and 21 mammalian NR genes have been found to be duplicated in zebrafish. The gene encoding ROR γ has even been triplicated. Figure was adapted with permission from [Bertrand et al. \(2007\)](#). Additional information was used from [Zhao et al. \(2015\)](#).

half of these articles describes research on ER (155). About one-third of zebrafish NR articles deals with the NR1 family of receptors (241), with RAR (63) and PPAR (50) being the most popular subjects to study. No publications were listed on NOR1 and EAR-2 in zebrafish. In this review, research will be highlighted on the three most studied zebrafish NRs, viz. ERs, RARs and PPARs. Studies on these NRs provide an interesting overview of the research areas in which the zebrafish is currently used, ecotoxicological, developmental and biomedical research, respectively.

The zebrafish estrogen receptors (ERs)

The ER is by far the most studied NR in zebrafish. Three ER genes have been identified in zebrafish ([Bardet et al. 2002](#), [Legler et al. 2002](#), [Menuet et al. 2002](#)): one encoding an orthologue of the mammalian ER α and two encoding orthologues of ER β (referred to as ER β 1 and ER β 2). These three receptors show distinct but largely overlapping expression patterns in tissues like the brain, pituitary, liver and gonads ([Menuet et al. 2002](#)). Particularly high expression levels were observed for all receptor isoforms in the neuromasts of the lateral line, a mechanoreceptive

system specific to aquatic vertebrates ([Tingaud-Sequeira et al. 2004](#)). The zebrafish ERs have been knocked down in several studies. Temporary knockdown by a morpholino of the ER β 2 expression resulted in the disrupted development of these neuromasts (absence of hair cells), which could be related to an aberrant activation of the Notch signaling pathway in the morpholino-treated embryos ([Froehlicher et al. 2009](#)). Knockdown of ER α expression during embryonic stages by morpholino-induced blocking of translation of maternal transcripts resulted in severe developmental defects and early mortality ([Celeghin et al. 2011](#)). Induction of specific target genes of ER α and ER β 2 could also be inhibited by morpholino knockdown of the receptor ([Griffin et al. 2013](#)). Recently, a mutant zebrafish line has been identified, carrying a mutation in the gene encoding ER β 2 (resulting in an 8 amino acid insertion in the ligand-binding domain ([Lopez-Munoz et al. 2015](#)). This mutation resulted in distorted sexual ratios (characterized by an increased adult male population), altered testicular morphology and increased testosterone and 17 β -estradiol (E2) levels. In addition, these fish show a decreased immune response to a viral infection ([Lopez-Munoz et al. 2015](#)).

Figure 2

Results of a PubMed (<https://www.ncbi.nlm.nih.gov/pubmedsearch>) literature search using 'zebrafish' and the name of the nuclear receptor (in as many variations as possible) as search terms. The pie chart indicates the relative number of articles on a specific NR within the total number of publications on the NR superfamily in zebrafish. The searches returned a total of 678 published articles, of which approximately fifty percent involves research on steroid receptors (298), and approximately half of these articles describe research on ER (155). Receptors of the NR1 family are studied in about one-third of zebrafish NR articles (241), with RAR (63) and PPAR (50) being the most studied NRs in this family. NOR1 and EAR-2 have not been studied in zebrafish.

Further analysis of the literature revealed that about half of the published articles on the zebrafish ER involves research on the role of this receptor as a mediator of the activity 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 (Colborn *et al.* 1993). This may ultimately lead to altered development and/or reproduction in humans and wildlife. EDCs form a heterogeneous group of substances, encompassing natural (e.g. E2) and synthetic hormones (e.g. diethylstilbestrol (DES),

and 17 α -ethinylestradiol (EE2)), phytoestrogens (e.g. genistein), and seemingly unrelated compounds like pesticides (e.g. dichlorodiphenyltrichloroethane (DDT)), polychlorinated biphenyls (PCBs), bisphenol A (BPA), phthalates, flavonoids and polycyclic musks (Frye *et al.* 2012, Gore *et al.* 2015). The main mechanism of action for these compounds is generally suggested to be agonistic or antagonistic interaction with the ER.

The zebrafish is widely used as a model organism to identify EDCs, and to perform risk assessment for both humans and wildlife (Segner 2009, Dang 2016). Research focuses on the development of screening assays for the activity of EDCs and their mechanism of action. Several *in vivo* biomarkers are used to identify EDCs in zebrafish.

The concentration of vitellogenin (VTG) is the most widely used biomarker for EDC activity, in particular for compounds that activate ER (Dang 2016). VTG is an egg yolk precursor protein, which is produced and secreted by the liver, taken up by the ovary and modified by developing eggs to form the egg yolk. VTG synthesis is under tight hormonal control. Hepatic production of VTG is induced by estrogens, and in male and immature fish, low levels of VTG can be detected under normal conditions. In the zebrafish genome, seven VTG genes are present, and their expression level can be monitored at the mRNA and protein level, in blood, organ and whole-body homogenates. Induction of VTG synthesis in male fish by estrogens occurs rapidly and can be detected within 24h, which makes this assay suitable for short-term screening of EDCs. However, induction of VTG does not provide conclusive evidence on the mechanism of action of the identified EDC. It has been well established that ER activation induces VTG production, but recent studies have shown that AR, PR and GR activation may result in altered VTG levels as well (Dang 2016).

To identify novel molecular biomarkers for ER activity, several transcriptomic analyses have been performed (Baker & Hardiman 2014). Adult male and female fish have been exposed to EE2, and their liver and telencephalon transcriptome have been determined using microarray analysis (Martyniuk *et al.* 2007, Hoffmann *et al.* 2008). In another study, whole-body transcriptome analysis was performed on male adults after exposure to E2 (Lam *et al.* 2011). Finally, a microarray was performed during early developmental stages (1, 2, 3 and 4 days post fertilization (dpf) (Hao *et al.* 2013)). In addition to well-known ER target genes like *vtg1* (the only gene induced by estrogens at all stages studied (Hao *et al.* 2013)), *vtg3*, *vtg4* and *esr1*, these studies also revealed many metabolic genes (Martyniuk *et al.* 2007, Hoffmann *et al.* 2008) and genes involved in cell cycle and DNA repair (Lam *et al.* 2011) as being regulated by ER.

An alternative approach for short-term screening of EDCs is the monitoring of transgenic reporter fish in which an ER-responsive promoter is genetically coupled to a gene encoding a reporter protein like luciferase or green fluorescent protein (GFP). Using a transgenic line with the luciferase gene coupled to a promoter with a single estrogen response element (ERE), estrogenic activity could be measured after 96-h exposure to EDCs by measuring the luciferase activity in tissue homogenates. This assay has been performed using adult zebrafish and juveniles (30 dpf). The latter, which are at the stage of gonad differentiation, appear to be more responsive (Legler *et al.* 2000,

Legler *et al.* 2002, Bogers *et al.* 2006). In addition, two transgenic zebrafish lines have been generated in which GFP is used, enabling microscopic analysis of the reporter activity. In the first line, a promoter with 5 EREs was utilized (Gorelick & Halpern 2011). After estrogen exposure, GFP expression was observed in embryonic and larval zebrafish in the brain, liver and pancreas (Gorelick & Halpern 2011, Hao *et al.* 2013), consistent with the location of *erb2* expression during these developmental stages (Gorelick & Halpern 2011). 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 appeared to be dependent on ER (it could be inhibited by the ER antagonist ICI 182,780 (fulvestrant)), although ER expression in these regions was not detectable by *in situ* hybridization at these stages. Interestingly, GFP induction by EDCs like EE2, BPA and genistein showed marked differences in the tissue specificity of the response (Gorelick & Halpern 2011). In adult fish, males showed no GFP expression in the liver, whereas displayed dim expression, 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 a second 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 (Tong *et al.* 2009). Before 9 dpf, the GFP expression is only observed after induction by estrogens, enabling screening estrogenic activity of EDCs using exposure from 0 to 5 dpf (Brion *et al.* 2012). To investigate which receptor isoform mediates the effect of EDCs, reporter zebrafish liver cell lines are available in which an ER:luciferase construct has stably been transfected together with an expression vector for ER α , ER β 1 or ER β 2 (Cosnefroy *et al.* 2012). These cell lines have now replaced assays in which reporter constructs and expression vectors were transiently transfected in human cells (Le Page *et al.* 2006, Sassi-Messai *et al.* 2009).

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 (Segner 2009, Dang 2016). Partial life-cycle studies may involve investigating the impact of short-term exposure in mature actively reproducing individuals, and in these studies, endpoints like fertilization success and fecundity are measured, which may be complemented by gonad histopathology or plasma levels of sex steroids

or VTG. Alternatively, fish may be exposed during a sensitive period in their development, generally between 0 and 60–70 dpf, and then the key endpoint is sexual differentiation, determined using parameters like gonad histopathology and sex ratio. Unlike other small fish model species like fathead minnow and medaka, zebrafish do not display secondary sex characteristics that are quantifiable and can be used as biomarkers for endocrine disruption. Full life-cycle testing involves exposure from fertilization until the reproductive age. These studies have the advantage of monitoring both developmental and reproductive effects of EDCs, but suffer from the disadvantage that they are more costly and resource intensive than partial life-cycle studies. In 2012, the Organisation for Economic Cooperation and Development (OECD) published a guidance document providing guidelines for various types of *in vitro* and *in vivo* EDC screening assays (OECD 2012). Among these assays, several standardized *in vivo* tests which may be performed in zebrafish are described, like the Fish Short-Term Reproduction Assay (OECD 2009a), the 21-Day Fish Assay (OECD 2009b), the Fish Sexual Development Test (FSDT (OECD 2011)) and the Fish Lifecycle Toxicity Test (FLCTT (EPA 1996)).

In recent years, zebrafish embryos and larvae have been used to unravel the mechanism of action of several EDCs. It was shown by using morpholinos and antagonists that the disruption of the migration and distribution of primordial germ cells by EE2 are mediated by ER β 1 (Hu *et al.* 2014). Using receptor antagonists, the behavioral effects of flavonoids were demonstrated to be independent of ER activity (Bugel *et al.* 2016). Finally, BPA has been shown to induce otolith malformations during development (Gibert *et al.* 2011). Although it is commonly suggested that ER is the receptor mediating this effect, it was shown that these malformations are ERR γ dependent using morpholino knockdown and overexpression by mRNA injection (Tohme *et al.* 2014).

The zebrafish retinoic acid receptors (RARs)

Retinoic acid (RA) is the active metabolite of vitamin A. The importance of vitamin A in embryonic development has been recognized since ancient times, even though its molecular identity was not elucidated until the twentieth century. Over the last century, the relevance of vitamin A in vertebrate development has been established experimentally in a large number of nutritional studies in mammalian and avian animal models (Mark *et al.* 2009,

Metzler & Sandell 2016, Wiseman *et al.* 2016). In all studies, newborns from mothers fed vitamin A-deficient diets showed an array of severe malformations. Collectively, these malformations are now known as vitamin A deficiency (VAD) syndrome, and include defects in the development of the eye and other organs like the lungs, kidneys and heart. Developmental abnormalities do not only occur under conditions of deficiency, but exposure to excess vitamin A or one of its metabolites can be equally detrimental to embryonic development, indicating that levels of these compounds should be tightly regulated.

The effects of RA are mediated by the RARs and RXRs, which form heterodimers. RARs are activated by RA and are only dimerized with RXRs, whereas RXRs do not require ligand binding for their activity and can also dimerize with other NRs like TRs, PPARs, VDRs and LXRs. In tetrapods, three RARs encoded by different genes have been identified: RAR α , RAR β and RAR γ . In the zebrafish genome, no gene encoding the RAR β isoform was found, whereas 2 genes encoding an RAR α isoform (Hale *et al.* 2006, Waxman & Yelon 2007) and two genes encoding an RAR γ isoform (Tallafuss *et al.* 2006, Waxman & Yelon 2007) were identified. *In situ* hybridizations for all zebrafish RAR and RXR paralogues showed overlapping and distinct areas of expression (Waxman & Yelon 2007).

The tight regulation of the RA concentration during embryogenesis is for a large part controlled through complementary expression of RA-synthesizing and RA-degrading enzymes. RA is biosynthesized from vitamin A in a two-step process. First, vitamin A is converted into retinaldehyde, and this step is catalyzed by alcohol dehydrogenases (ADHs) or retinol dehydrogenases (RDHs). Second, retinaldehyde is oxidized to RA, and this step requires retinal dehydrogenases, members of the aldehyde dehydrogenase (ALDH) family. Whereas humans express three ALDH enzymes that catalyze this reaction, only two are present in zebrafish (Aldha2 and Aldha3 (Pittlik *et al.* 2008)). Biodegradation of RA into 4-hydroxy-RA is controlled by enzymes of the Cytochrome P450 26 (Cyp26) subfamily. Zebrafish, like mammals, express three subtypes: Cyp26a1, Cyp26b1 and Cyp26c1.

The importance for vertebrate embryogenesis of these enzymes controlling the RA concentrations has clearly been demonstrated in a variety of studies (reviewed in Samarut *et al.* 2015). In general, these studies show that during early embryogenesis RA is involved in anterior/posterior patterning, promoting the formation of posterior structures like the rhombomeres in the hindbrain, at the expense of anterior structures like eyes

and the forebrain. To control early embryogenesis, RA concentrations are high in posterior tissues, mainly due to high Aldh1a2 expression in the posterior mesoderm, and low RA levels are found in anterior regions, mainly as a result of Cyp26a1 activity. This gradient has elegantly been visualized using a RARE:YFP transgenic line in which the expression of Yellow Fluorescent Protein (YFP) is driven by retinoic acid response elements (RAREs) (Perz-Edwards *et al.* 2001, White *et al.* 2007). More recently, a transgenic line was generated expressing so-called genetically encoded probes for RA (GEPRAs) that can be used to measure RA levels directly (Shimozono *et al.* 2013). These probes are made of the RAR LBD fused to a pair of fluorescent proteins. A conformational change in the LBD upon RA binding induces fluorescence resonance energy transfer (FRET) between the two fluorescent proteins, and this FRET signal can be readily detected. In addition to studies using these reporter lines, the relevance of enzymes that regulate the RA gradient has been studied using chemical inhibitors, selective knockdown of genes using morpholinos or CRISPR/Cas9 technology or using mutants identified in forward-genetic screens, in which the offspring zebrafish, randomly mutagenized by treatment with N-ethyl-N-nitrosourea (ENU), was screened for developmental abnormalities.

In two forward-genetic screens, mutant lines were generated with a mutation in the gene encoding the RA biosynthesis enzyme Aldh1a2 (*neckless* (Begemann *et al.* 2001) and *no-fin* (Grandel *et al.* 2002)). These mutants displayed defects in the hindbrain and midline mesendodermal tissues, and absence of pectoral fins and cartilaginous gill arches, which is highly reminiscent of VAD in other animal models. However, the hindbrain phenotype was less severe than observed in these other models, which was explained by maternal Aldh1a2 activity. This was demonstrated in a study in which two *aldh1a2* morpholinos were compared (Alexa *et al.* 2009): a translation-blocking morpholino (affecting expression from all mRNAs) which induced a similar phenotype as chemical inhibition of Aldh1a2, and a splice-blocking morpholino (affecting expression from only zygotic mRNAs) which displayed a less severe phenotype. Morpholino knockdown of the expression of another enzyme of this subfamily, Aldh1a3, did not result in hindbrain defects, but aberrant eye development was observed (Yahyavi *et al.* 2013).

Three more mutant lines, with mutations in the *cyp26a1* gene (*giraffe*) and *cypb1* gene (*stocksteif* and *dolphin*), were derived from forward-genetic screens (Emoto *et al.*

2005, Laue *et al.* 2008, Spoorendonk *et al.* 2008). In the *giraffe* mutant, the expression of Cyp26a1 affected the main RA-metabolizing enzyme during early embryonic development. This mutant displayed patterning defects in various organs including the common cardinal vein, pectoral fin, tail, hindbrain and spinal cord (Emoto *et al.* 2005). The lines in which the *cyp26b1* gene was mutated, *stocksteif* and *dolphin*, showed severe over-ossification, which appeared to be a result of increased osteoblast activity in these mutants (Spoorendonk *et al.* 2008). This effect could be phenocopied by RA treatment between 84 and 96 hpf, but treatment between 48 and 54 hpf resulted in decreased bone mineralization, probably due to inhibited osteoblast differentiation, suggesting a dual role for RA in bone development (Li *et al.* 2010).

To study which RAR isoform mediates the developmental effects of RA, individual isoforms were knocked down using morpholinos (Linville *et al.* 2009). This study showed that all four isoforms are required for anterior-posterior patterning of rhombomeres in the hindbrain. In contrast, RAR γ A was uniquely required in the cranial mesoderm for hindbrain patterning, and RAR α B in lateral plate mesoderm for specification of the pectoral fins. Morpholino knockdown of specific receptor isoforms combined with transcriptome analysis revealed isoform-specific transcriptional activity. Knockdown of the two RAR α isoforms, the two RAR γ isoforms and all four RAR isoforms demonstrated that three classes of RA-responsive genes exist. The first class of genes requires activation of one specific isoform for their regulation, the second class of genes respond to activation of either isoform, and the third class require activation of both isoforms (Samarut *et al.* 2014).

The zebrafish peroxisome proliferator-activated receptors (PPARs)

PPARs are NRs which are activated by dietary lipids including saturated and unsaturated fatty acids, and by lipids involved in intracellular signaling pathways like prostaglandins and leukotrienes. By binding these ligands, they play an important physiological role as lipid sensors regulating energy metabolism and adipocyte differentiation. In addition, involvement of PPARs in vascular tone and atherosclerotic plaque formation, anti-inflammatory effects and a regulatory role in myelination have been proposed. Polymorphisms in the PPAR genes have been shown to result in disturbed lipid metabolism and insulin resistance (Yong *et al.* 2008), and synthetic

PPAR ligands like fibrates and thiazolidinediones have proven to be effective therapies against metabolic disorders like diabetes. They have been shown to decrease dyslipidemia and insulin resistance. As a result, PPARs are a widely studied class of NRs, in particular as drug targets for novel therapies against disease like diabetes and obesity (Berger & Moller 2002, Feige *et al.* 2006).

In vertebrates, three PPARs have been identified which are encoded by three different genes: PPAR α , PPAR β (also called PPAR δ) and PPAR γ . They regulate gene expression by binding to PPAR response elements (PPREs) as a heterodimer with RXR. *In silico* analysis showed that the zebrafish genome contains five genes encoding PPARs: duplicate PPAR α and PPAR β genes and a single gene for PPAR γ (Bertrand *et al.* 2007, McPartland *et al.* 2007, Den Broeder *et al.* 2015). Studies on PPREs in the promoter regions of zebrafish fatty acid-binding protein (*fabp*) genes showed that different PPAR isoforms activate specific target genes (Laprairie *et al.* 2016).

An *in situ* hybridization study showed no detectable expression of the PPAR α -encoding genes until 5 dpf. At this time point mRNA for PPAR β -A and PPAR γ was detectable around the swim bladder, in the liver and the intestine, and PPAR β -B mRNA around the swim bladder (Bertrand *et al.* 2007). At 15 dpf, expression of the gene for PPAR γ was detected in terminally differentiated adipocytes located in the pancreas (which shows that it can be used as a marker for these cells), and in cells of the intestinal epithelium (Flynn *et al.* 2009). In adult fish, expression of this gene was observed in liver, pancreas and visceral adipose tissue (Imrie & Sadler 2010). Immunohistochemistry revealed that PPAR α , PPAR β and PPAR γ are all widely expressed after 7 dpf, and that for all isoforms, the expression level in liver is higher in early developmental stages than in adults (Ibabe *et al.* 2005).

In line with the variety of physiological systems affected by PPARs, many effects of these NRs have been investigated in the zebrafish model. For example, the role of PPAR γ has been studied in a zebrafish xenograft model of gastric cancer (Cho *et al.* 2015), PPAR α activation appeared to stimulate angiogenesis in zebrafish larvae (Rizvi *et al.* 2013) and PPAR agonists were shown to enhance myelination, as measured by whole larvae myelin basic protein transcript levels (Buckley *et al.* 2010). The majority of research on the effects of PPAR activation in zebrafish has focused on lipid metabolism to develop drugs against obesity (Den Broeder *et al.* 2015). In most of these studies, the optical transparency of the zebrafish larvae allows

for the visualization of adipocyte formation and fatty acid uptake *in vivo* using fluorescent lipophilic dyes like Nile Red or LipidGreen. Non-fluorescent dyes like Oil Red O or Sudan dyes are used as well, but require fixation of the larvae. Using Nile Red, a standard assay has been developed for chemical screening of potential drugs or toxic compounds (Jones *et al.* 2008, Tingaud-Sequeira *et al.* 2011). In this zebrafish obesogenic test (ZOT), larvae are fed a standard or high-fat diet for one day, and are subsequently starved for two days with chemical treatment during the second day. Subsequently, as a measure for adiposity, whole-body lipid droplet formation was measured using Nile Red staining and fluorescence signal quantification.

In this assay, the PPAR γ agonist rosiglitazone showed a clear obesogenic effect (Tingaud-Sequeira *et al.* 2011). The effect was blocked by a PPAR γ antagonist, which on its own showed anti-obesogenic activity in the ZOT (Ouadah-Boussouf & Babin 2016). Similar data were observed for a PPAR β agonist and antagonist, whereas a PPAR α antagonist did not elicit an effect (Ouadah-Boussouf & Babin 2016). In a similar study, treatment with a PPAR γ antagonist between 3 and 5 dpf resulted in decreased lipid droplet accumulation in the head and heart regions, suggesting decreased adipocyte differentiation (Li *et al.* 2010). The PPAR γ antagonist bisphenol A diglycidyl ether (BADGE) showed a similar effect between 26 and 55 hpf, which could be inhibited by RA or endocannabinoid treatment (Fraher *et al.* 2015). Recently, it was suggested that the adipogenic activity of PPAR γ agonists involves activation of the expression of the enzyme cytosolic NADP(+) isocitrate dehydrogenase (cICDH) (Chun *et al.* 2014).

Although rosiglitazone was demonstrated to induce obesogenic effects in zebrafish, which were inhibited by the PPAR γ antagonist T0070907 (Ouadah-Boussouf & Babin 2016), several studies have shown that this compound binds very weakly to the zebrafish PPAR γ . It appeared to be unable to activate a zebrafish PPAR γ LBD construct in transient transfection experiments (Riu *et al.* 2011), and it was suggested that the replacement of human PPAR γ Gly284 and Cys285 by serine and tyrosine residues in the zebrafish PPAR γ causes a weak binding affinity of rosiglitazone for the zebrafish receptor (Grimaldi *et al.* 2015). Apparently, the ligand specificity of zebrafish receptors does not necessarily resemble the situation in humans, and this has been shown for other NRs as well (Escriva *et al.* 2006, Pippal *et al.* 2011). These cases seem to be exceptions, since most often ligand specificity is well conserved between humans and zebrafish, but care should

be taken when pharmacological research is performed in zebrafish.

Conclusion

In this review, an overview is presented of research on NRs in which the zebrafish is used as a model system. The research on the three NRs that are most studied in zebrafish, ERs, RARs and PPARs, illustrates the versatility and advantages of this animal model system. The work performed on the action of ERs illustrates that the zebrafish is widely used in ecotoxicological research as a model for monitoring of the toxicity of environmental pollutants, due to its potential for *in vivo* phenotypical screening at relatively high throughput. Furthermore, the research on RARs reflects that the zebrafish model is ideally suited for developmental studies, because of its rapid external development and the suitability for forward and reverse genetic studies. The investigations performed on the action of PPARs in zebrafish display the possibilities of the zebrafish as a model system for human disease in biomedical research, since most processes underlying these diseases are well conserved between humans and zebrafish. However, the limited use of the zebrafish in NR research as compared to other animal models shows that the full potential of the zebrafish model system has not been reached in this research area. A good example of the exploitation of the advantages of the zebrafish model is the engineering of a screening system for human NR ligands and cofactors (Tiefenbach *et al.* 2010). In this system, an affinity tag and a GAL4 DBD were fused to a human NR LBD, and this fusion protein was expressed in zebrafish containing a UAS:GFP reporter construct. This 'Ligand Trap' system allows for high-throughput visual screening of endogenous and exogenous ligand activity, and the identification of novel NR-specific ligands and cofactors. In the next decade, it is expected that further characterization, advancement of technology and a wider availability of mutant and transgenic fish lines will increase the role of the zebrafish model system in NR research.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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