Functional characterization and expression analysis of the androgen receptor in zebrafish (*Danio rerio*) testis

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Abstract

The biological activity of androgens, important for male sexual differentiation and development, is mediated by the androgen receptor (AR) that binds to specific DNA recognition sites regulating the transcription of androgen target genes. We investigated androgen production by adult zebrafish testis tissue, and identified 11 β -hydroxyandrostenedione, 11-ketoandrostenedione (OA), and 11-ketotestosterone (11-KT) as main products, and hence potential ligands, for the zebrafish Ar. These androgens were then included in the pharmacological characterization of the zebrafish Ar. The zebrafish Ar responded well in terms of binding and transactivation to synthetic androgens as well as to testosterone and 11-KT, and reasonably well to OA and androstenedione. *In situ* hybridization analysis of zebrafish testis revealed that *ar* mRNA expression was detected in the subpopulation of Sertoli cells contacting early spermatogonia.

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Introduction

The androgen receptor (AR) is a member of the nuclear receptor family of proteins that function as ligand-activated transcription factors. The AR mediates the masculinizing effects of androgens on different parts of the reproductive system at different stages of ontogenesis. For example, androgenic sex steroids are involved in male differentiation of the efferent duct system for germ cells (Hannema & Hughes 2007), spermatogenesis (De Gendt *et al.* 2004), reproductive behavior, and secondary sexual characteristics (Sato *et al.* 2004, Soma 2006). The AR shows a widespread expression pattern over different tissues, suggesting a broad spectrum of androgen-induced biological activities. Also in females, androgens are important for reproductive function, as indicated by the folliculogenesis phenotype in female *Ar* knockout mice (Shiina *et al.* 2006).

Teleost fish are no exception to this general vertebrate pattern, as exemplified by the effects of androgens on secondary sexual characteristics and behavior (Pall *et al.* 2002a, 2002b), spermatogenesis (Miura *et al.* 1991, Cavaco *et al.* 2001), or Leydig cell androgen production (Cavaco *et al.* 1999). As regards sex differentiation, fish appear to be particularly sensitive to androgen action, considering that fully functional female-to-male sex reversal can be induced by exposure of juvenile (Baroiller & Guiguen 2001) and even adult fish

(Kobayashi *et al.* 1991) to androgens; in some species, sex change is part of the normal life cycle (Baroiller & Guiguen 2001).

To further our work on zebrafish male sex differentiation and on the development to functional maturity and adult regulation of the two main testicular functions, spermatogenesis and steroidogenesis, and to be able to proceed to studies on the identity and regulation of the expression of AR target genes relevant for these processes, we cloned the full-length zebrafish *ar* cDNA and studied *ar* mRNA expression by real-time, quantitative PCR and *in situ* hybridization. Moreover, we wanted to identify the physiological ligand(s) for the zebrafish Ar in males. In this context, it is important to note that teleost fish express 11 β -hydroxylase (Cyp11b) (Wang & Orban 2007) and 11 β -hydroxysteroid dehydrogenase (Hsd11b) (Kusakabe *et al.* 2006) activities in the testis, so that 11-ketotestosterone (11-KT) is a prominent circulating androgen next to testosterone (T) in many species (Schmidt & Idler 1962, Borg 1994). Although respective data are not available in zebrafish, a close relative, the common carp (*Cyprinus carpio*), showed the typical teleost pattern with 11-KT levels being twice as high as the T levels in the plasma of mature males (Koldras *et al.* 1990). We have, therefore, analyzed the main androgens produced by zebrafish testis tissue, which were then included in the pharmacological characterization of the zebrafish Ar. Cloning and

quantitative expression analysis of zebrafish *ar* have been published also by others (Jørgensen *et al.* 2007, Hossain *et al.* 2008) very recently, but these studies did not include a detailed comparison between the ligand-binding characteristics and transactivation properties of the zebrafish Ar. Such a comparison is, however, reported in the present study.

Results

Zebrafish *ar* cDNA cloning, phylogenetic and expression analyses

The results section describing the full-length zebrafish *ar* cDNA cloning, the phylogenetic analysis of the zebrafish Ar, and the tissue distribution and ontogeny of zebrafish *ar* mRNA expression are presented as Supplementary Information, along with Supplementary Figures 1–3, which can be viewed online at www.reproduction-online.org/supplemental/.

Androgen production by zebrafish testis tissue

To study the steroid specificity and transactivation capacity of the zebrafish Ar in a targeted manner, we first investigated to which products adult zebrafish testis tissue fragments metabolized 100 nM [3 H]-androstenedione ([3 H]-A2) during 15-, 30-, or 60-min incubation. Separation of the products by thin layer chromatography showed that the substrate remained largely unconverted in the absence of tissue (Fig. 1A), but a minor impurity of the substrate (Unk-2) was found. In all cases where [3 H]-A2 was incubated with tissue, at least 96% of the radioactivity co-migrated with known carrier steroids. Densitometry of the autoradiogram showed that the substrate was progressively metabolized with time (Fig. 1B). A major metabolite was 11 β -hydroxyandrostenedione (OHA) that appeared quickly and was prominently present (11–22%) at all time points. The pattern of appearance of 11-ketoandrostenedione (OA) differed from that of OHA by showing a steady increase with time from 7 to nearly 27% of the total product. This pattern was similar to the one of 11-KT except that the latter accumulated at a lower rate (1.5–11%). Minor products (<2% at all time points) were T and 11 β -hydroxytestosterone (OHT), and 1–2% of the radioactive products that did not co-migrate with the non-radioactive carrier steroids were assigned to Unk-1 and -3 respectively, while up to 1% was represented by Unk-2, a minor impurity of the substrate also present in the control incubation without tissue. Taken together, these data suggest that androstenedione (A2) is quickly and effectively metabolized to OHA by Cyp11b activity, before being converted to OA by Hsd11b activity. OA is further converted to 11-KT by 17 β -hydroxysteroid dehydrogenase (Hsd17b) activity. It appears, however, that neither OHA nor A2 are readily accepted as

Figure 1 Androgen production in zebrafish testis tissue. (A) Representative autoradiogram of a thin layer chromatogram of dichloromethane-extracted L15 medium containing [3 H]-A2 in the presence of zebrafish testis fragments for 15, 30, or 60 min or in the absence of zebrafish testis fragments for 60 min (con 60 min), after which non-radioactive carrier steroids (androstenedione (A2), 11-ketoandrostenedione (OA), testosterone (T), 11 β -hydroxyandrostenedione (OHA), 11-ketotestosterone (11-KT), 11 β -hydroxytestosterone (OHT)) were added. Unk-1, -2, and -3 indicate unknown products. (B) Quantification of densitometrically scanned bands of the autoradiogram (shown in Fig. 1A), representing the steroid metabolites of [3 H]-A2 conversion in zebrafish testis tissue; each column represents the average radioactivity ($\pm$ S.E.M.) for a given metabolite, expressed as percentage of the total amount of radioactivity per lane, determined in three independent experiments.

substrate by the testicular Hsd17b activity, considering that only trace amounts of T or OHT have been found. These results are summarized schematically in Fig. 2.

Ligand-binding characteristics of the zebrafish Ar

To determine the ligand-binding characteristics of the zebrafish Ar, we first performed saturation ligand-binding assays on human embryonic kidney cell line (HEK 293T cells), transfected with the zebrafish *ar* expression vector construct, using [3 H]-testosterone ([3 H]-T) as a tracer. High-affinity and saturable binding

Figure 2 Schematic representation of steroidogenic processing of androstenedione in zebrafish testis (based on the data shown in Fig. 1). The main products of zebrafish testis are 11 β -hydroxyandrostenedione (OHA), 11-ketoandrostenedione (OA), and 11-ketotestosterone (11-KT). The main steroidogenic pathway is indicated by thick arrows. Steroidogenic enzymes indicated are: 11 β -hydroxylase (Cyp11b), 11 β -hydroxysteroid dehydrogenase (Hsd11b), and 17 β -hydroxysteroid dehydrogenase (Hsd17b).

of [3 H]-T was observed (Fig. 3A), while no specific binding was observed using non-transfected or mock-transfected cells (data not shown). Our analysis clearly indicated that the zebrafish Ar behaved as a single class of high-affinity [3 H]-T-binding protein with a K_d of 1.7 nM.

Zebrafish Ar steroid specificity

To determine the relative affinity of the zebrafish Ar for the most prominent androgens produced in zebrafish testis in comparison with other (synthetic) androgens and non-androgenic steroids, competitive binding assays were performed, using 2.7 nM [3 H]-T as a tracer (Table 1 and Fig. 3B). The curves were parallel, indicating competitive binding between the androgens and [3 H]-T, allowing IC_{50} values to be determined and K_i values to be calculated. The zebrafish Ar showed the highest affinity for the two synthetic androgens 17 α -methyltestosterone (MT) and 17 α -dimethyl-19-nortestosterone (mibolerone, MB) (Table 1). Natural androgens, like T and 11-KT, showed nanomolar affinities for the receptor, with T and 5 α -dihydrotestosterone (DHT; an important androgen in higher vertebrates, but not produced in fish) showing slightly higher affinities for the receptor than 11-KT. Of the androgenic steroids that are biochemical precursors of 11-KT, A2 had a higher affinity for the zebrafish Ar than OA or OHA. Of the non-androgenic steroids, progesterone (P) and its hydroxylation derivatives (17 α -hydroxyprogesterone (OHP) and 17 α -hydroxy, 20 β -dihydroprogesterone (OHH $_2$ P)) showed affinities for the receptor in the high nanomolar range, while 17 β -estradiol (E_2) and cortisol showed clearly lower affinities to the zebrafish Ar.

Transactivation of the zebrafish Ar

To determine the steroid-induced transactivation properties of the zebrafish Ar, we co-transfected the zebrafish Ar expression vector construct together with an androgen-regulated reporter vector construct (MMTV-Luc vector, containing the luciferase gene under the control of the mouse mammary tumor virus (MMTV) promoter) into HEK 293T cells (see below). When HEK 293T cells were transfected only with the MMTV-Luc vector, androgens did not increase luciferase activity, indicating that HEK 293T cells do not express endogenous ARs (data not shown).

Dose-dependent, zebrafish Ar-mediated activation of the MMTV promoter was shown for several androgens, e.g., MT, DHT, 11-KT and T (Table 1 and Fig. 3C). The synthetic androgen MT was the most potent steroid for the zebrafish Ar ($EC_{50} = 0.03 \pm 0.01$ nM), reaching maximal activation at 1 nM (not shown). The main circulating androgens in fish, 11-KT and T, were somewhat less potent in activating the zebrafish Ar, T being more potent than 11-KT. A2 and OA showed medium to high nanomolar EC_{50} as well as K_i values. OHA, albeit showing a certain binding to the zebrafish Ar, was a weak androgen in terms of Ar-mediated transactivation of the MMTV promoter. The EC_{50} values for all steroids tested are shown in Table 1.

To determine the relative potency of various non-androgenic steroids to transactivate the MMTV promoter via the zebrafish Ar, they were tested at a fixed concentration of 100 nM (Fig. 3D). The androgens 11-KT and T (positive controls) induced clear responses, increasing luciferase activity by 32- and 23-fold respectively. Of the non-androgenic steroids, only P and OHH $_2$ P were able to induce small but statistically non-significant increases in luciferase activity, whereas E_2 , cortisol, and OHP were inactive. Hence, the surprisingly low K_i concentrations found for some of the non-androgenic steroids (e.g., P, OHH $_2$ P) were not associated with the capacity to activate the zebrafish Ar. We can conclude that low K_i concentrations only coincide with low EC_{50} concentrations for activation as well as with effective induction of reporter gene expression in the case of androgens. Among these C19 steroids, the 17 β -hydroxylated configuration was most effective while the status of the C-atom 11 (with or without an oxygen function, viz. T and 11-KT) seemed less relevant. However, when a keto group was present at C-atom 17, the status of C-atom 11 did matter, since androgens with either no oxygen (A2) or a keto group (OA) showed an intermediate affinity and transactivational capacity, while an 11 β -hydroxy group (OHA) further reduced binding affinity and abolished biological activity.

Transactivation of the MMTV promoter via the 11-KT-stimulated zebrafish Ar was inhibited by an AR antagonist. The antagonistic effect of flutamide on the zebrafish Ar-mediated MMTV-promoter transactivation

via increasing doses of 11-KT (1 pM to 10 μ M) was clearly demonstrated (Fig. 3E), since a 4- or 60-fold higher concentration of 11-KT was needed to reach 50% of the maximal activation with 11-KT in the presence of 1 (EC₅₀ = 4.3 nM) or 10 μ M (EC₅₀ = 64 nM) flutamide respectively, compared with the condition where no flutamide was included (EC₅₀ = 1.2 nM).

Localization of *ar* mRNA in zebrafish testis

To identify the cell types in zebrafish testis that express *ar* mRNA, we performed *in situ* hybridization on 10 μ m thick cryosections. At low power magnification, a clear signal was observed in discrete cells scattered throughout the testis, in the sections that were hybridized with the antisense cRNA *ar* probe (Fig. 4A). No signal was observed with the sense cRNA *ar* probe (Fig. 4B), indicating the specificity of the antisense probe generated against the sequence of zebrafish *ar* mRNA. At a higher magnification (Fig. 4C), the *in situ* hybridization signal was observed in the cytoplasm of Sertoli cells, judged by the shape and intratubular position of the

signal. The Sertoli cell is the only intratubular somatic cell type and differs from the germ cells by showing a triangular or kidney-shaped nucleus, in contrast to the round or oval nuclear shape of germ cells. Not all Sertoli cells showed the same level of *ar* mRNA expression, since only a subset of Sertoli cells were stained (Fig. 4C), compared with the higher number of Sertoli cells lining a tubule in a histological section at the same magnification (see for comparison Fig. 4D). Based on the size, shape, number, and position close to the tubular basement membrane of the germ cells enveloped by the sub-population of *ar* mRNA positive Sertoli cells, these germ cells were identified as early spermatogonia present as single cells or in small groups. No clear *in situ* hybridization signal was observed for peritubular myoid and interstitial Leydig cells (data not shown).

Discussion

This study describes the cDNA cloning and expression analysis (see Supplementary Information) of the zebrafish *ar*. Moreover, we identified the physiologically relevant androgens, produced in the zebrafish testis, and included these – together with other steroids – in the functional characterization of the zebrafish Ar.

Figure 3 Binding and transactivation analysis of the zebrafish Ar.

(A) Saturation analysis of zebrafish Ar. HEK 293T cells were transfected with the zebrafish *ar* expression vector construct and incubated with increasing amounts of [³H]-T in the presence or absence of a 1000-fold excess of unlabeled T. Total, non-specific, and specific [³H]-T binding are shown. (B) Competitive binding analysis of the zebrafish Ar. HEK 293T cells were transiently transfected with the zebrafish *ar* expression vector construct, and incubated with [³H]-T as a tracer in the absence (not shown) or presence of increasing concentrations (10 pM to 1 μ M) of various androgens. (C) Ligand-induced transactivation properties of the zebrafish Ar. HEK 293T cells were transiently co-transfected with the MMTV-luciferase vector together with the zebrafish *ar* expression vector construct. The cells were incubated with increasing concentrations of various androgens (1 pM to 10 μ M). (D) Ligand-induced, zebrafish Ar-mediated transactivation of the MMTV promoter at a fixed concentration of 100 nM of various steroids. As a control, the ratio of the fold induction of OHA at 100 nM (no appreciable induction observed) divided by the fold induction of OHA at 1 pM (no active ligand) is given. In addition, OHA has a very low affinity for the zebrafish Ar. Each column represents the mean ratio of luciferase activity at 100 nM of the steroid divided by the luciferase activity at 1 pM of OHA of three independent experiments, with the vertical bars representing the S.E.M. Lack of error bars is due to the errors being too small to show graphically. Asterisks represent fold induction significantly different from control, $P < 0.001$, using one-way ANOVA with Newman-Keuls post test. (E) Inhibition of 11-KT-induced, zebrafish Ar-mediated transactivation of the MMTV promoter by flutamide. The cells were incubated for 24 h with increasing concentrations of 11-KT (1 pM to 10 μ M) with or without 1 μ M or 10 μ M flutamide. Percentage (%) of response: values are given relative to the maximal amount of luciferase activity for each condition. Each point (Fig. 3B, C, and E) represents the mean $\pm$ S.E.M. of three independent experiments. Curves were generated using non-linear regression (GraphPad Prism 4.0).

Table 1 Comparison of ligand competition data and transactivation data of the zebrafish Ar.

Competitive binding		Transactivation		
Steroid	K _i (nM)	Steroid	EC ₅₀ (nM)	Max. fold induction
MT	0.73 ± 0.20	MT	0.03 ± 0.01	40.81 ± 3.93
MB	0.82 ± 0.01	DHT	0.13 ± 0.04	39.69 ± 2.22
DHT	1.65 ± 1.55	T	0.42 ± 0.20	39.33 ± 4.64
[³ H]-T (K _d value)	1.70 ± 0.50	11-KT	1.16 ± 0.60	43.70 ± 2.52
OHH ₂ P	4.64 ± 2.80	MB	1.63 ± 2.79	34.86 ± 3.79
11-KT	4.77 ± 2.26	OA	50.8 ± 34.7	36.38 ± 5.01
P	6.99 ± 1.61	A2	58.0 ± 31.1	16.84 ± 4.17
A2	19.6 ± 1.84	OHH ₂ P	116 ± 103	8.44 ± 2.19
OHP	21.8 ± 1.34	P	467 ± 615	4.50 ± 1.61
E ₂	71.6 ± 96.7	OHP	951 ± 1543	4.50 ± 1.46
OA	71.7 ± 5.24	Cortisol	1855 ± 702	10.07 ± 2.55
OHA	128 ± 24.0	OHA	ND	2.16 ± 0.26
Cortisol	352 ± 162	E ₂	ND	1.87 ± 0.26

K_i values (nM) of several steroids for the zebrafish Ar are given. For reference, the K_d value of radiolabeled testosterone ([³H]-T) is also included. The zebrafish Ar was expressed in HEK 293T cells and [³H]-T was used as tracer. K_i values represent average ± S.E.M. of three independent experiments. EC₅₀ values of ligand-induced Ar transactivation of the MMTV promoter are also shown. Luciferase activity was measured after incubation with increasing concentrations of various steroid hormones (1 pM to 10 μM). EC₅₀ values represent average ± S.E.M. of three independent experiments.

In the present study, a positive *ar in situ* hybridization signal was detected in a subpopulation of Sertoli cells in zebrafish testis. The majority of Sertoli cells, however, remained unlabeled, indicating that not all Sertoli cells have the same level of *ar* mRNA expression. Spermatogenesis in zebrafish, as in all other fish and amphibians, occurs within spermatogenic cysts. The cysts are formed when Sertoli cells envelop a germ cell clone by cytoplasmic extensions. Within each cyst, germ cell development occurs synchronously and different cysts contain clonal lines of germ cells at different

developmental stages (for review, see Schulz & Miura 2002). From our evaluation of the morphology and position of the germ cells surrounded by the zebrafish *ar* mRNA-positive subpopulation of Sertoli cells, we conclude that Sertoli cells in contact with early spermatogonia express the highest levels of *ar* mRNA in zebrafish testis. Interestingly, testicular explants from immature Japanese eel (*Anguilla japonica*) containing only early spermatogonia responded to incubations with 11-KT by showing full spermatogenesis (Miura *et al.* 1991), starting with several rounds of rapid proliferation of spermatogonia. A high level of expression of *ar* mRNA in Sertoli cells surrounding early spermatogonia would be consistent with the notion that these Sertoli cells are the target of stimulatory effects of 11-KT, resulting in a stimulation of spermatogonial proliferation and differentiation (Miura & Miura 2001). Likewise, the level of Ar protein in the zone of the salamander testis that contains predominantly spermatogonia was 1.5- to 5-fold higher than in zones containing further advanced germ cell types (Singh & Callard 1992). Future work has to demonstrate whether 11-KT has similar effects on zebrafish spermatogenesis as in Japanese eel, and whether progress of spermatogenesis beyond the stage of early spermatogonia is associated with a down-regulation of *ar* mRNA levels in the Sertoli cells contacting later germ cell stages. Although in mammalian testis a particular Sertoli cell supports germ cells in different stages of development simultaneously, differences in *Ar* mRNA levels among Sertoli cells that depend on the stage of the seminiferous epithelial cycle have been described in rat (Shan *et al.* 1995). In the same study, *Ar* mRNA has been detected in Leydig cells and peritubular myoid cells, albeit at much lower levels than in Sertoli cells at adulthood. In the present study no prominent positive *in situ* hybridization signal was found

Figure 4 *In situ* hybridization analysis of *ar* mRNA localization in zebrafish testis. Lower magnification (10×) of a 10 μm cryosection of zebrafish testis, hybridized with (A) the *ar* antisense cRNA probe, showing signal in discrete cells, and (B) the *ar* sense cRNA probe, showing no specific signal. Higher magnification (60×) of a cryosection of zebrafish testis, hybridized with (C) the *ar* antisense cRNA probe, revealed staining in the cytoplasm of a subpopulation of Sertoli cells. (D) Resin-embedded histological section (4 μm) stained with toluidine blue shows the position of different germ cell stages and Sertoli cells. Indicated are the nuclei of Sertoli cells (arrows) and early spermatogonia (arrowheads), while late spermatogonia are indicated with ISg, spermatocytes by Sc, spermatids by St, and spermatozoa by Sz (in C and D). Scale bars are shown in each panel.

in somatic cell types other than Sertoli cells, indicating that the levels of *ar* mRNA in Leydig cells and peritubular myoid cells in zebrafish testis are too low to be detected by the present *in situ* hybridization approach.

Naturally occurring androgens as well as synthetic androgens are potential ligands for ARs. Our studies suggest that OHA, OA, and 11-KT are the major A2 metabolites of the adult zebrafish testis. Although we did not study the production of A2, it seems unlikely that this steroid is a quantitatively important end product of the zebrafish testis, viz. its rapid and effective conversion to OHA. Considering the low affinity and marginal transactivational capacity of OHA, it is unlikely to be a relevant AR ligand in zebrafish. However, OA and in particular 11-KT accumulate at the end of the biosynthetic chain and show respectively reasonable and high binding affinity and transactivation properties. We propose to consider 11-KT as the physiologically most important androgen of the group of 11-oxygenated steroids produced by zebrafish testis, in particular because 11-KT holds the most downstream position in the steroidogenic pathway. In closely related species, such as goldfish (*Carassius auratus*; Abdullah & Kime 1994) or common carp (Barry *et al.* 1990), 11-oxygenated teleost androgens have been identified as main products of testicular steroidogenesis, such as 11-KT in goldfish, or OA in common carp, which can either be converted to 11-KT by Hsd17b activity residing in erythrocytes of many fish species (Mayer *et al.* 1990), or is directly produced by carp testis tissue with an efficiency increasing during pubertal maturation (Consten *et al.* 2002), suggesting that an increasing Hsd17b activity (i.e., conversion of OA to 11-KT) is one of the factors associated with puberty. A testicular *hsd17b* type 3 cDNA has been identified recently in zebrafish (Mindnich *et al.* 2005), which converted OA to 11-KT. Although the same enzyme also has the catalytic capacity to convert OHA to OHT and A2 to T when transfected into a cell line (Mindnich *et al.* 2005), these conversions are not occurring to a noteworthy degree in zebrafish testis tissue fragments. A possible explanation may be the competition for the substrates in the primary tissue culture: we showed that A2 is rapidly converted to OHA by Cyp11b activity, possibly restricting the A2 to T conversion, while Hsd17b-mediated conversion of OHA to OHT may be hampered by the Hsd11b-catalyzed conversion of OHA to OA.

The very low levels of T production in zebrafish testis tissue may seem surprising, also considering that circulating levels of T reach ~50% of those of 11-KT in adult male carp (Koldras *et al.* 1990). However, a similar situation has been described in African catfish (*Clarias gariepinus*) where the testicular production of T is at least 200-fold lower than one of the 11-oxygenated androgens (Vermeulen *et al.* 1994), while T plasma levels are in the same order of magnitude as 11-KT (Schulz *et al.* 1994). The possibility that circulating T might be derived from

extra-testicular sources was excluded for the catfish (Vermeulen *et al.* 1994) since castration decreased T plasma levels below the detection limits. We therefore speculate that the relatively high T plasma levels reflect the high-affinity and high-capacity binding of T to sex steroid-binding globulin (SGB), protecting T from rapid breakdown and thereby prolonging its biological half-life time. An SGB-like protein has been identified in zebrafish (Miguel-Queralt *et al.* 2004), and steroid-binding characteristics have been studied in a number of species, including the close zebrafish relatives goldfish (Pasmanik & Callard 1986) and carp (Chang & Lee 1992), showing that T (and E₂) but not 11-KT are bound with high affinity and capacity.

While no information on circulating androgens is available in zebrafish, respective data have been published from closely related bigger species. In common carp, 11-KT and T were quantified at different stages of the reproductive cycle, and the concentrations varied between 3–6 and 1.5–2.5 ng/ml respectively (Koldras *et al.* 1990). In goldfish (Rosenblum *et al.* 1985), 11-KT and T levels varied at different stages of testis development between 0.5–8.5 and 0.6–10 ng/ml respectively. Taking the above consideration and our pharmacological and steroidogenesis data, we conclude that 11-KT is likely to be the main androgen in adult male zebrafish, while T may fulfill specific roles as well. As regards the K_d , K_i , and EC₅₀ values for 11-KT in the range of 2–5 nM (see below), the plasma concentrations of 11-KT ranging from 0.5 to 10 ng/ml – i.e., 1.5–30 nM – in male carp and goldfish would be well suited to activate the Ar in zebrafish.

We have shown that the zebrafish Ar is a functional AR, which is supported by high-affinity androgen-binding and androgen-dependent transactivational capacity. Comparison of the ligand-binding and transactivation properties of the zebrafish Ar revealed that steroids with a high affinity for the receptor (i.e., MT, MB, DHT, T, OHH₂P, and 11-KT) also gave a high induction of zebrafish Ar-mediated transactivation. The exception is OHH₂P, which could only induce transactivation in the high nanomolar range.

Similar binding affinities for the zebrafish Ar have been obtained by Jørgensen *et al.* (2007) for DHT, 11-KT, T, and A2. In the regard of high-affinity binding to synthetic androgens as well as 11-KT, T, and DHT, the zebrafish Ar protein is similar to a number of other piscine Ar proteins cloned from rainbow trout (*Oncorhynchus mykiss*; i.e., Ara; Takeo & Yamashita 2000), fathead minnow (*Pimephales promelas*; Wilson *et al.* 2004), and three-spined stickleback (*Gasterosteus aculeatus*; Olsson *et al.* 2005). Studies on androgen binding to tissues extracts indicated that in some species two distinct patterns of androgen binding were found – one with rather specific binding of T and the other more similar to the broader specificity found for the zebrafish Ar in the present study. Binding of a broad range of synthetic and natural androgens, as found for the zebrafish Ar, was shared by

one of the Ar types present in Atlantic croaker (*Micropogonias undulatus*; Sperry & Thomas 1999) and coho salmon (*O. kisutch*; Fitzpatrick *et al.* 1994) gonad tissue.

The transactivation properties of zebrafish Ar relate well to those of rainbow trout Ara, which did not distinguish between T and 11-KT (Takeo & Yamashita 2000). Transactivation studies with both Japanese eel Ar proteins using a fixed concentration (100 nM) of the steroids tested) revealed that 11-KT, DHT, MB, and MT were the most potent steroids in terms of transactivation of eel Ara (Todo *et al.* 1999), and 11-KT, MB, and MT of eel Arb (Ikeuchi *et al.* 1999).

Data on zebrafish Ar transactivation, but not on receptor binding, have been reported very recently (Hossain *et al.* 2008) with regard to five androgens we have studied as well, however, using a zebrafish liver cell line. While similar data have been obtained as regards the two main androgens (11-KT and T), EC₅₀ values for MT and DHT were reported to be ~100-fold lower than the results presented here, while A2 that we found to have reasonable transactivation activity was reported to be inactive. The relatively low activity of DHT and MT does not appear to be in line with the studies that reported on the transactivation profiles of other fish Ar proteins (Ikeuchi *et al.* 1999, Todo *et al.* 1999, Takeo & Yamashita 2000). Moreover, the well-established use of MT as a compound to induce female-to-male sex reversal in zebrafish research (Westerfield 2000) or salmonid aquaculture (Donaldson & Hunter 1982) provides evidence for the biological activity of this compound. It also seems important to note that Hossain *et al.* (2008), when modeling the zebrafish Ar-binding site to calculate the interaction energy between Ar and different ligands, reported that DHT and A2 showed interaction energies similar to 11-KT.

The pharmacological characterization of Ar subtypes from the different species (Ikeuchi *et al.* 1999, Todo *et al.* 1999, Takeo & Yamashita 2000, Wilson *et al.* 2004, Olsson *et al.* 2005) does not show sufficient overlap to draw a general conclusion at present, because of differences in experimental set up (i.e., the use of different cell lines, different tracers, and different promoter-reporter constructs). Taken together, however, it seems that the zebrafish Ar described here groups well with Ar proteins characterized in fathead minnow, rainbow trout (i.e., Ara), Japanese eel (both Ara and Arb), and Atlantic croaker (type 2 Ar), presenting a broad androgen-binding specificity.

In summary, we found a single gene coding for a nuclear Ar in the zebrafish, and no indications exist for another *ar* gene in the zebrafish genome. A similar conclusion, supported by Southern blot analysis, has been drawn by Hossain *et al.* (2008). The zebrafish *ar* mRNA is expressed in all tissues examined and our *in situ* hybridization studies revealed that high levels of expression in adult testis are found in the subpopulation of Sertoli cells that contact early spermatogonia.

Furthermore, the receptor has been characterized *in vitro* to respond well in terms of binding as well as transactivation to 11-KT and T, two natural androgens proposed to be the physiologically most relevant androgens in zebrafish. The pharmacological characteristics and the tissue distribution pattern of the zebrafish Ar will allow us to further study the role of this receptor in male sex differentiation and spermatogenesis.

Materials and Methods

The material and methods section describing the cloning of the full-length zebrafish *ar* cDNA, the phylogenetic analysis of the zebrafish Ar as well as the real-time, quantitative PCR analysis of zebrafish *ar* tissue distribution and ontogeny are presented as Supplementary Information, which can be viewed online at www.reproduction-online.org/supplemental/.

Animals and source of steroid hormones

Zebrafish (*Danio rerio*; Tübingen AB strain) were kept at a 12 h light:12 h darkness cycle under standard conditions (Westerfield 2000). Animal culture and handling was consistent with the Dutch national regulations; the Life Science Faculties Committee for Animal Care and Use approved the experimental protocols.

All non-radioactive steroids and the AR antagonist flutamide were purchased from Sigma-Aldrich. Steroids used in this study were T, 11-KT, MT, MB, A2, OA, OHA, OHT, DHT, E₂, P, OHP, OHH₂P, and cortisol.

Zebrafish *ar* expression vector construct, cell lines, and transfections

The full-length open-reading frame of the zebrafish *ar* was PCR amplified using primers 1943 and 1944 (Supplementary Table 1, which can be viewed online at www.reproduction-online.org/supplemental/), cloned into pcDNA3.1/V5-His TOPO vector (Invitrogen), and the insert was sequence verified by DNA sequence analysis.

Since zebrafish is a small species (body weight of an adult male ~0.5 g), it is not feasible to perform ligand-binding studies on target tissue homogenates. Therefore, HEK 293T cells (DuBridge *et al.* 1987) were used to express the zebrafish *ar*. HEK 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v fetal bovine serum (FBS), non-essential amino acids, glutamine, and penicillin/streptomycin (all from Gibco) at 37 °C in a CO₂ incubator. The cells were transfected using a standard calcium phosphate precipitation method (Graham & Van der Eb 1973).

Binding assay

For saturation ligand-binding analysis, HEK 293T cells were seeded in 10 cm dishes (~1 × 10⁶ cells per dish) and after 24 h co-transfected with 0.1, 1, or 6 µg zebrafish *ar* expression vector construct and up to 11 µg carrier plasmid. One day after transfection, the cells were transferred to 24-well plates, coated

with poly-L-lysine hydrobromide (Sigma-Aldrich). Two days after the transfer, the cells were incubated for 2 h with binding assay medium (DMEM without phenol red, supplemented with glutamine, non-essential amino acids, and charcoal-stripped 0.2% v/v FBS (to remove any steroids originating from the FBS)) at 37 °C. Then, radioactive tracer ($[^3\text{H}]\text{-T}$; specific activity 77.0 Ci/mmol; Perkin-Elmer, Waltham, MA, USA) was added, either alone or in the presence of 1 μM unlabeled T, dissolved in binding medium. After 90-min incubation at room temperature, the cells were quickly washed twice with ice-cold PBS to remove unbound tracer. The cells were harvested in 200 μl sodium hydroxide (1 M) per well and radioactivity was counted in a β -counter (Packard 1900 TR liquid scintillation counter; Packard Instruments, Meriden, CT, USA). Specific $[^3\text{H}]\text{-T}$ binding over a range of increasing concentrations was calculated by subtracting non-specific binding (binding of tracer in the presence of unlabeled T) from total binding (binding of tracer in the absence of unlabeled T); in all cases, bound $[^3\text{H}]\text{-T}$ could be displaced by increasing concentrations of unlabeled T (data not shown). Pilot experiments with HEK 293T cells, transfected with various amounts of zebrafish *ar* expression vector construct, revealed that the zebrafish *Ar* displayed nanomolar affinity for $[^3\text{H}]\text{-T}$ (data not shown); in all other experiments, including the ligand competition assays (see below), HEK 293T cells were transfected with 1 μg zebrafish *ar* expression vector construct and 10 μg carrier plasmid. Binding remained unchanged over a period up to 8 h, indicating that $[^3\text{H}]\text{-T}$ is not metabolized in HEK 293T cells (data not shown). Moreover, both non-transfected and mock-transfected HEK 293T cells did not show any specific binding of $[^3\text{H}]\text{-T}$ (data not shown). Non-linear curve fitting procedures (GraphPad PRISM 4.0; GraphPad Software Inc.; San Diego, CA, USA) were used to calculate the K_d .

Ligand competition assay

To determine the affinity of other steroids for the zebrafish *Ar*, HEK 293T cells transfected with the zebrafish *ar* expression vector construct were incubated with increasing concentrations (10 pM to 1 μM) of each steroid, mixed with tracer ($[^3\text{H}]\text{-T}$; final concentration 2.7 nM) at room temperature, followed by measurement of tracer binding to the transfected cells. The IC_{50} values were calculated with non-linear regression (GraphPad PRISM 4.0). To allow the calculation of K_i values from IC_{50} values, a dose-response curve of non-labeled testosterone was included in each experiment. Assuming that ARs possess the same affinity for T and $[^3\text{H}]\text{-T}$, K_i values were calculated using the formula: $K_i = (\text{IC}_{50} \text{ steroid} / \text{IC}_{50} \text{ T}) \times K_d [^3\text{H}]\text{-T}$.

Transactivation assay

HEK 293T cells were seeded in 10 cm dishes ($\sim 1.25 \times 10^6$ cells per dish). After 24 h, the cells were co-transfected with 500 ng zebrafish *ar* expression plasmid and 10 μg of MMTV-Luc plasmid (Stocklin *et al.* 1996). After 1 day, the cells were transferred to 24-well plates coated with poly-L-lysine hydrobromide (Sigma-Aldrich). The next day, the medium was replaced with transactivation assay medium (DMEM without phenol red, supplemented with charcoal-stripped 0.2% v/v FBS, glutamine, and non-essential amino acids) containing

steroid at end concentrations ranging between 1 pM and 1 μM . After 24–36 h of incubation at 37 °C, the cells were harvested in lysis mix (100 mM potassium phosphate (pH 7.7), 1% v/v Triton X-100 (Sigma-Aldrich), 15% v/v glycerol, and 2 mM dithiothreitol (DTT)) and stored at –80 °C. Luciferase activity was determined by adding an equal volume of substrate mix (100 mM potassium phosphate (pH 7.7), 250 mM D-luciferin (Invitrogen), 1 mM DTT, 2 mM ATP (Roche) and 15 mM magnesium sulfate (Promega)) to thawed samples and luminescence was measured in a Perkin-Elmer luminometer.

Analysis of androgen production in zebrafish testis

Except for a study on the production of steroid glucuronides and their possible role as pheromones (Van den Hurk *et al.* 1987), no information has been published on the identity of the main androgenic steroids produced by adult zebrafish testis tissue. To address this caveat, the following experiment was performed in triplicate: testis tissue was collected from eight adult males (28.7 ± 3.6 mg total wet weight). Each testis was divided into two fragments, and the tissue fragments were pooled, rinsed with L15 medium, and transferred into 2 ml L15 medium containing tritiated A2 (7- $[^3\text{H}]\text{-A2}$; specific activity 24.5 Ci/mmol; NET1001, NEN Dupont, Boston, MA, USA) at a final concentration of 100 nM. After 15-, 30-, and 60-min incubation at 28 °C in a gently shaking waterbath (5 revolutions per min), 0.25 ml medium was removed, added to a tube containing a mixture of 5 μl of each of the following non-radioactive carrier steroids (20 $\mu\text{g}/\text{ml}$ ethanol): T, A2, OHA, OHT, OA, and 11-KT. Steroids were immediately extracted twice with 0.5 ml dichloromethane. The two aliquots of dichloromethane were combined, evaporated, and the extracts were transferred, dissolved in a few drops of ethanol, to thin layer chromatography plates (10 $\times$ 10 cm HPTLC silica-coated glass plates with a 10 $\times$ 2.5 cm concentrating zone; Merck). The plates were first developed in toluene:cyclohexane = 1:1 to concentrate the samples, and steroids were then separated by developing the plate with chloroform:ethanol = 95:5. The non-radioactive carrier steroids, added just before extraction, were localized under u.v. light at 254 nm. The plate was then treated with a scintillation spray (Enhance Spray, NEN Dupont), and radioactivity was localized as photons using Hyperfilm MP (Amersham Life Science). To relate bands on the film to the amount of radioactivity associated with the different fractions, the bands were quantified densitometrically using a PC-based image analysis system, using a program developed in the KS400 version 3.0 software package (Carl Zeiss Vision, Göttingen, Germany). Results are expressed as percentage of the total amount of radioactivity of the respective sample. Steroids were identified by co-migration with non-radioactive carrier steroids that were visualized under u.v. light.

In situ hybridization

A zebrafish *ar*-specific PCR product was generated with primers 2430 and 2431 (see Supplementary Table 1). The ~ 465 bp PCR product was gel purified, and served as a template for digoxigenin-labeled cRNA probe synthesis, as described previously (Vischer *et al.* 2003).

Zebrafish testes were dissected and fixed in 4% w/v paraformaldehyde in PBS, immersed in 25% w/v sucrose at 4 °C for 16 h, and then frozen in Neg-50 frozen section medium (Richard Allen Scientific, Kalamazoo, MI, USA). The protocol used for *in situ* hybridization was previously described (Weltzien *et al.* 2003) with the following modifications. Cryostat sections were cut at 10 µm thickness, and probe was added in a final concentration of 800 ng/ml. After staining, sections were rinsed in 96% ethanol for 40 s and in MilliQ water for 15 min, before mounting in Aquamount (Merck).

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice their impartiality.

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