Expression of the SET protein in testes of mice at different developmental stages

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INTRODUCTION

Mammalian spermatogenesis is a unique and complex process of cell division and differentiation. Germ cells of the seminiferous epithelium ultimately form spermatozoa through three stages: proliferation of spermatogonia, meiosis of spermatocytes, and spermiogenesis. The whole process of spermatogenesis is controlled by a complete hypothalamic-pituitary-gonadal axis. Gonadotrophin-releasing hormone, one of the more important neuropeptides released from the hypothalamus, regulates reproduction by inducing pulsatile discharges of gonadotrophins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from the anterior pituitary gland. LH stimulates testicular Leydig cells to secrete testosterone (T). It is T, not LH directly, that stimulates certain phases of spermatogenesis. FSH with T stimulates mitosis of spermatogonia. Many testicular factors and cytokines are even more importantly involved in regulating spermatogenesis, and Sertoli cells play central roles in this process. Much is unknown on the local regulation of spermatogenesis.

SET (patient “SE translocation”), also termed I2PP2A, PHAP-II, and TAF-1b, an inhibitor of protein phosphatase 2A (PP2A), was first identified in 1992 in a study of leukemia. SET has multiple cellular functions, including control of cell cycle, gene transcription, apoptosis, cell migration, and epigenetic regulation. SET has an important role in facilitating cellular growth and proliferation and interacts with pathways that promote tumorigenesis and metastasis. SET protein is expressed at high levels in the developing gonads, gonadal steroid-hormone-producing cells, mature oocytes and spermatocytes. PP2A, a serine/threonine phosphatase, is a key regulator of diverse signaling pathways. SET-mediated PP2A inhibition is an important regulatory mechanism for a number of physiological and pathological processes, including cell differentiation, apoptosis and carcinogenesis. One study demonstrated that SET protein in oocytes is essential for the faithful sister chromatid segregation by removing the protection of centromeric cohesion in meiosis II. Our evidence suggests that in the ovary SET protein regulates T production by regulating both the promoter activity of CYP17 and the biological activity of P450c17. However, it remains still unknown the roles that SET protein plays in testicular spermatogenesis and androgen production.

In this study, we observed the expression of SET protein in the testes of mice at different developmental stages, and have discussed its potential function in regulating spermatogenesis and steroidogenesis. This increases our knowledge of the paracrine and autocrine regulation of testicular functions.

MATERIALS AND METHODS

Animals and protocol

Male ICR mice (named by the Institute for Cancer Research of American) of Specific Pathogen Free Grade II were purchased from Medical Animal Center of Jiangsu Province in Nanjing Medical University. ICR mice have a life span of about 18 months. Mice aged 4 weeks are at the stage of prepubertal development or early maturity.
Mice aged 8 weeks have attained sexual maturation; while mice over 12 months are ageing (almost equal to humans over 50 years). Twelve mice aged 1 week were assigned to the infant group (sexual infantilism); 12 mice aged 4 weeks were used as the prepubertal group (early maturity); 12 mice aged 12 weeks were considered as the adult group (sexual maturation) and 12 mice aged over 12 months were used as the ageing group. This study was approved by the Animal and Human Ethics Board of Nanjing Medical University.

After 5 days' adaptation in the animal house, mice were killed under narcosis with 50 mg kg\(^{-1}\) pentobarbital sodium intraperitoneal (1% (w/v) concentration, 100 µl for 20 g mouse). One testis (left side) from a mouse was fixed in Bouin’s solution for pathological exam and immunostaining, another one (right side in general) was freshly washed in ice-cold saline and immediately stored in liquid nitrogen. The fresh testis frozen in liquid nitrogen was then divided into two pieces for RNA isolation and protein extraction, individually. Four testes from the infant group were too small to be divided into two pieces, so two samples were pooled for RNA isolation and other two for protein extraction only. There were 10 samples in the infant group for real-time polymerase chain reaction (RT-PCR) and 10 for Western blot, but 12 samples in other three groups.

**Immuno-histochemical analysis**

To observe the cellular localization of SET expression and qualitative comparisons, immuno-histochemistry was performed as described previously. After Bouin-fixation, the paraffin-embedded testicular tissue was cut in 4-µm sections, deparaffinized, and then incubated in 3% (v/v) H\(_2\)O\(_2\) to inhibit endogenous peroxidase activity. The sections were blocked with 5% (w/v) bovine serum albumin and incubated overnight at 4° with primary antibody to SET (Santa Cruz Biotechnology, Dallas, USA, sc-25564, 1:1000 dilution). Negative control sections were incubated with the corresponding nonimmunized IgG. Following three PBS washes, the sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody. Immuno-reactive sites were visualized brown by using an enhanced chemiluminescence detection kit according to the manufacturer’s instructions (Amersham Biosciences, London, UK). Western blot experiments were performed with 10 samples in the infant group and 12 samples in other three groups.

**RESULTS**

**Cellular localization and qualitative comparison of SET expression in testes**

Results of immuno-histochemical analysis showed that SET protein was mainly located in the cytoplasm and nucleus of spermatogenic cells, as well as in Leydig cells (Figure 1). There was also low expression of SET protein in Sertoli cells. There were different expressions in the testes of mice at different developmental stages, with a consistent cellular localization. SET protein was mainly expressed in spermatogonia and spermatocytes, with the highest expression in haploid and tetraploid cells of 4-week-old mice and 12-week-old mice, and the Leydig cells of 12-week-old mice. Qualitatively, the DAB density in the adult group appeared stronger than those of the infant group and the ageing group (Figure 1).

**Expressions of Set mRNA in the four groups**

To examine quantitatively the expression levels of the Set gene in the four groups, real-time PCR was performed (Figure 2a). Expression of Set mRNA was very low in the infant group. In general, the prepubertal group had the highest level of Set expression in the four groups. The prepubertal group had significantly higher expression than the infant group (P < 0.05). Expression of Set mRNA in the adult group was maintained at a high level. Interestingly, expression of Set mRNA in the ageing group was significantly lower than that in the adult group (P < 0.05).

**Expressions of SET protein in the four groups**

The different expressions of SET protein in the testicular tissues of the four groups were analyzed by Western blot (Figure 2b). The expression...
of SET protein in the infant group was very low. The expression of SET protein in the prepubertal group was significantly increased than that in the infant group, with a 4-fold increase ($P < 0.05$). The expression in the adult group was highest among the four groups ($P < 0.05$). When compared with the adult group, there was a large decrease in expression of SET protein in the ageing group, with a 2-fold decrease ($P < 0.05$).

**Expression of SET protein in the seminiferous epithelium at different stages**

SET protein was expressed in all of the seminiferous epitheliums at different stages (Figure 3). However, it was mainly expressed in spermatogonia and spermatocytes. There was less expression of SET protein, or even no expression, in testicular spermatozoa.

**DISCUSSION**

Testosterone is primarily produced by testicular Leydig cells, which is mainly regulated by LH. By binding to the membrane LH receptor, LH induces cAMP synthesis in Leydig cells. cAMP catalyzes PKA synthesis, and PKA transports cholesterol from the cytoplasmic pool to the mitochondria to promote steroidogenesis by steroidogenic enzymes (e.g., CYP11A1, HSD3B) and StAR. PP2A has been shown to dephosphorylate P450c17; this in turn is regulated by SET, a phosphoprotein, that inhibits its activity. SET is expressed in human NT2 neuronal precursor cells, suggesting a role for it in...
neuro-steroidogenesis.\(^{20}\) In this study, it was found that SET protein was expressed in the cytoplasm of testicular Leydig cells, suggesting that SET plays a role in regulating androgen production. So it is necessary to investigate the pathway(s) of SET protein in Leydig cells. Binding of bacterially-expressed SET protein to the DNA site at 2418/2399 of rat P450c17 gene trans-activates P450c17 in \textit{in vitro} cultured Leydig cells.\(^{20}\) The central role of PP2A in SET-mediated regulation of T production was confirmed in our previous study by the findings that SET promoted the lyase activity of P450c17 and that PP2A inhibited its lyase activity.\(^{11}\) The spermatogenic tubule exhibits a production line of germ cells; the earlier stages of spermatogenesis (spermatocytia) are found at the basal side of the tubule; towards the tubular lumen, germ cells are in progressively later stages of differentiation.\(^{21}\) Maturing spermatids are nearest the center of the seminiferous tubule, with their tails extending into its lumen. Spermatogenesis in seminiferous tubule from primitive stem cell, the type A spermatogonial, to spermatocytes and mature sperm passes through several complex transformations (Figure 3). In the seminiferous epithelium, the immature germ cells lying next to the wall (basement membrane) are the spermatogonia. These cells multiply by mitosis, and then continue to divide mitotically. Some of them change in appearance, which is called primary spermatocyte. The latter then completes two meiotic divisions to produce haploid cells, the spermatids. As shown in Figure 3, multi-layered spermatocytes were arranged in the middle of seminiferous epithelium, while the spermatocytes and subsequently transformed spermatocytes (spermatids) and matured spermatids arranged near to the lumen at the defined stages. The cell associations, also called stages of the cycle of the seminiferous epithelium, are most accurately defined by the morphology of the developing acrosomes and of the nuclei of early spermatids.\(^{22}\) Along the seminiferous tubules, these stages follow each other in a wave-like fashion. This makes it possible to study the effect of different regulators on spermatogenesis, by collecting tubular segments from different stages of the cycle.

Pituitary FSH and testicular androgen are the main hormones that regulate spermatogenesis.\(^{21,24}\) Sertoli cells are generally considered the main targets of these hormones in the seminiferous epithelium. In the rat, the various generations of germ cells form the specific associations, labeled I through XIV, follow each other in a fixed sequence in a given area of seminiferous tubules.\(^{21}\) In 1956, Oakberg proposed that the cycle of the mouse seminiferous epithelium was subdivided into 12 stages based on the first 12 steps of the development of spermatids.\(^{25}\) Stages VII and VIII appear to be androgen-dependent.\(^{26}\) The secretory activity of Sertoli cells significantly changes during the cycles, suggesting that Sertoli cells play key roles in the cycle of the seminiferous epithelium. In our previous studies, we found that SET protein was predominantly expressed in theca cells and oocytes in human and mouse ovaries, which located in nucleus, cytoplasm, and mitochondria. Here, we infered that the role (s) of the expressed SET protein in Sertoli cells was related to metabolism, cell activation, cytokines synthesis and secretion, etc., However, spermatogenic cells are mainly dependent on those factors produced by Sertoli cells under stimulation by FSH.\(^{21,24}\) The local mechanisms are poorly understood. In this study, we found that SET protein was expressed in spermatogonia and spermatocytes of the seminiferous epithelium at different stages. The highest expression of SET protein mainly occurred in haploid and tetraploid cells. There was less expression of SET protein, or even none, in testicular spermatocytes. In recent years, a large number of testicular genes have been claimed to be involved in the chromatin dynamics and DNA repair during mitosis and meiosis. Our findings suggest potential effects of SET protein in spermatogenesis.
SET roles in spermatogenesis and androgen production

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