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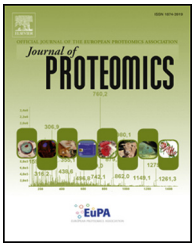

Unraveling the proteomic profile of mice testis during the initiation of meiosis

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ABSTRACT

In mice, once primordial germ cells (PGCs) are generated, they continue to proliferate and migrate to eventually reach the future gonads. They initiate sexual differentiation after their colonization of the gonads. During this process, retinoic acid (RA) induces meiosis in the female germ cells, which proceeds to the diplotene stage of meiotic prophase I, whereas the male germ cells initiate growth arrest. After birth, meiosis is initiated in mice spermatogonia by their conversion to preleptotene spermatocytes. There are evidences showing the roles of RA in the regulation of spermatogonial differentiation and meiosis initiation. However, it is still not well known on what responds to RA and how RA signaling engages meiosis. Thus, we constructed a proteomic profile of proteins associated with meiosis onset during testis development in mouse and identified 104 differentially expressed proteins (≥1.5 folds). Bioinformatic analysis showed proteins functioning in specific cell processes. The expression patterns of five selected proteins were verified via Western blot, of which we found that *Tfrc* gene was RA responsive, with a RA responsive element, and could be up regulated by RA in spermatogonial stem cell (SSC) line. Taken together, the results provide an important reference profile for further functional study of meiosis initiation.

Biological significance
Spermatogenesis involves mitosis of spermatogonia, meiosis of spermatocytes and spermiogenesis, in which meiosis is a unique event to germ cells, and not in the somatic cells. Till now, the detailed molecular mechanisms of the transition from mitosis to meiosis are still not elucidated. With high-throughput proteomic technology, it is now possible to systemically identify proteins possibly involved. With TMT-6plex based quantification, we identified 104 proteins differentially between testes without meiosis (day 8.5) and those that were meiosis initiated (day 10.5). And a well-known protein essential for meiosis initiation, stra8, was identified to be differentially expressed in the study. And bioinformatic analysis and functional studies revealed several proteins regulated by retinoic acid, a chemical known to regulate the meiosis initiation. Thus, this quantitative proteomic

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approach can identify meiosis initiation regulating proteins, and further functional studies of these proteins will help elucidate the mechanisms of meiosis initiation.

1. Introduction

Meiosis is a type of cell division that is necessary for sexual reproduction in eukaryotes. In mammals, spermatogonia and oogonia undergo species-specific rounds of mitotic divisions after which meiosis occurs [1,2]. The timing of meiotic initiation differs dramatically in spermatogonia and oogonia. Female germ cells enter prophase I of meiosis at approximately E13.5, and remain arrested in the diplotene stage of prophase I until after puberty [3,4]. On the other hand, male germ cells remain arrested at the G1/G0 stage of mitosis at approximately E13.5, resume active mitosis perinatally, and initiate meiosis at puberty [5,6].

In recent years, independent investigations from numerous laboratories have yielded evidences pointing to the role of the active vitamin A metabolite retinoic acid (RA) in triggering the onset of meiosis in both male and female mammals [7,8]. In mice, RA acts in the fetal ovary to trigger the onset of meiosis in germ cells [4,9]. In the fetal testis, endogenous RA is cleared by CYP26B1, ensuring that the onset of meiosis in male germ cells is delayed until after birth [7,10,11]. In early postnatal and adult testes, RA is required to induce the expression of stimulated by retinoic ccid gene 8 (*Stra8*) and sustain meiosis [7,12]. In the absence of *Stra8*, germ cell progression through meiosis is blocked in both the fetal ovary and postnatal testis; therefore, it has been suggested that *Stra8* might play a critical role in oogenesis and spermatogenesis [10,13,14].

Kumar et al. have challenged this widely adopted view. They concluded that RA is not present in the developing mouse ovary and is therefore not required for the initiation of meiosis in XX fetal germ cells and that in the developing fetal testis, CYP26B1 degrades a non-retinoid meiosis-inducing factor that diffuses in from the mesonephros [15,16]. Obviously, these data do not fit comfortably with the established model for RA-driven control of germ cell entry into meiosis. Although some people question their experimental design and findings, skepticism regarding an established model is not a bad thing. Currently, however, the molecular pathway by which RA induces meiosis remains to be clarified.

Therefore, in this study, we aimed to establish a proteomic profile at the initiation of meiosis during testis development in mice and to identify the specific proteins and pathways involved downstream towards RA signaling. After confirming the specific time points by hematoxylin and eosin (H&E) staining, a comparative proteome analysis was carried out on mouse testis samples obtained on postnatal day (PND) 8.5 and PND 10.5. We identified 104 proteins that showed significantly differential expression ($\geq$1.5 fold and $P < 0.05$) and used bioinformatic analysis to annotate the differentially expressed proteins. Then, five selected proteins that were considered to be involved in RA signaling and meiosis were verified via Western blot analysis, and their relationship with RA signaling was further investigated by qRT-PCR. Finally, we further studied the cellular localization of PDCD4, whose role in spermatogenesis is not well known.

2. Materials and methods

2.1. Animals

Pregnant ICR mice were obtained from the laboratory animal center of Nanjing Medical University (Nanjing, China) and maintained in a controlled environment under a 12/12-h light/dark cycle at 20–22 °C and 50–70% humidity with food and water available ad libitum. Testes of the male offspring were collected at day 8.5 and 10.5 postpartum, and fixed in Bouin's solution. The fixed tissues were embedded in paraffin, sectioned, and stained with H&E for histological examination.

2.2. Sample preparation, protein digestion, and tandem mass tag (TMT) labeling

The testes were collected from the male offspring of three mice at two time points postpartum: PND 8.5 and PND 10.5. The collected testes were then divided into three separate samples to obtain three biological replicates. Therefore, we analyzed a total of six biological samples across both time points. The tissues were lysed using protein extraction buffer (7 M urea, 2 M thiourea, 65 mM dithiothreitol and 1% (v/v) protease inhibitor cocktail), and centrifuged at 40,000 ×*g* for 1 h at 4 °C. Protein concentrations were measured by the Bradford assay, and 100 μg of each sample was used for TMT labeling (Pierce, Rockford, IL, USA).

The proteins collected from mouse testes at PND 8.5 and PND 10.5 were used in quantitative proteomic analysis. The proteins were labeled according to the manufacturer's protocol with minor modifications (Pierce). In brief, cysteine residues were reduced with dithiothreitol for 1 h at 56 °C, then alkylated in 375 mM iodoacetamide (IAA) for 45 min at room temperature in the dark, and digested with trypsin overnight at 37 °C. The peptides were then labeled with isobaric tags and pooled before further analysis.

2.3. Strong cation exchange (SCX) fractionation

Peptide mixtures were resuspended in strong cation exchange (SCX) chromatography buffer A (10 mM NH4COOH and 5% ACN, pH 2.7) and loaded onto cation exchange columns (1 mm internal diameter × 10 cm packed with Poros 10S, DIONEX, Sunnyvale, CA, USA) and analyzed in an UltiMate® 3000 HPLC system at a flow rate of 50 μL/min. The following gradient was employed: 0% to 56% of chromatography buffer B (800 mM NH_4COOH and 5% ACN, pH 2.7) for 20 min; 56% to 100% of chromatography buffer B for 1 min; 100% of chromatography buffer B for 5 min; 100% to 0% of chromatography