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Cloning and expression of *StAR* during gonadal cycle and hCG-induced oocyte maturation of air-breathing catfish, *Clarias gariepinus*☆

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ABSTRACT

Complementary DNAs encoding steroidogenic acute regulatory protein (*StAR*) have been isolated from different fish species, yet the relevance of *StAR* during gonadal cycle and more importantly in final oocyte maturation has not been assessed so far. A cDNA encoding *StAR* was isolated from the ovarian follicles of air-breathing catfish, *Clarias gariepinus*. Catfish *StAR* exhibited 55 to 72% identity at nucleotide level with other vertebrate orthologs. RT-PCR analysis of tissue distribution pattern demonstrated the presence of *StAR* mRNA in various tissues including gonads, kidney, liver, brain and intestine of catfish. Real-time RT-PCR analysis revealed high expression of *StAR* mRNA in the pre-spawning phase of ovary while it was low in preparatory, spawning and regressed phases. In testis, maximum expression was noticed during the preparatory phase. During human chorionic gonadotropin (hCG)-induced oocyte maturation, both *in vitro* and *in vivo*, *StAR* mRNA levels were augmented by 2 h and then declined gradually to reach basal levels by 12 h as that of saline-treated controls. Taken together, high level of expression during hCG-induced oocyte maturation vis-à-vis in spawning suggests a role for *StAR*, in addition to the steroidogenic enzyme genes in final oocyte maturation.

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1. Introduction

Steroid hormones play a crucial role in the regulation of growth, development, differentiation, reproduction and several other functions in vertebrates. Production of different classes of steroids occurs from a common precursor, cholesterol and involves a battery of oxidative enzymes (Payne and Hales, 2004). The first committed step in steroid hormone biosynthesis is the conversion of cholesterol to pregnenolone, which occurs in mitochondria by the action of P450 side chain cleavage enzyme (P450scc). However, cholesterol cannot cross the mitochondria from cytoplasm and is delivered by a sterol transfer protein, steroidogenic acute regulatory protein (StAR; Stocco, 2000). Now, it has been accepted that the true rate-limiting step in steroidogenesis is the delivery of cholesterol across mitochondrial membrane. This is an important target for acute steroidogenesis by tropic hormones (Stocco, 2001), few other mediators and some endocrine disruptors (Walsh and Stocco, 2000) as well. Therefore, StAR is indispensable for mediating cholesterol transfer vis-à-vis steroidogenesis. Perhaps the most compelling evidence came from the identification of mutations in *StAR* gene during congenital adrenal hyperplasia (CAH), a condition in which cholesterol and cholesterol esters accumulate and the newborn fails to synthesize adequate levels of steroid hormones (Lin et al., 1995). This is further evidenced by *StAR* knockout mice which showed phenotypic mirrors of human lipoid CAH (Caron et al., 1997).

A cDNA encoding a 30 kDa mouse *StAR* was first characterized by Clark et al. (1994). StAR is believed to transfer cholesterol across mitochondria either by forming a transport tunnel (Tsujishita and Hurley, 2000) or by a cavity (Mathieu et al., 2003). It is plausible that StAR interacts with contact sites where the inner and outer mitochondrial membranes are in close proximity (Thomson, 2003). Further, studies have shown that StAR is rapidly synthesized in response to stimulation of several hormones such as luteinizing hormone (LH), adrenocorticotropic hormone (ACTH; Clark et al., 1995) typically with activation of the cAMP second messenger system. Although StAR appears to be critical for steroidogenesis in the adrenal and gonads, some of the tissues that do not express StAR, including placenta, synthesize large amounts of pregnenolone suggesting for the

☆ Catfish ovarian StAR nucleotide sequence has been submitted to GenBank under the accession no. FJ793811.

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existence of StAR-independent mechanisms for movement of cholesterol to P450scc enzyme (Stocco, 2001).

Complementary DNA-encoding proteins with high homology to StAR of mammals were cloned from zebrafish, rainbow trout, eel, cod and stingray (Bauer et al., 2000; Kusakabe et al., 2002; Li et al., 2003; Goetz et al., 2004; Nunez et al., 2005). In teleosts, besides correlating increase in *StAR* mRNA to acute interrenal or gonadal steroid production, very little is known about the dynamics of *StAR* transcripts in relation to gonadal cycle and more importantly final oocyte maturation. A shift in steroidogenesis from estradiol-17β (E_2) to 17α,20β-dihdroxy-4-pregnen-3-one (17α,20β-DP) is important for final oocyte maturation that is associated with pre-ovulatory LH surge (Nagahama, 1997; Senthilkumaran et al., 2004; Nagahama and Yamashita, 2008). This steroidogenic shift is governed by the down regulation of ovarian P450 aromatase and up-regulation of 20β-hydroxysteroid dehydrogenase, the enzymes that produce E_2 and 17α,20β-DP, respectively (Yoshiura et al., 2003; Senthilkumaran et al., 2004; Sreenivasulu et al., 2005, Sreenivasulu and Senthilkumaran, 2009a). However, involvement of *StAR* during shift in steroidogenesis would be possible, owing to the reason that *StAR* is rapidly synthesized in response to trophic hormone stimulation. In the present study, a cDNA encoding *StAR* was isolated from ovarian follicles of the air-breathing catfish, *Clarias gariepinus*. We then analyzed *StAR* transcript abundance during hCG-induced oocyte maturation, *in vitro* and *in vivo* by real-time RT-PCR. To complement our results, expression of *StAR* was also analyzed during different stages of gonadal cycle.

2. Materials and methods

2.1. Animals and treatments

Adult catfish, *Clarias gariepinus* (Actinopterygii, Siluriformes), of about 400–500 g were purchased live from local fish markets (Hyderabad, India) and acclimated for 2–3 weeks in aquaria filled with filtered tap water and maintained at normal photoperiod and ambient temperature conditions. Fish were fed *ad libitum* with minced goat liver/commercial food pellets during acclimation and experimentation. Catfish gonad undergoes four broadly distinguishable phases including an extensive preparatory phase followed by pre-spawning, spawning and regressed/resting phases (Swapna et al., 2006). For seasonal cycle analysis, gonads at different phases of reproductive cycle were collected, snap frozen in liquid nitrogen and stored at −80 °C. For *in vitro* oocyte maturation studies, animals were killed by decapitation and oocytes with centrally located germinal vesicles were collected. About 100 oocytes were then incubated (separately for each time point) in triplicate in catfish oocyte incubation medium (Senthilkumaran and Joy, 2001) with 100 IU/ml of human chorionic gonadotropin (hCG; Pubergen, Uni-Sankyo Pvt. Ltd., India). Controls were treated with saline. Follicles were collected at different time points and used for real-time RT-PCR. For *in vivo* oocyte maturation studies, fish were injected intra-peritoneally with hCG (1000 IU/kg body mass) and about 100 follicles at different time points were collected by gently stripping from ovipore and utilized for real-time RT-PCR. Controls were maintained similarly except for injecting with physiological saline. Both *in vitro* and *in vivo* studies are repeated thrice ($n=3$–5) with different batch of female fish in two consecutive years. Dosages of hCG for *in vitro* and *in vivo* induction were chosen based on standardization done earlier (Sreenivasulu and Senthilkumaran, 2009a).

2.2. Cloning of catfish StAR cDNA and sequence analysis

Total RNA was prepared from 100 mg of catfish ovarian follicles using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) as per manufacturer's instructions. One μg of total RNA was reverse transcribed using Superscript III (Invitrogen, Carlsbad, CA, USA) and cDNA was used as template in PCR with a set of degenerate primers (listed in Table 1) designed by aligning existing fish *StAR* cDNA sequences. RT-PCR products were separated on agarose gel, a band corresponding to *StAR* was sub-cloned into pGEM-T Easy (Promega, Madison, WI, USA) vector and subsequently sequence was determined from both the ends. The identity of cDNA clone was confirmed by BLAST analysis.

5′ and 3′ rapid amplification of cDNA ends (RACE) were subsequently performed using RNA-ligase mediated RACE kit (Invitrogen) as per the manufacturer's protocol. The gene specific primers used in RACE were given in Table 1 and were designed from partial cDNA sequence. The PCR products were sub-cloned and sequenced as mentioned above. The full-length cDNA of catfish ovarian *StAR* was obtained by aligning the overlapping sequences of 5′, 3′ RACE and partial cDNA sequences. Finally, the open reading frame (ORF) was amplified using gene specific primers and the sequences were confirmed bi-directionally. Phylogenetic analysis was performed with MEGA (4.1) software.

2.3. Northern blot analysis

Twenty five μg of total RNA from ovary and testis were separated on a 1% denaturing formaldehyde-agarose gel and transferred on to a positively charged nylon membrane (Amersham, Buckinghamshire, England) by capillary transfer. Gel was stained with ethidium bromide and RNA ladder was used to track the size of transcripts. The membrane was hybridized under high stringency conditions overnight with a catfish *StAR* ORF labeled with ^{32}P-dCTP by random primer labeling kit (Perkin Elmer, Boston, MA, USA). Following hybridization, membranes were washed and signals were detected by Phosphor-imager (Typhoon, GE Healthcare). Northern blot analysis was repeated thrice independently.

2.4. RT-PCR analysis of tissue distribution

To analyze tissue distribution pattern of *StAR* in catfish tissues, 1 μg of total RNA obtained from different tissues was reverse transcribed and RT-PCR was performed as mentioned above using catfish *StAR* ORF primers.

2.5. Real-time RT-PCR

Transcript abundance of *StAR* was quantified by real-time RT-PCR of total RNA isolated from the ovarian follicles collected at different durations of hCG-induced oocyte maturation, *in vitro* and *in vivo* as well as ovary and testis at different stages of reproductive cycle. One μg of total RNA was reverse transcribed using random hexamers and MMLV-reverse transcriptase (Invitrogen). Gene-specific primers (Table 1) for PCR amplification were designed according to the requirements set forth by Primer Express software and the developer

Table 1
Primers used for cloning and expression analysis of *StAR*.

Primer[§]	Sequence 5′–3′	Annealing position[*]	Purpose
DF1	TGTGYGCTGGCATYTCYTAC	23–42	Degenerate RT-PCR
DR1	GGTGRTTKRCRAARTCCACCT	785–805	Degenerate RT-PCR
GSP-F1	CCAACATCCTAGCATGCCTGACCA	606–629	5′ RACE
GSP-F2	GACCAAGTTCACCTGGTTACTCAG	702–725	3′ RACE
GSP-R1	GGATAGCTTGCTCAGCTCGTGGTG	94–117	3′ RACE
GSP-R2	ATGGCCATCATGGCGTTCCTCCTC	69–92	3′ RACE
ORF-F	ATGCTACCTGCAACTTTTAAG	1–21	ORF cloning & tissue distribution
ORF-R	GCAGGCCATTGCCTCCTCCA	836–855	ORF cloning & tissue distribution
qRT-F	TGGCCATCCACCACGAGCTG	86–105	Real-time RT-PCR
qRT-R	CAATCTCAGTTTGCCAGCCATC	274–295	Real-time RT-PCR

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