Increased expression of dermatopontin and its implications for testicular dysfunction in mice

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Abstract. An array of specific and non-specific molecules, which are expressed in the testis, have been demonstrated to be responsible for testicular function. Our previous study revealed that dermatopontin (DPT) is expressed in Sertoli cells of the testis, however, its roles in testicular function remains somewhat elusive. In the present study, CdCl2- and busulfan-induced testicular dysfunction models were used to investigate the implications of DPT expression for testicular function. The mRNA and protein expression levels of DPT were detected using reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. A negative correlation was observed between testicular damage and the expression of DPT, which suggested that an increase in DPT expression may be a marker for testicular dysfunction. This result was corroborated by the finding that transgenic mice exhibiting Sertoli cell-specific overexpression of DPT exhibited damage to their testicular morphology. Additionally, DPT overexpression in the testis affected the expression levels of claudin-11 and zonula occludens-1, which indicated that DPT may affect testicular function by affecting the integrity of the blood-testis barrier (BTB). In conclusion, the present study provided evidence to suggest that DPT may be indicative of mouse testicular dysfunction, since increased expression may be associated with damage to the BTB.

Introduction

Infertility is a growing problem worldwide. The term ‘infertility’ refers to the failure of a couple to conceive following 12 months of regular intercourse. Infertility affects 10-15% of couples, and a male factor is responsible for ~50% of infertility cases (1,2). Numerous reports have shown that greater exposure to electromagnetic radiation (3), smoking (4), heat (5), the consumption of certain drugs (6), exposure to heavy metals (7-9), exposure to pesticides (10,11), and biological hazards may partly explain the mechanisms underlying male infertility.

The majority of causes of male infertility are considered to be associated with abnormal spermatogenesis and failures in sperm function (12). Mammalian spermatogenesis, which is a complex and highly regulated process of germ cell proliferation and differentiation, which occurs within the seminiferous tubules of the testes, involves dynamic interactions between the developing germ cells and the Sertoli cells. The Sertoli cells provide structural and nutritional support for the germ cells. An array of specific and non-specific molecules that are expressed by the Sertoli cells, including proteases, protease inhibitors, signaling molecules, growth factors and cell adhesion molecules, have been identified, which are responsible for the regulation of spermatogenesis (13-15). Numerous harmful factors can target the molecules that are expressed by the Sertoli cells, and disruptions to these molecules may arrest germ cell production and maturation or dysfunctional spermatozoa may be produced, resulting in male infertility (16,17). For example, the dibutyl phthalate-induced collapse of Sertoli cell vimentin filaments may lead to the detachment of spermatogenic cells, and the detached cells may undergo apoptosis since they have lost support from the Sertoli cells (18). Zhang and Lui (19) showed that nectin-2 is a direct molecular target for cadmium and proposed that the dysregulation of nectin-2 in the Sertoli cells may explain cadmium-induced male infertility (19).

Dermatopontin (DPT), a 22 kDa non-collagenous extracellular matrix protein, which promotes cellular adhesion and extracellular matrix assembly, initially co-purifies with decorin from bovine dermal extracts (20-22). Western blotting and northern blot analysis indicates that DPT is expressed in the skin, skeletal muscle, bone, cartilage and other tissue types (22,23). DPT reportedly modifies the behavior of transforming growth factor (TGF)-β on mink lung epithelial cells via its interaction with decorin within the extracellular matrix in vivo (24). Furthermore, a reduction in the expression of DPT is a molecular link between uterine leiomyomas and keloids.

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Light microscopy has demonstrated that Dpt-null corneas from 2-month-old mice exhibit a 24% reduction in the average stromal thickness compared with the corneas from wild-type mice, which suggests that DPT is important in collagen fibril organization (25). Additionally, DPT may be involved in the pathogenesis and growth of prostate cancer cells (26).

Our previous study demonstrated that DPT is expressed in the Sertoli cells within the testis (27). An in vitro study showed that DPT is a novel regulator of the CdCl₂-induced reduction in claudin-11 expression, which suggests that DPT may be associated with testicular dysfunction. Therefore, the present study aimed to further investigate the implications of DPT expression for testicular function.

Materials and methods

Materials. The protein extraction kit and the Beyo-ECL Plus western blotting reagents were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). The mouse monoclonal DPT antibody (cat. no. sc-376863) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The anti-claudin-11 (cat. no. 36-4500) and anti-zonula occludens (ZO-1; cat. no. 61-7300) antibodies were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). All secondary antibodies were purchased from ZsBio (Beijing, China). The FLAG-tag antibody (cat. no. 20543-1-AP) was purchased from Proteintech (Wuhan, China). The SYBR® PrimeScript® RT-PCR kit (Perfect Real Time) was purchased from Takara Bio, Inc. (Nanjing, China). CdCl₂ and busulfan were purchased from Sigma-Aldrich (St. Louis, MO, USA). The remaining chemicals were purchased from Sangon Biotech (Shanghai, China).

Animal experiments. A total of 90 C57BL mice, aged between 3 and 4 months (weight, 25-32 g) were obtained from the Experimental Animal Centre at Chongqing Medical University (Chongqing, China). The animals were maintained on a 12 h light/dark cycle, with free access to food and water. The mice were randomly divided into groups containing 6 mice. The mice received 30 mg/kg¹ busulfan or 3.5 mg/kg¹ CdCl₂, which were administered via intraperitoneal injection. Mice were sacrificed 5, 15, 25, 35 or 70 days following treatment with an overdose of 6% chloral hydrate. The left testes were removed and were fixed in a formaldehyde solution at room temperature. The testes were sacrificed 5, 15, 25, 35 or 70 days after injection. The remaining tissues were washed briefly with Tris-buffered saline containing 0.1% Tween-20 (100mM Tris, 0.9% NaCl, 0.1% Tween-20; pH 7.4), and were subsequently incubated for 2 at room temperature with the following corresponding secondary antibody: Horseradish peroxidase (HRP) conjugated-anti-rabbit immunoglobulin G (IgG; 1:3,000; cat. no. ZB-2301) and HRP-anti-mouse IgG (1:3,000; cat. no. ZB-2305). The proteins were detected using the Beyo-ECL kit, according to the manufacturer's protocol. The densities of bands were analyzed using Quantity One (version 4.6.2; Bio-Rad Laboratories, Inc.) and were normalized against β-actin, which served as the loading control.

Histopathological examination. Routine histology was performed to assess the statuses of the testes. The formalin-fixed testes were embedded in paraffin and 4 µm sections were cut using a microtome (Leica RM2135; Wetzlar, Germany). The sections were de-waxed and were re-hydrated through a descending series of alcohol concentrations to distilled water. The tissue sections were subsequently stained with hematoxylin and eosin. The samples were analyzed using light microscopy (Olympus BX51; Olympus, Tokyo, Japan).

Vector constructs. To generate transgenic mice with a Sertoli cell-specific overexpression of DPT, a fragment of the Müllerian-inhibiting substance (MIS) upstream promoter, which was previously validated to be sufficient for the expression of growth hormone (28), nuclear receptor subfamily 0, group B, member 1 (29) and Smad4 (30) in Sertoli cells, was used. The transgenic construct contained the following components: The upstream promoter region (-180 to +1) of the MIS, FLAS tag, the complete open reading frame of mouse DPT and the simian virus (SV)40 polyadenylation poly (A) sequence. The MIS promoter fragment was amplified from the

Western blotting. The total protein was extracted from the testes using radioimmunoprecipitation assay lysis buffer, and the concentrations were determined using the bicinchoninic acid assay. Protein samples (40 µg) were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and were transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat milk for 2 h at 37°C, then incubated with the primary antibodies against DPT (1:500, mouse monoclonal), claudin-11 (1:300; rabbit polyclonal), ZO-1 (1:300, rabbit polyclonal) and FLAG (1:1000, rabbit polyclonal) overnight at 4°C. Following incubation, the membranes were washed briefly with Tris-buffered saline containing 0.1% Tween-20 (100mM Tris, 0.9% NaCl, 0.1% Tween-20; pH 7.4), and were subsequently incubated for 2 at room temperature with the following corresponding secondary antibody: Horseradish peroxidase (HRP) conjugated-anti-rabbit immunoglobulin G (IgG; 1:3,000; cat. no. ZB-2301) and HRP-anti-mouse IgG (1:3,000; cat. no. ZB-2305). The proteins were detected using the Beyo-ECL kit, according to the manufacturer's protocol. The densities of bands were analyzed using Quantity One (version 4.6.2; Bio-Rad Laboratories, Inc.) and were normalized against β-actin, which served as the loading control.
C57BL mice genomic deoxyribonucleic acid (DNA) using the primers MIS-FLAG-F: 5'-GTCGACCTCAGGCTCTGCAG TTATGGG-3' and MIS-FLAG-R: 5'-GAGGTTTACCTTGTCT ATCGTGTCTTGTAGTCCATGTGTTGACACAGAAG-3'. To clone DPT from the testes of C57BL mice and to add the FLAG epitope (DYKDDDDK) to the DPT protein, the primers FLAG-Dpt-F: 5'-CCACCATGACTACAAGGACAGC AGATGGACCTCAGCCAGTTCTTGTA-3' and FLAG-Dpt-R: 5'-TGGCTGCAACTAGAAGGCAACAGC TAAACGTGTTTCTGACAGCAGTCTGTA-3' were used. The SV40 fragment was amplified from the pcDNA3.1 vector using the primers Dpt-SV40-F1: 5'-GAA TTCCAGAACGTAGTCTAGTCTAT GCAGTGT-3' and Dpt-SV40-R1: 5'-GAT CTCCTGGAGTACAGACATGATAGACATTG-3'. Subsequently, the transgenic cassette, MIS-FLAG-DPT-SV40, was generated by in-fusion PCR using the primers MIS-Dpt-F1: 5'-ATAATCC ATGTCAACCTCAGGCCTCTGCAGTTACA-3' and Dpt-SV40-R1: 5'-GATCCCTGAGGATAACAGACATGATAAGTACATTG-3'. The produced fragment was cloned into an 18-T simple vector (Takara Bio, Inc.) for sequencing.

**Generation of mice with overexpression of DPT.** The purified transgenic constructs were microinjected into the pronuclei of fertilized oocytes from C57BL mice. To identify the founder transgenic mice, tail biopsies were collected for genomic DNA isolation using the Universal Genomic DNA Extraction kit (Takara Bio, Inc.) when the mice were 2 weeks of age. The resulting genomic DNA samples were screened by PCR using the primers, MIS-FLAG-F and Dpt-SV40-R1. The amplified products were resolved using 1.5% agarose gel electrophoresis.

**Immunohistochemical detection of the FLAG-tagged DPT protein.** Testicular sections (5 µm) were deparaffinized in xylene and were hydrated in a descending series of ethanol concentrations, which was followed by antigen retrieval in 0.01 mol/l citrate buffer (pH 6.0). Non-specific background was eliminated by blocking with goat serum buffer (ZsBio) at room temperature for 1 h, followed by incubation of the slides with anti-FLAG antibody (1:100; rabbit polyclonal) overnight at 4°C. The samples were subsequently incubated for 1 h at room temperature with goat-anti-rabbit immunoglobulin G secondary antibody (1:1,000; polyclonal; cat. no. ZB-2301). Following washing with phosphate-buffered saline, containing 0.1% Tween-20, the sections were incubated with diaminobenzidine and were counterstained with hematoxylin. The tissue sections were examined and images were captured using a light microscope (Olympus).
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Statistical analysis. The data are expressed as the mean ± standard error. The statistical differences were evaluated using one-way analysis of variance and Newman-Keuls test. P<0.05 was considered to indicate a statistically significant difference.

Results

CdCl₂- or busulfan-induced testicular morphology damage. Routine histology was performed to assess spermatogenesis in the mice following 30 mg/kg⁻¹ busulfan or 3.5 mg/kg⁻¹ CdCl₂ treatment (Fig. 1). No histopathological changes were observed in the testes of the mice in the control groups (Fig. 1A and G). After 5 days of treatment with busulfan, the mice exhibited no obvious changes in the morphology of the epithelium of the seminiferous tubules compared with the control mice (Fig. 1B). After 15 days treatment with busulfan, the seminiferous tubule epithelium had become thinner, and exhibited the detachment of the spermatogenic cells (Fig. 1C). At 25 days following treatment with busulfan, 15% of the seminiferous tubules contained no germ cells (Fig. 1D) and 35 days following treatment with busulfan, 40% of the seminiferous tubules contained no germ cells (Fig. 1E). At 70 days following treatment with busulfan, a histological analysis determined that the majority of the testes contained germ cells and that regeneration was occurring in the seminiferous tubules (Fig. 1F).

In the CdCl₂-treated mice, the histological analysis determined that the basement membrane of the seminiferous tubules was discontinuous, and that secondary spermatocytes and round spermatids were present in the tubules' lumens at 5 days post-treatment (Fig. 1H). At 15 days following treatment with CdCl₂, severe necrosis and degeneration of the seminiferous tubules were apparent, with 20% of the seminiferous tubules exhibiting germ cell losses (Fig. 1I). At 25 days following treatment with CdCl₂, the majority of the seminiferous tubules exhibited severe histopathological changes (Fig. 1J). Between 35 and 70 days following treatment with CdCl₂, the structure of the testes exhibited more severe disorganization and the degree of fibrosis within the testes appeared to increase in a time-dependent manner (Fig. 1K and L).

Effects of busulfan or CdCl₂ treatment on the expression of DPT in the testes of mice. The effects of busulfan or CdCl₂ treatment on the expression levels of DPT in the testes of mice were determined using PCR and western blotting. At 5 days following treatment, the busulfan-treated group exhibited no change in the expression of DPT (P>0.05). Between 15 and 25 days
following treatment with busulfan, the DPT levels gradually increased with time. Between 35 and 70 days following treatment with busulfan, the DPT levels gradually normalized (Fig. 2A, C, and E).

The pattern of DPT expression in the mice administered CdCl₂ differed from that observed in the busulfan-treated mice. Time-dependent changes in the expression of DPT mRNA (Fig. 2B) and protein (Fig. 2D and F) were observed in the mouse testes in response to treatment with CdCl₂.

Establishment of a transgenic mouse with DPT overexpression. Two males (lines 37 and 45) positive for the mouse DPT transgene (Fig. 3A) were selected for further breeding. The 4-month-old mice, originally derived from the two male founder mice, exhibited an upregulation of the protein expression of DPT in their testes, however, the control litter mate did not (Fig. 3B and C). No apparent differences were observed in the expression of testicular DPT between the transgenic lines. Since the transgenic construct contains a FLAG tag,
the present study also tested for the expression of the FLAG epitope in the testes, and the results revealed that while no expression of the FLAG epitope occurred in the control mice, its protein was detected in the testes from the transgenic animals at a molecular weight that was appropriate for DPT (Fig. 3B). Histological analysis also immunolocalized the FLAG epitope in the Sertoli cells that were within the sections of the testicular tissue from the DPT overexpression mice (Fig. 3D), which further substantiated the expression of the transgenic construct within the testes.

Overexpression of DPT in the Sertoli cells results in germ cell losses and vacuole formation in the seminiferous tubules. Testes from the 4-month-old transgenic mice exhibited histopathological changes in the seminiferous tubules, which included the desquamation of the spermatogenic cells (Fig. 4). In addition, a significant proportion of the tubules within the testes of the 4-month-old transgenic mice exhibited impairments of the spermatogenesis process, which included the formation of vacuoles, suggesting that endogenous DPT overexpression in the Sertoli cells also negatively impacts upon reproductive function.

Overexpression of DPT in the Sertoli cells leads to a decrease in the expression levels of ZO-1 and claudin-11 in the testes. To determine whether the integrity of the blood-testis barrier (BTB) is affected by DPT overexpression in vivo, the present study assessed the steady-state levels of claudin-11 and ZO-1 by immunoblotting. As shown in Fig. 5, the relative expression levels of ZO-1 and claudin-11 in the DPT overexpression mice were significantly reduced as compared with the control mice (Fig. 5), which suggested that DPT may be associated with injury to the BTB.

Discussion

DPT is a gene with unknown function in male reproduction, which was previously shown to be expressed in the testes. The present study demonstrated that increased expression of DPT has an affect on testicular dysfunction in mice. To investigate changes in the expression of DPT in mice with testicular dysfunction, an animal model of testicular dysfunction was established by treating mice with a single dose of busulfan or CdCl₂. Findings from previous research have demonstrated that busulfan or CdCl₂ can damage reproductive function in males by disrupting the endogenous balance within the testicular microenvironment, which leads to germ cell losses and somatic cell damage (31-33). In the present study, busulfan or CdCl₂ injections damaged the structure of the testicles of the mice, which concurs with results reported previously. The histological data suggested that the degree of damage to the testes was considerably lower in the busulfan-treated mice compared with the CdCl₂-treated mice. At 70 days following busulfan treatment, the testicular histology appeared to be recovering to an almost normal profile, while exposure to CdCl₂ resulted in progressive testicular injury. Subsequently, the expression pattern of DPT in the testis with regard to busulfan or CdCl₂ treatment was characterized. In busulfan treated groups, the expression of DPT was normal up to day 5, however, was significantly increased on days 15, 25 and 35, and with recovery up to day 70. Taking the results from the histological analyses of the testes following busulfan treatment together with the levels of DPT expression in the testes at different time points following busulfan treatment, which were determined using PCR and western blotting, suggested that an increase in the expression of DPT may be a good marker for male testicular dysfunction. Therefore, a high level of DPT expression may indicate a low level of reproductive function.

Time-dependent changes in the expression of DPT were observed in the mouse testes in response to treatment with CdCl₂. The pattern of DPT expression in the mice administered CdCl₂ differed from that observed in the busulfan-treated mice, which further supports the concept that an increase in the expression of DPT may be indicative of reproductive dysfunction in male mice.

Since the increases in the expression of DPT in testis were induced by exogenous detrimental factors, namely CdCl₂ or busulfan administration, the present study hypothesized that endogenous increases in the levels of DPT expression were directly associated with testicular function. Our previous study revealed that DPT is expressed in the Sertoli cells (27), after which DPT transgenic mice, which had a Sertoli cell-specific overexpression of DPT, were established to facilitate studies into the effects of endogenous DPT...
overexpression on testicular function in mice. A significant proportion of the tubules within the testes of the 4-month-old transgenic mice exhibited impairments of the spermatogenesis process suggested that just as exogenous factors induce increases in the levels of DPT expression, endogenous DPT overexpression in the Sertoli cells also implicates testicular dysfunction.

The present study further investigated the possible mechanisms underlying testicular injury in mice with Sertoli cell-specific DPT overexpression. Alterations in the BTB is associated with male fertility (34). Sertoli cell tight junctions (TJ) are an essential component of the BTB, which create an immunologically balanced microenvironment, and they provide structural support for spermatogenesis. TJ are multimolecular membrane specializations comprising several integral membrane proteins, including occludin, claudins, and ZO-1. The findings from previous in vitro and in vivo investigations show that particular factors can affect the integrity of the BTB by targeting TJ (35). For example, TGF-β3 and tumor necrosis factor-α, which are secreted by the Sertoli cells and the germ cells, can induce reversible disruption to the BTB in vivo by reducing the steady-state levels of occludin and ZO-1 (36).

Our previous data from in vitro investigations showed that CdCl₂-induced downregulation of claudin-11 is partially counteracted by DPT silencing in 15P-1 Sertoli cells, which suggests that DPT is a novel regulator of CdCl₂-induced BTB damage in vitro (27). In the present study, the steady-state levels of claudin-11 and ZO-1 were assessed by immunoblotting to determine whether the integrity of the BTB is affected by DPT overexpression in vivo. It was revealed that compared with the control mice, the relative expression levels of ZO-1 and claudin-11 in the DPT overexpression mice were significantly reduced, which suggested that DPT overexpression may be associated with injury to the BTB, and, in turn, testicular dysfunction.

In conclusion, the expression patterns of DPT in mice treated with CdCl₂ or busulfan indicated that an increase in DPT expression has implications for testicular dysfunction in mice. This finding was corroborated by the findings in the testicles of mice exhibiting endogenous overexpression of DPT, rather than increases in DPT expression induced by busulfan or CdCl₂. The increase in the expression of DPT may harm the integrity of the BTB, which may, in turn, be injurious to testicular function.

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