Theriogenology 118 (2018) 46–56

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Pig StAR: mRNA expression and alternative splicing in testis and Leydig cells, and association analyses with testicular morphology traits

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ARTICLE INFO

Article history:
Received 10 February 2018
Received in revised form
6 May 2018
Accepted 25 May 2018
Available online 31 May 2018

Keywords:
Pig
Steroidogenic acute regulatory protein (StAR)
Alternative splicing
Genetic variants
Reproductive traits

ABSTRACT

Steroidogenic acute regulatory protein (*StAR*), primarily expressed in Leydig cells (LCs) in the mammalian testes, is essential for testosterone biosynthesis and male fertility. However, no previous reports have explored the expression profiles, alternative splicing and genetic variations of *StAR* gene in pig. The aim of current study was to explore the expression profiles in different tissues and different types of testicular cells (LCs; spermatogonial stem cells, SSCs; Sertoli cells, SCs), to identify different splice variants and their expression levels, as well as to detect the indel polymorphism in pig *StAR* gene. Expression analysis results revealed that *StAR* was widely expressed in all tested tissues and the expression level in testis was significantly higher than that in other tissues ($P < 0.01$); among different types of testicular cells, the *StAR* mRNA expression level was significantly higher in LCs than others ($P < 0.05$). Furthermore, three splice variants, *StAR*-a, *StAR*-b and *StAR*-c, were first found in pig. Further study showed *StAR*-a was highly expressed in both testis and LCs when compared with other variants ($P < 0.01$), suggesting *StAR*-a was the primary variant at *StAR* gene post-transcription and may facilitate the combination and transportation of cholesterol with *StAR*. In addition, a 5-bp duplicated deletion (NC_010457.5:g.5524-5528 delACTTG) was verified in the porcine *StAR* gene, which was closely related to male testicular morphology traits ($P < 0.05$), and we speculated that the allele "D" of *StAR* gene might be a positive allele. Briefly, the current findings suggest that *StAR* and *StAR*-a play imperative roles in male fertility and the 5-bp indel can be a potential DNA marker for the marker-assisted selection in boar.

1. Introduction

Testosterone not only develops the male reproductive organs and spermatogenesis, but also controls the physiological function of androgen-dependent tissues [1,2]. Testosterone is secreted by Leydig cells (LCs), which are the mainly steroid-producing cells in mammalian testes [3]. Except for producing testosterone, LCs also influence spermatogonial stem cells (SSCs) through secreted bioactive factors [4], which in turn affects spermatogenesis and male fertility. Heretofore, numerous studies about testosterone and LCs have been published on rat [5,6], mouse [7,8], and human [9,10], but the studies in pig are still deficient. As an important economic livestock and an ideal mammal model, pig and its fertility should receive considerable attention [11].

Testosterone biosynthesis is a complex physiological process regulated by many genes, such as cytochrome P450 family 11

Abbreviations: LCs, Leydig cells; SSCs, spermatogonial stem cells; CYP11A1, cytochrome P450 family 11 subfamily A member 1; HSD3B, 3β-hydroxysteroid dehydrogenase; CYP17A1, cytochrome P450 17A1; HSD17B3, type 3 17β-hydroxysteroid dehydrogenase; StAR, steroidogenic acute regulatory protein; START, StAR-related lipid transfer; AS, alternative splicing; Indel, insertion/deletion; LY, Landrace×Yorkshire; GZB, Guanzhong black; TW, testicular weight; TLG, testicular long girth; TSG, testicular short girth; SCs, Sertoli cells; EDS, ethane dimethane sulphonate; qRT-PCR, quantitative real-time PCR; PIC, polymorphism information content; Ne, effective allele numbers; Ho, homozygosity; He, heterozygosity; HWE, Hardy-Weinberg equilibrium; SE, standard error; II, insertion/insertion; ID, insertion/deletion; DD, deletion/deletion; SNPs, single nucleotide polymorphisms; SVs, structural variants.

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https://doi.org/10.1016/j.theriogenology.2018.05.031
0093-691X/

subfamily A member 1 (*CYP11A1*), 3β-hydroxysteroid dehydrogenase (*HSD3B*), cytochrome P450 17A1 (*CYP17A1*), type 3 17β-hydroxysteroid dehydrogenase (*HSD17B3*) and steroidogenic acute regulatory protein (*StAR*) [12]. Among these regulators, *StAR* is especially important due to its function: the transportation of cholesterol into the mitochondria, which is a rate-limiting step for testosterone synthesis [13,14].

The *StAR* gene (also named *STARD1*) belongs to the StAR-related lipid transfer (START) domain protein superfamily [13]. The *StAR* gene encodes a rapidly synthesized labile phosphoprotein, contributing to the acceleration of cytoplasmic cholesterol transfer into the mitochondria [13,14]. Inhibition of *StAR* destroyed the steroidogenic machinery and decreased testosterone production in LCs [15–17]. The absence of testosterone led to poor libido and sexual function, as well as caused hypogonadism; additionally, it could be harmful to other tissues and organs, including decreased bone mineral density and muscle mass, induced cardiovascular disease, or even increased mortality [18,19]. These studies have clearly demonstrated the indispensable role of the *StAR* gene in testosterone synthesis in LCs and male reproduction.

It is well known that the expression of gene may be affected by the DNA level and mRNA level regulation, thereby affecting gene function. The pre-mRNA by splicing produces different transcripts during the transcription process to increase the diversity of gene, which known as alternative splicing (AS) [20]. The AS events are widespread in eukaryotes and some can regulate physiological process by producing functional protein variants or spurious transcripts [21]. Several studies investigated that AS was associated with male reproduction, including testosterone synthesis [22], sperm maturation [23], semen quality [24], and male fertility [25]. Furthermore, DNA genetic variations (such as insertion/deletion, indel) could adjust gene transcription and translation due to binding to some transcription factors, nucleotide sequences or histone modifications [26]. Our previous studies have demonstrated that the association between indel mutations and male pig reproduction traits, such as *SPEF2* [27], *Oct4* [28], *KDM1B* [29] and *KDM5B* [30]. Given the above analyses, the correlation between *StAR* gene expression and male pig fertility both in mRNA and DNA levels is worthy of in-depth study.

In this study, the expression profiles in different tissues and different types of testicular cells of *StAR* gene in pigs were investigated; the splice variants and their expression levels were explored; as well as the 5-bp duplicated deletion associated with testicular morphology traits were also detected. These data would contribute to thoroughly understanding the role of *StAR* gene in boar reproduction, and further facilitate the development of pig industry.

2. Materials and methods

2.1. Animal and tissue sample collection

All animal experiments adhered to the relevant laws and institutional guidelines and approved by the Institutional Animal Care and Use Committee of the Northwest A&F University. All efforts were made to minimize any discomfort during the pig slaughtering process.

Tissue samples, including large intestine, liver, small intestine, lung, spleen, heart, muscle, brain, epididymis and testis, were obtained in seven 7-day-old male Landrace × Yorkshire (LY) pigs from the national swine foundation seed farm, Ankang, Shaanxi, China. In addition, in the existing resources of our laboratory, the same tissue samples from four adult Guanzhong black (GZB) pigs were tested, which were raised in the animal farm of Northwest A&F University, Yangling, Shaanxi, China. Each tissue was immediately frozen in liquid nitrogen and stored at −80 °C for subsequent study.

Moreover, for DNA experiment, a total of 263 testis samples of male piglets from Yorkshire breed were collected, which reared in the national swine foundation seed farm. Testicular morphology parameters from 15-day-old (n = 178) and 40-day-old (n = 85) Yorkshire piglets were measured, including testicular weight (TW), testicular long girth (TLG) and testicular short girth (TSG), which would be used for association analysis. Indeed, as important reproductive traits, testicular morphology traits were particularly prevalent in evaluating male pig reproductive ability [27–31].

2.2. Isolation of porcine different types of testicular cells

The fresh testis samples of 7-day-old piglets (crossbred of Landrace and Yorkshire) were acquired from Besun agricultural industry group Co., Ltd. (Yangling, Shaanxi, China), and LCs, SSCs and Sertoli cells (SCs) were separated by previous manipulation [11,32]. Briefly, the fresh testes were dispersed with 0.75 mg/mL collagenase type IV (Invitrogen, Massachusetts, USA) and DNase I (0.1 mg/mL; Sigma) in DMEM/F12 medium at 37 °C for 20 min. The separate fragments of seminiferous tubules were collected by natural sedimentation and washed with DPBS. Then the supernatant and tubules were separated.

For LCs, the collected supernatant was filtered through the 160 μm and 59 μm monofilament nylon meshes (Solarbio, Beijing, China). Freshly isolated cells were diluted with 1 mg/mL hyaluronidase (Invitrogen, Massachusetts, USA) and centrifugation at 500 *g* for 5 min. The LCs were gathered and cultured from the suspension. At the same time, in previous studies, ethane dimethane sulphonate (EDS) could selectively eliminate LCs to withdraw the testosterone [33,34]. According to our previous study [11], after EDS treatment for 24 h the LCs were lysed with TRIzol® reagent (TaKaRa, Dalian, China) for total RNA extraction.

Moreover, the tubules were treated with 0.25% Trypsin-EDTA (Gibco) at 37 °C for 5 min. The single cell suspension was obtained by a 40 mm mesh filter and centrifugation at 300 *g* for 5 min, then resuspended in DMEM/F12 supplemented with 2% (v/v) FBS (Gibco). The cell suspension was plated into 10 cm plastic culture dishes. Next day, the SSCs within the surface area and the SCs from the bottom of the dish were harvested, respectively.

2.3. Total RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol total RNA extraction reagent (TaKaRa, Dalian, China) according to the manufacturer's instruction. The cDNA was synthesized by reverse transcription PCR using the PrimeSript™ RT reagent Kit (TaKaRa, Dalian, China) and then was preserved at −20 °C.

2.4. Identification of StAR splice variants

Based on the known swine *StAR* reference sequence (GenBank: NM_213755.2), three primer pairs named AS-1, AS-2, and AS-3 (Table 1) were designed to amplify the full-length transcript and splice variants. The PCR reaction mixture contained 1 μL cDNA (0.5 ng/μL), 7.5 μL 2 × *Taq* PCR Mastermix (+dye) (BIOSCI, Hangzhou, China), 0.3 μL each primer (10 pmol/μL) and 5.9 μL ddH_2O. PCR program as follows: denaturation at 98 °C for 5 min, followed by 35 cycles at 98 °C for 10 s, 59.4 °C for 30 s and 72 °C for 1 min; with a final extension at 72 °C for 10 min. PCR products were separated by 1.0% agarose gel, purified using a Gel Extraction Kit (Omega Bio-tek, Georgia, USA) according to the manufacturer's instruction. And then the purified products were cloned into the pDM19-T Vector (TaKaRa, Dalian, China) and transferred into *Escherichia coli* competent DH5α cells for sequencing by Sangon Co., Ltd (Shanghai,