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Testosterone-retinoic acid signaling directs spermatogonial differentiation and seasonal spermatogenesis in the Plateau pika (Ochotona curzoniae)

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ABSTRACT

During evolution, animals optimize their reproductive strategies to increase offspring survival. Seasonal breeders reproduce only during certain times of the year. In mammals, reproduction is tightly controlled by hypothalamus-pituitary-gonad axis. Although pathways regulating gametogenesis in non-seasonal model species have been well established, molecular insights into seasonal reproduction are severely limited. Using the Plateau pika (*Ochotona curzoniae*), a small rodent animal species native to the Qinghai-Tibetan plateau, as a model, here we report that seasonal spermatogenesis is governed at the level of spermatogonial differentiation. In testis of the reproductively dormant animals, undifferentiated spermatogonia failed to differentiate and accumulated in the seminiferous tubules. RNA-seq analyses of the active and dormant testes revealed that genes modulating retinoic acid biogenesis and steriodogenesis were differentially regulated. A single injection of all-trans retinoic acid (ATRA) reinitiated spermatogenesis and inhibition the function of RA-degrading enzyme CYP26B1 for 10 days induced spermatogonial differentiation. Strikingly, testosterone injection reinitiated spermatogenesis in short day adapted animals. Testosterone provides a permissive environment of RA biogenesis and actions in testis, therefore, indirectly controls spermatogonial differentiation. Collectively, these findings provide a key mechanistic insight regarding the molecular regulation of seasonal reproduction in mammals.

1. Introduction

During evolution, animals optimize the reproductive strategies to increase the survival rate of their offspring. Short day breeders are sexually active in autumn to winter while long day breeders successfully reproduce in spring and summer [1–3]. In males, fertility relies on normal spermatogenesis. Mammalian spermatogenesis is a complex process that includes proliferation and differentiation of spermatogonia, meiosis of spermatocytes and spermiogenesis [4]. In adult testis, during steady-state, spermatogenesis initiates when a subpopulation of undifferentiated spermatogonia becomes differentiating spermatogonia, a process termed spermatogonial differentiation [4]. Differentiating spermatogonia then continue to develop into spermatocytes and spermatozoa. Deciphering molecular pathways that govern fertility

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and spermatogenesis in seasonal animals is of paramount importance for understanding animal adaptation, population dynamics and germ cell fate decisions in general.

Spermatogenesis is tightly regulated by gonadotropins and testosterone [5–7]. While male mice deficient in follicle stimulating hormone (FSH) signaling were fertile and FSH signaling only quantitatively affects spermatogenesis [8], luteinizing hormone (LH) is absolutely required for spermatogenesis. LH acts on Leydig cells to control testosterone production. Testosterone binds to androgen receptor (AR) in Sertoli cells, peritubular myoid (PM) cells and Leydig cells and signaling elicited in these cells are all crucial for normal spermatogenesis [9,10]. Androgen signaling in Sertoli cells is crucial for the survival of spermatocytes and development of round spermatids [11,12]. Recent studies showed that testosterone signaling also plays a key role in spermatogonial fate decisions, because spermatogonial differentiation was impaired in LH receptor knockout mice [13] and progressive loss of spermatogonia was evident in the global *Ar* knockout or PM specific *Ar* knockout mice [14]. In contrast to the well-established roles of testosterone in non-seasonal model animals, molecular mechanisms connecting steroids and development of spermatogenic cells in seasonal breeders are largely unknown.

Spermatogenesis shows dynamic patterns in seasonal breeders. Non-seasonal adult animals have continual spermatogenesis throughout the year, interestingly, many photoperiod sensitive mammals exhibit a marked annual cycle of testis size and hormone levels, including hamster [15,16], Prairie vole [17], mink [18], bear [19], roe deer [20], etc. Serum levels of FSH, LH and testosterone decrease significantly in seasonal animals during non-breeding season [21–23]. A pioneer study by Russell et al., reported that in nonbreeding season, testis weight of adult hamster decreased by 90%, and histological analysis further revealed that the seminiferous tubules predominately contained Sertoli cells and spermatogonia [24]. Spermatogenesis in roe deer and many other animals exhibits the similar pattern [20]. It appears that spermatogenesis is arrested at the undifferentiated spermatogonia stage in these animals in non-breeding season and resumption of spermatogenesis is regulated by hormones; however, a direct test of this hypothesis has not been reported.

The Plateau pika (*Ochotona curzoniae*) is a small rodent species native to the Qinghai-Tibetan Plateau and surrounding areas [25]. The adult Plateau pikas breed in April–July and enter a reproductively inactive state in winter [26]. In this study, we hypothesized that spermatogenesis is arrested due to the spermatogonial differentiation failure in Plateau pika during non-breeding season. We employed histological analysis, RNA-seq technology and functional experiments to test this hypothesis. We found that testosterone-dependent retinoic acid signaling is the fgatekeeper of spermatogonial differentiation and seasonal spermatogenesis. This study uncovers a previous unknown mechanism that bridges testosterone and spermatogenesis in Plateau pika. We propose that similar regulatory machinery may work in other seasonal animals to direct seasonal reproduction.

2. Materials and methods

2.1. Animal

Animal experiments were approved by animal ethic and welfare committee at Northwest Institute of Plateau Biology, Chinese Academy of Sciences. Plateau pikas were captured in Haibei Experimental Station, Chinese Academy of Sciences (37°29′-37°45′N,101°12′-101°23′E) in 2015–2017. The age of animals were estimated based on the body weight according to previously published study [47]. To harvest tissue, animals were sacrificed on site and tissues were snap-frozen in liquid nitrogen. To conduct shot-day treatment, animals were transported to the laboratory at Northwest Institute of Plateau Biology, Chinese Academy of Science. Animals were housed in polypropylene cages with standard bedding and litter. Animals were provided with food and tap water *ad libitum*.

2.2. Treatment

Adult males were randomly assigned into each experimental groups and treated with testosterone (0.5 mg/kg b.wt, i.p.), retinoic acid (2.5 mg, i.p.), or talarozole (2.5 mg/kg b.wt, i.p.) respectively (n = 3 to 5, each group). Information of testosterone, retinoic acid and talarozole were provided in supplemental table 4. Animals were captured between September to early December and maintained in SD condition (8Light:16Dark), control animals were maintained in the same conditions and treated with vehicles.

2.3. Tissue collection and processing

After various treatments, animals were weighed and sacrificed after injecting 10% chloral hydrate anesthesia. Testes, epididymis, epididymal fats and seminal vesicle were immediately removed and weighed. For histological and immunohistological analyses, tissues were fixed in 4% paraformaldehyde or Bouin's solution for 10h at 4 °C. For RNA analyses, tissues were frozen in liquid nitrogen and stored at −80 °C. Blood samples were collected and serum samples were stored at −80 °C for Elisa analyses.

2.4. Histology and immunohistochemistry

For histological studies, paraffin embedded tissues were cut into 5 μm sections andstained using hematoxylin and eosin as described previously [48]. Histology was examined under a microscope (Nikon, E 200, Japan) in randomly selected sections. Morphology of Sertoli cells and spermatogonia were identified as described previously [49]. Expressions of PLZF and STRA8 were detected by immunohistochemical staining. Sections were sequentially rehydrated and endogenous peroxidase activity was blocked by 3% H_2O_2 for 10 min at room temperature (RT). The sections were washed three times with phosphate buffered saline (PBS: NaH_2PO4, Na2HPO4, NaCl; pH 7.4) and incubated with 10% goat blocking serum for 1h. Then the sections were incubated with primary antibodies (Supplemental Table 3) overnight at 4 °C.Sections were washed three times with PBS and incubated with biotinylated secondary antibody. Sections were washed thrice with PBS and visualized using the 3,3-diaminobenzidine (DAB, ZSGB-BIO, Beijing, China) and counterstained with Ehrlich's hematoxylin. Sections were dehydrated, mounting and observed under research microscope (Nikon, E 200, Japan).

2.5. RNA isolation and quantitative RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration and purity were quantified using a Nanodrop ND-1000 Spectrophotometer (Biolab, Scoresby, Vic., Australia). Samples were incubated with RNase-free DNase for 30 min at 37 °C. After heat inactivating the DNase (75 °C for 10 min), RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster, CA, USA). The SYBR Green Detection System was used in combination with primer pairs (100 nM) (Supplemental Table 2). A ViiA7 Real Time PCR System (Applied Biosystems) was used to quantify the relative abundance of specific transcripts. The optimized parameters for the thermal cycler were as follows: activation at 95 °C for 2 min followed by 40