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Characterization of two paralogous *StAR* genes in a teleost, Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

Steroidogenic acute regulatory protein (*StAR*) transports cholesterol, the substrate for steroid synthesis, to the inner membranes of mitochondria. It is well known that estrogen is essential for female sex determination/differentiation in fish. However, no reports showed that the conventional *StAR*, which was supposed to be essential for estrogen production, was expressed in female gonads during the critical timing of sex determination/differentiation. In this study, two different *StAR* isoforms, named as *StAR1* and *StAR2*, were characterized from the gonads of Nile tilapia (*Oreochromis niloticus*). Phylogenetic and synteny analysis revealed that two *StAR* genes existed in teleosts, *Xenopus* and chicken, indicating that the duplication event occurred before the divergence of teleosts and tetrapods. Real-time PCR revealed that *StAR1* was dominantly expressed in the testis, head kidney and kidney; while *StAR2* was expressed exclusively in the gonads. *In situ* hybridization and immunohistochemistry demonstrated that StAR1 was expressed in the interrenal cells of the head kidney and Leydig cells of the testis; while StAR2 was expressed in both the Leydig cells of the testis and the interstitial cells of the ovary. Ontogenic analysis demonstrated that StAR2 was expressed abundantly from 5 d after hatching in the somatic cells in XX gonads, whereas in XY gonads, both StARs could be detected from 30 dah until adulthood. Intraperitoneal injection of human chorionic gonadotropin experiments showed that expression of StAR1 and 2 was significantly elevated at 8 h and persisted until 24 h after injection in the testis. Taken together, our data suggested that StAR1 is likely to be required for cortisol production in the head kidney, and StAR2 is probably involved in estrogen production during early sex differentiation in XX gonads. In contrast, both StARs might be required for androgen production in testes. For the first time, our data demonstrated that two fish StARs might be involved in steroidogenesis in a tissue and developmental stage dependent manner.

1. Introduction

Cholesterol is the starting point for biosynthesis of steroids, oxysterols and bile acids, and is also an essential component of cellular membranes. Steroids and sterols derived from cholesterol activate a broad spectrum of nuclear and membrane-based receptors (Miller, 2007). Previous reports in mammalian species revealed that cholesterol transport from the outer to the inner mitochondrial membrane is the first event of steroidogenesis. Multiple lines of evidence showed that the delivery of cholesterol depends on the vital factor StAR (Simpson and Boyd, 1966, 1967; Churchill and Kimura, 1979; Clark et al., 1994; Lin et al., 1995; Stocco and Clark, 1996a; Wang et al., 1998; Stocco, 2001; Tsuchiya et al., 2003). Therefore, StAR is the single most important factor regulating the timing and rate of steroidogenesis (Stocco and Clark, 1996b; Tsuchiya et al., 2003). STAR is known to be an essential factor for steroidogenesis in both the hypothalamo–pituitary–adrenal (HPA) axis and the hypothalamo–pituitary–gonadal (HPG) axis in mammals (Patchev et al., 2007; Tkachenko et al., 2011; Zempo et al., 2013). Previous clinical studies and *in vitro* assay have proved that mutations in the

Abbreviations: StAR, steroidogenic acute regulatory protein; hCG, human chorionic gonadotropin; dah, days after hatching; m, month after hatching; *ISH*, *In situ* hybridization; IHC, immunohistochemistry; ACTH, adrenocorticotropic hormone; E2, 17β-estradiol; DHP, 17α, 20β–Dihydroxy-4-pregnen-3-one.

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StAR gene impaired the conversion of cholesterol to pregnenolone, which in turn caused the lipoid congenital adrenal hyperplasia (lipoid CAH) with defects in the synthesis of all adrenal and gonadal steroid hormones (Lin et al., 1995; Bose et al., 1996; Caron et al., 1997; Saenger, 1997). It is well known that both human chorionic gonadotropin (hCG) and adrenocorticotropic hormone (ACTH) could promote steroidogenesis by stimulating the transcription and translation of several genes encoding steroidogenic enzymes, including *StAR* (Sewer and Waterman, 2003; Tsuchiya et al., 2003; Hoegg et al., 2004; Sugawara et al., 2006). Furthermore, increase of STAR transcripts and protein in response to cAMP has been shown to be implicated in the protein kinase A intracellular signaling pathway (Jones et al., 2000).

In fish, glucocorticoids (cortisol and corticosterone), synthesized by interrenal tissue in the head kidney (the piscine counterpart of the mammalian adrenal), are essential for growth, reproduction and stress-homeostasis, intermediary metabolism, ionic and osmotic regulation, and immune function (Gallo and Civinini, 2003). Gonad-derived sex steroids are essential for sex determination, differentiation, gametogenesis, gamete-maturation and sexual behavior (Garcia-Lopez et al., 2011; Murata et al., 2011). Recently, cloning and expression of *StAR* genes have been studied from several teleosts including zebrafish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*), Atlantic croaker (*Micropogonias undulatus*), fathead minnows (*Pimephales promelas*), sea bream (*Sparus aurata*), sturgeon (*Acipenser transmontanus*), Senegalese sole (*Solea senegalensis*), medaka (*Oryzias latipes*) and Japanese eel (*Anguilla japonica*) (Bauer et al., 2000; Geslin and Auperin, 2004; Nunez and Evans, 2007; Villeneuve et al., 2007; Kusakabe et al., 2009; Marin-Juez et al., 2011; Nakamoto et al., 2012). As in mammalian species, several reports were available on fish *StAR* expression stimulated by acute disturbance (Kusakabe et al., 2002), ACTH injection, change in salinity and hCG induction (Kim et al., 1997; Li et al., 2003; Ings and Van Der Kraak, 2006; Nunez and Evans, 2007; Sreenivasulu et al., 2009). In croaker, *in vitro* treatment of ovarian follicles with hCG could efficiently induce a 16-fold increase of *StAR* mRNA level by 24 h, whereas it was unable to alter *StAR* expression in testicular tissues (Nunez and Evans, 2007). However, *StARs* were significantly increased in the testis of Senegalese sole by the *in vivo* administration of hCG (Marin-Juez et al., 2011). Therefore, hCG induced *StAR* gene expression between different fish species displayed considerable discrepancy which needs further clarification.

It is well known that estrogen, which requires the action of StAR for its synthesis, is a natural inducer of fish ovarian differentiation during early female sex determination/differentiation (Nagahama, 2005). However, the reports in medaka and catfish showed that *StAR* was barely detectable during early gonadal differentiation, indicating the absence or inactive role of conventional StAR in steroidogenesis during early sex differentiation period (Raghuveer et al., 2011; Nakamoto et al., 2012). This led us to hypothesize that there could be a second *StAR* gene, responsible for the production of estrogen in the fish gonad during early female sex determination/differentiation. There were reports describing another *StAR-like* gene in Senegalese sole (Marin-Juez et al., 2011; Marin-Juez et al., 2013), however, reports about the expression of *StAR-like* gene in the gonad during early female sex determination/differentiation are not available.

Tilapia (*Oreochromis niloticus*), with a XX/XY sex determination system, availability of mono sex fish and open genome database, is a good model for the study of steroidogenesis in fish. Moreover, we accomplished the sequencing of eight gonadal transcriptomes of tilapia at different developmental stages, which gave us a better understanding of the early sex determination and differentiation process (Tao et al., 2013). To provide further insights into the molecular mechanisms of steroidogenesis in teleosts, we performed an *in silico* analysis of tilapia genome (http://www.ensembl.org/Oreochromis_niloticus/Info/Index) and isolated a novel *StAR* gene. The expression profiles of two *StARs* in different tissues and ontogenic stages were checked to elucidate their possible roles in steroidogenesis and sex determination/differentiation. Moreover, the expression profiles of two *StARs* in testis were also examined under *in vivo* hCG administration.

2. Materials and methods

2.1. Fish

Tilapias were reared in large tanks with a re-circulating aerated freshwater system. Fish were maintained at ambient temperature (26 °C) under natural photoperiod. All genetic females (XX) and males (XY) were obtained by artificial fertilization of eggs from normal female (XX) with sperms from either sex reversed male (XX) or super male (YY), respectively. The super males (YY) were obtained by crossing the normal XY-male with the XY-female which was sex-reversed hormonally by E2 treatment. All animal experiments conformed to the Guide for Care and Use of Laboratory Animals and were approved by the Committee of Laboratory Animal Experimentation at Southwest University, China.

2.2. Identification of two StARs

Two *StAR* genes (*StAR1:* ENSONIG00000010793; *StAR2:* ENSONIG00000016122) were retrieved from different scaffolds from the available genome database of tilapia (http://www.ensembl.org/Oreochromis_niloticus/Info/Index). The deduced sequences including the open reading frame (ORF) and untranslated regions for both *StARs* were isolated from the 3-month old tilapia gonadal transcriptome. Gene specific primers were designed to amplify the ORFs (*StAR1*-oF,-oR; *StAR2*-oF,-oR) from the testis. RNA isolation and cDNA synthesis were carried out according to the methods reported previously (Zhou et al., 2007). All PCR products were ligated into the pGEM-T easy vector (Promega, USA) and sequenced at Life Technologies Corporation (Shanghai, China).

2.3. Phylogenetic and synteny analyses

The deduced amino acid sequences of tilapia StARs and their counterparts from other vertebrates, including medaka, zebrafish, *Xenopus*, human and so on, were aligned using Clustal W. The neighbor-joining method was used to construct the phylogenetic tree by MEGA5.0 (Tamura et al., 2011) by using tilapia MLN64 (Metastatic Lymph Node, clone 6) (XP_003458111.1) as an outgroup. The credibility of the branching was tested using bootstrap resampling with 1000 pseudo replicates. The GenBank accession Nos. of the sequences used in this study are as follows, lizard (*Anolis carolinensis*) StAR (ENSACAP00000000450), coelacanth (*Latimeria chalumnae*) StAR1 (ENSLACP00000010485) and StAR2 (ENSLACP00000015423), medaka StAR1 (NP_001098380.1) and StAR2 (ENSORLP00000011263), zebrafish StAR1 (NP_571738.1) and StAR2 (XP_002664090.2), *Xenopus* (*Xenopus laevis*) StAR1 (XP_002932770.1) and StAR2 (ENSXETT00000006725), human (*Homo sapiens*) STAR (NP_000340.2), chicken (*Gallus gallus*) StAR1 (NP_990017.1) and StAR2 (ENSGALT00000007165), fugu (*Takifugu rubripes*) StAR1 (XP_003976115.1) and StAR2 (XP_003961286.1), stickleback (*Gasterosteus aculeatus*) StAR1 (ABG34343.1) and StAR2 (CBN81516.1), *Tetraodon* (*Tetraodon nigroviridis*) StAR1 (CAF91743.1) and StAR2 (XP_003961286.1), Senegalese sole StAR1 (HQ392856) and StAR2 (EU921450), tilapia StAR1 (XP_003445653.1) and StAR2 (XP_003441954.1).

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