Gene 592 (2016) 215–220

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Ubiquitin ligase gene *neurl3* plays a role in spermatogenesis of half-smooth tongue sole (*Cynoglossus semilaevis*) by regulating testis protein ubiquitination

Wenteng Xu, Hailong Li, Zhongdian Dong, Zhongkai Cui, Ning Zhang, Liang Meng, Ying Zhu, Yang Liu, Yangzhen Li, Hua Guo, Jialu Ma, Zhanfei Wei, Nianwei Zhang, Yingming Yang, Songlin Chen *

[a] Yellow Sea Fisheries Research Institute, CAFS, Key Lab for Sustainable Development of Marine Fisheries, Ministry of Agriculture, Qingdao 266071, China
[b] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237, China

ARTICLE INFO

Article history:
Received 10 March 2016
Received in revised form 20 July 2016
Accepted 25 July 2016
Available online 30 July 2016

Keywords:
Cynoglossus semilaevis
neurl3
Expression pattern
RNA interference
Spermatogenesis
Ubiquitination

ABSTRACT

E3 ubiquitin ligases are a large gene family that plays a diversity of roles in spermatogenesis. In this study, the functional characterization of a neuralized E3 ubiquitin protein ligase 3 (*neurl3*) revealed its potential participation in spermatogenesis. Firstly, we found that *neurl3* exhibited male-biased transcription and that its translation was predominant in testis germ cells. The knockdown of *neurl3* by RNA interference caused increased transcription of spermatogenesis-related genes. These results corroborate previous studies indicating a role for *neurl3* in spermatogenesis. Moreover, the levels of *neurl3* transcription and testis protein ubiquitination were closely correlated. Based on these findings, we speculate that *neurl3* modulates testis protein ubiquitination in a dosage-dependent manner and that this influences spermatogenesis.

1. Introduction

Half-smooth tongue sole (*Cynoglossus semilaevis*) is an economically important marine fish widely cultivated in northern parts of China. This fish exhibits significant sexual dimorphism, *e.g.* females usually grow 2–4 times larger than males. However, in aquaculture populations the female proportion is usually very low and this substantially reduces industrial productivity. There are many reasons behind the low female ratio in aquaculture, an important one of which is the frequent occurrence of sex-reversal. The half-smooth tongue sole has a ZW/ZZ sex determining system and the females contain heterogametes (female: ZW; male: ZZ), although under some conditions sex-reversal occurs and genetic female fish become phenotypic male fish, designated as pseudomale (ZW). The pseudomale fish can develop mature testis, produce sperm and have male growth characteristics (Chen et al., 2014; Shao et al., 2014), and so is disadvantageous in aquaculture. Nonetheless if pseudomales are crossed with normal female, this should produce super females (WW), which can be crossed with normal male (ZZ) for full-female offspring. Attempts to generate super females have been unsuccessful due to the phenomenon of W sperm absence since the pseudomales only produce Z sperm (Chen et al., 2014). Characterization of the molecular mechanism behind spermatogenesis in male and pseudomale fish should provide insight into this intriguing phenomenon.

The availability of the half-smooth tongue sole genome has facilitated studies of gene function and led to the proposal that male-determining genes are mainly localized on the Z chromosome (Chen et al., 2014). Several Z chromosome localized genes have proved to be important players in sex determination, sex differentiation and spermatogenesis, such as doublesex and mab-3 related transcription factor 1 (*dmrt1*) and testis-specific protein kinase 1 (*tesk1*) (Chen et al., 2014; Meng et al., 2014). Neuralized E3 ubiquitin protein ligase 3 (*neur13*) was identified on the Z chromosome and proposed as an intriguing candidate in spermatogenesis since it was highly expressed in testis at 116 days post hatch after gonad differentiation (Chen et al., 2014). Furthermore, the ubiquitination pathway is a conserved and complex regulatory network that participates in many physiological processes including spermatogenesis (Pickart, 2001; Shmueli and Oren, 2005; Dikic et al., 2009; Jiang and Chen, 2012). Several E3s have been suggested to function in spermatogenesis, including *huwe1* that is involved in spermatogenesis by regulating histone ubiquitination and modification; mouse *ubr2* that interacts and metabolically stabilizes

Abbreviations: A, adenosine; Bp, base pair(s); C, cytidine; G, guanosine; min, minute; *neurl3*, neuralized E3 ubiquitin protein ligase 3; PCR, polymerase chain reaction; RNAi, RNA interference; s, second; T, thymidine.

* Corresponding author.
E-mail address: chensl@ysfri.ac.cn (S. Chen).

http://dx.doi.org/10.1016/j.gene.2016.07.062
0378-1119/

a germ cell-specific protein Tex 19.1, which is necessary for normal spermatogenesis; *rnf8* and *rnf168*, which are two RING-type ubiquitin ligases that participate in spermatogenesis by targeting histone H2A and H2AX (Liu et al., 2005; Yang et al., 2010; Bohgaki et al., 2011; Sin et al., 2012).

The present study uses genomic and epigenetic data to carry out functional characterization of *neurl3* in the half-smooth tongue sole. In 1-year-old and 1.5-year-old fish, *neurl3* transcripts were present in testis but not in the ovary, although the level was lower in pseudomales compared to males. The Neurl3 protein was mainly localized in germ cells and the expression level in males was much higher than pseudomales. After *neurl3* knockdown, the transcription levels of *tesk1* and *wt1a* were significantly increased in 1.5-year-old fish testis, suggesting *neurl3* was involved in spermatogenesis. Furthermore, males and pseudomales exhibited different protein ubiquitination patterns in testis, and the pattern was closely related to *neurl3* transcription levels. Based on these observations, we propose that *neurl3* can regulate ubiquitination in a dosage-dependent manner, which in turn influences spermatogenesis in an as yet undefined manner.

2. Materials and methods

2.1. Ethics statement

All experiments were approved by the Yellow Sea Fisheries Research Institute's animal care and use committee. Efforts were made to minimize the suffering of fish during experiments.

2.2. Sample preparation

Cynoglossus semilaevis were purchased from the Haiyang High-Tech Experimental Base (Haiyang, China). Genetic and phenotypic sex was determined using established methods (Chen et al., 2012). Dorsal gonads from 1-year and 1.5-year fish (3–5 individuals) were collected and frozen in liquid nitrogen. RNA was extracted using a Trizol isolation kit (Qiagen, Dusseldorf, Germany). After extraction, the quality was check with agarose gel and stored at −80 °C for further use.

2.3. Bioinformatic analysis

The deduced protein sequence of Neurl3 was established using ExPASy (http://www.expasy.ch/) and domains and motifs predicted using SMART (http://smart.embl-heidelberg.de/).

2.4. Quantitative RT-PCR (qRT-PCR)

Genomic DNA eraser was employed to avoid potential genomic contamination, then five micrograms (μg) of total RNA was used for the first-strand cDNA synthesis with cDNA synthesis Kit (TaKaRa, Dalian, China). Oligo dT primer and random 6 mers were employed for cDNA synthesis. Transcription levels of *neurl3*, *star*, *p450scc*, *tesk1* and *wt1a* were determined using specific primers (Table 1) by qRT-PCR using an ABI 7500 fast realtime thermal cycler (Applied Biosystems, CA, USA). The qRT-PCR was performed using SuperReal Premix Ex Plus SYBR Green Kit (Tiangen, Beijing, China) and following the protocol of the manufacturer. In brief, a total volume of 20 μl was prepared containing 10 μl of 2× Premix Ex Taq, 8.4 μl ddH_2O, 1 μl cDNA, 0.3 μl each of the forward and reverse primers (10 μM) (Table 1). The amplification procedure consisted of an initial denaturation step at 95 °C for 15 min, then 40 cycles of 95 °C for 10 s, 60 °C for 30 s, followed by a melting curve step from 60 °C to 95 °C. *β-Actin*, which did not significantly vary between samples, was used as internal reference for normalization. Reactions were performed in triplicate and the transcription level was analyzed by the comparative CT method. Using *t*-test, significant difference was only accepted when $P < 0.05$.

Table 1
Primer sequences in the study.

Primer	Sequences (5′–3′)	T_m/°C	Purpose	Product size
ExpF	CGCGGATCCATGGTGAAG CAGAGCAG	69.5	Recombinant expression	891 bp
ExpR	CGCAAGCTTTCAGACACA CACGTCTCTG	69.5		
neurl3 F	CTGGTGTTTAGCAGCCGTCCT	60.0	qRT-PCR	234 bp
neurl3 R	CCAGAACTCCAGCACTGACCC	62.0		
tesk1 F	GCAGAAACTCTCTCACCCCAACA	62.0	qRT-PCR	290 bp
tesk1 R	CCAGACCAAAGTCCGTCACCA	61.9		
star F	AGGACGGCTGGACCACTGAAAT	64.5	qRT-PCR	232 bp
star R	ACCTCGTGGGTGACCATCGTGT	66.5		
p450scc F	GGATACGGGCGTGGTGAA	60.0	qRT-PCR	177 bp
p450scc R	TGAATGGCCGGGTGCTTA	59.5		
wt1a F	ACCGCCGTTTCCCCTTAC	59.6	qRT-PCR	267 bp
wt1a R	GGGCTGGTGGTGATGTGC	61.9		
β-Actin F	CCTTGGTATGGAGTCCTGTGGC	63.8	qRT-PCR	150 bp
β-Actin R	TCCTTCTGCATCCTGTCGGC	61.9		

2.5. In vivo RNA interference (RNAi) and analysis of putative interacting genes

Small interfering RNA (siRNA) were designed and synthesized by Raybiotech Co. Ltd. (Raybiotech, Guangzhou, China), including a nonspecific siRNA as the negative control (NC) and two pairs of *neurl3*-specific siRNAs (si-*neurl3*-001 and si-*neurl3*-002). After comparison of the knockdown effect in half-smooth tongue sole testis cell line in *in vitro* culture, si-*neurl3*-001 was selected for *in vivo* RNAi experiment. For the *in vivo* RNAi experiments, the siRNA dosage (0.05–0.25 nmol/g) was determined according to the suggested of Raybiotech with some modifications. Briefly, 1.5-year-old male and pseudomale fish (~300 g) were injected with 10 nmol NC or si-*neurl3*-001 (equivalent to 0.033 nmol/g) through the dorsal side of the body into the testis, which could be clearly distinguished when the torch light was applied from ventral side. For 1-year-old male and pseudomale fish (~16 g), 2.5 nmol NC or si-*neurl3*-001 were used for injection (0.156 nmol/g). Each of the experimental groups (NC males, RNAi males, NC pseudomales, RNAi pseudomales) contained three individuals. The testes on dorsal side were collected 48 h after treatment and split into several parts for fixation, RNA or protein extraction. The transcription levels of *neurl3* (Gene ID: 103398139, chromosome Z), *star* (Gene ID: 103398034, chromosome Z), *tesk1* (Accession number: KF939086.1, chromosome Z), *wt1a* (Gene ID: 103379534, chromosome 6) and *p450scc* (Gene ID: 103380225, chromosome 6) were quantified by qRT-PCR as described above. For statistical analysis of transcription levels, the average value from the NC group was set to 1, the six individuals (three in the NC group, three in the RNAi group) were then compared. For analysis of the sperm types, sperm was obtained by squeezing the testis and analyzed using established protocols (Chen et al., 2012).

2.6. Recombinant expression and purification

A pair of primers (ExpF and ExpR) was designed to amplify the *neurl3* coding sequence (Table 1). The resulting amplicon was digested using by *Bam*HI and *Hin*dIII and then inserted into a pET-28a vector. The recombinant vector was sequenced to confirm that the cloned *neurl3* insert was in frame and then used to transform *Escherichia coli* BL21 (DE3) (Transgen, Beijing, China). Transformed *E. coli* were grown in shake cultures to an OD_{600} of 0.6 and then induced to express the recombinant protein by adding isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 1 mM. The culture was then incubated at 37 °C for another 4 h and then protein that was expressed as inclusion bodies was purified using a B-PER 6XHis-tag protein purification kit (Thermo Fisher Scientific, MA, USA). The purified protein was dialyzed