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·*Original Article*·

Differential expression and regulation of integral membrane protein 2b in rat male reproductive tissues

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Abstract

Aim: To examine the expression and regulation of integral membrane protein 2b (Itm2b) in rat male reproductive tissues during sexual maturation and under different treatments by *in situ* hybridization. **Methods:** Testis, epididymis, and vas deferens were collected on days 1–70 to examine Itm2b expression during sexual maturation. To further examine the regulation of Itm2b, adult rats underwent surgical castration and cryptorchidism. Ethylene dimethane sulfonate and busulfan treatments were carried out to test the regulation of Itm2b after destruction of Leydig cells and germ cells. **Results:** In testis, Itm2b expression was moderately detected in the adluminal area of seminiferous cords on days 1–10, and detected at a low level in the spermatogonia on days 20 and 30. The Itm2b level was markedly increased in Leydig cells from day 20 to day 70. In epididymis and vas deferens, Itm2b was detected from neonate to adults, and the signal gradually increased in accordance with sexual maturation. Itm2b expression was significantly downregulated in epididymis and vas deferens of castrated rats, and strongly stimulated when castrated rats were treated with testosterone. Cryptorchidism led to a significant decline of Itm2b expression in testis and caput epididymis. Itm2b expression in epididymis and vas deferens was significantly decreased after the Leydig cells were destroyed by ethylene dimethane sulfonate. Busulfan treatment produced no obvious change in Itm2b expression in epididymis or vas deferens. **Conclusion:** Our data suggested that Itm2b expression is upregulated by testosterone and might play a role in rat male reproduction. *(Asian J Androl 2008 May; 10: 503–511)*

Keywords: testis; epididymis; vas deferens; integral membrane protein 2b

1 Introduction

The objective of the present study was to examine the expression patterns and regulation of integral membrane protein 2b (Itm2b) in rat testis, epididymis, and vas deferens under sexual maturation, castration, induced cryptorchidism, ethylene dimethane sulfonate (EDS) treatment, and busulfan treatment by *in situ* hybridization. The type II integral membrane protein (Itm2) family consists of Itm2a, Itm2b, and Itm2c [1, 2]. The homology of amino acid sequences in Itm2 family members between mouse and human was very high, up to 91%–

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94% [3]. Itm2a is 38% identical to Itm2b and 49% identical to Itm2c, and Itm2b is 49% identical to Itm2c [2, 3]. All three members of the Itm2 family show different expression patterns. Itm2b expression is strongly detected in different tissues tested in mouse and human, but its function in mouse is still unknown [1, 2, 4]. Human ITM2B is also designated as BRI2 and located on chromosome 13 [1]. Point mutation or decamer duplication at the stop codon of wild-type BRI2 is associated with autosomal dominant disorders familial British dementia and familial Danish dementia, respectively [4–6]. ITM2B is located in the region most frequently deleted in prostate carcinoma, and showed a significant difference in expression pattern between normal and neoplastic prostate tissues [7].

Amyloid deposition in testis is considered a cause of secondary azoospermia [8]. Amyloid beta protein precursor (APP) is localized only in the acrosome and tail of spermatids in the seminiferous tubules during spermatogenesis [9, 10]. Amyloid beta peptides are the major components of amyloid and derived from APP processing. ITM2B can regulate APP processing to reduce the level of amyloid beta peptides [6]. In sporadic Alzheimer's disease, both ITM2B and APP were co-localized with amyloid beta peptide [11]. We assumed that Itm2b might be important for spermatogenesis. The expression pattern and regulation of the integral membrane protein family in mammalian male reproductive tissues have not been reported.

2 Materials and methods

2.1 Animals

Male rats of Wistar strain were used in this study. All animals were maintained in a controlled environment (14 h:10 h Light:Dark cycle) and provided with standard pellet diet and clean water *ad libitum*. All animal procedures were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University (Harbin, China). Every experiment was repeated at least three times. To study the expression pattern during sexual maturation, testis, epididymis and vas deferens were collected on postnatal days 1, 10, 20, 30, 40 and 70. Epididymis was further divided into caput, corpus and cauda. All tissues were frozen in liquid nitrogen and stored at –70ºC until cryosectioning. The tissues on days 1 and 10 were first embedded in Tissue-tek OCT freezing medium (Sakura Finetek USA Inc., Torrance, CA, USA) and frozen in liquid nitrogen. Hereafter, adult male rats (70 days old) weighing 300–350 g were used for all treatments and controls.

2.2 Castration and hormonal replacement

Adult rats were bilaterally orchidectomized under ether anesthesia, and then epididymis was carefully returned into scrotum. Castrated rats were rested for 3 days to eliminate the endogenous testosterone. Three days later, the castrated rats were injected s.c. with testosterone propionate (TP) (3 mg/kg body weight; Sigma, St. Louis, MO, USA) dissolved in 0.2 mL sesame oil for 1, 5 and 12 consecutive days. The sham-operated control rats were injected with 0.2 mL sesame oil. Twenty-four hours after the last injection, epididymis and vas deferens of treated and control rats were collected, frozen in liquid nitrogen and stored at –70ºC until cryosectioning.

2.3 Unilateral induced cryptorchidism

Adult rats were surgically excised with unilateral induced cryptorchidism under ether anesthesia as previously described [12]. Briefly, a small incision was made on the lower abdomen, and the right testis was displaced into the abdominal cavity. After cutting the gubernaculum, the inguinal canal was closed to prevent the descent of testis. The left testis remained within the scrotum as the euthermic control [13]. Thirty days after the cryptorchidism surgery, testis, epididymis and vas deferens were collected from cryptorchid and euthermic sides, frozen in liquid nitrogen and stored at –70ºC until cryosectioning.

2.4 EDS treatment

EDS treatment was used to eliminate testicular Leydig cells from male rats [14]. Adult rats were treated with a single i.p. injection of EDS dissolved in dimethylsulfoxide (DMSO) and water (1:3) at a concentration of 37.5 mg/mL in a dose of 75 mg/kg body weight. Seven days later, the EDS-treated rats were injected s.c. with either TP (3 mg/kg body weight) or sesame oil (vehicle) for 1 and 5 consecutive days, respectively. Twenty-four hours after the last injection, testis, epididymis and vas deferens of treatment and control rats were collected, frozen in liquid nitrogen and preserved at –70ºC for cryosectioning.

2.5 Busulfan treatment

Adult rats were given a single i.p. injection of busulfan at 10 mg/kg dissolved in 50% DMSO to deplete the

germ cell populations in testis [15, 16]. Control rats were injected with a comparable volume of 50% DMSO as the vehicle. Thirty days after the injection, testis, epididymis and vas deferens were collected and preserved as described above.

2.6 In situ hybridization

The total RNA of rat uterus was reverse transcribed, and the cDNA was amplified with forward primer GTGGCGGTGGATTGCAAGGA and reverse primer GGGCGGCATAACGATGGAAG designed from mouse Itm2b (432 bp; GenBank Accession No. U76253). The amplified polymerase chain reaction (PCR) fragment was recovered from the agarose gel and cloned into pGEM-T plasmid. The sequence of Itm2b clone was verified by sequencing. These recombinant plasmids were amplified with the primers for T7 and SP6 to prepare the templates for labeling sense and antisense probes. Digoxigenin (DIG)-labeled antisense or sense cRNA probes were transcribed *in vitro* using a DIG RNA labeling kit (Roche Diagnostics, Mannheim, Germany) and stored at –70ºC for use.

Cryosections (10 μm) of frozen tissues were mounted on 3-aminopropyltriethoxy-silane (Sigma) coated slides and dried on a 50ºC hot plate for 3 min. The sections were fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h at room temperature. After fixation, the sections were washed in PBS twice, treated in 1% Triton-100 for 20 min, and washed again in PBS three times. The sections were incubated in prehybridization buffer containing 50% formamide and 5 × SSC (1 × SSC includes 0.15 mol/L sodium chloride and 0.015 mol/L sodium citrate) at room temperature for 15 min. Following prehybridization washes, the sections were incubated in the hybridization buffer as previously described [17]. After washed for stringency, the sections were incubated with sheep anti-DIG antibody conjugated to alkaline phosphatase in 1% Blocking reagent (Roche Diagnostics, Mannheim, Germany) overnight at 4ºC. The signal was visualized with 0.4 mmol/L 5-bromo-4-chloro-3-indolyl phosphate and 0.4 mmol/L nitroblue tetrazolium. Endogenous alkaline phosphatase activity was inhibited with 2 mmol/L levamisole (Sigma). Sections were counterstained with 1% methyl green in 0.12 mol/L glacial acetic acid and 0.08 mol/L sodium acetate for 30 min, and positive signals were detected as a dark brown color.

3 Results

3.1 Itm2b expression in rat testis, epididymis, and vas deferens during sexual maturation

Itm2b expression was differentially detected in rat testis during sexual maturation from neonate to adult stage. On days 1 and 10, Itm2b was detected at a moderate level in the adluminal area of seminiferous cords. On days 20 and 30, Itm2b was detected at a low level in basal compartmental cells, particularly spermatogoina and Leydig cells. On day 40, Itm2b was strongly detected in Leydig cells and increased up to day 70. The Itm2b signal was barely detected in the basal compartmental area of seminiferous tubules from days 40 to 70 (Figure 1).

Itm2b expression was basally detected in all three regions of epididymis on day 1. On day 10, the signal was moderate in caput and cauda regions, but relatively low in the corpus region. Itm2b expression was mainly located in the apical region of tubular epithelium until day 20 in caput, and until day 10 in corpus and cauda epididymis. The tubular epithelium of all three regions showed a strong level of Itm2b from day 30. In caput, the signal became slightly higher up to day 70. In corpus and cauda, the signal was slightly higher up to day 40

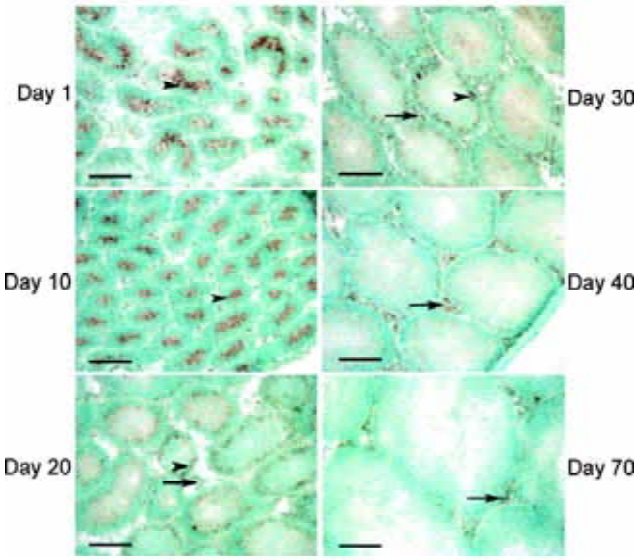

Figure 1. Integral membrane protein 2b (Itm2b) expression in rat testis during sexual maturation on postnatal days 1, 10, 20, 30, 40, and 70. Itm2b signal was detected at a moderate level in the adluminal area of seminiferous cords on days 1 and 10, and at a low level in the spermatogonia on days 20 and 30 (arrow heads). There was a marked increase of Itm2b signal seen in Leydig cells from day 20 to day 70 (arrows). Bars = 30 μm.

and maintained the same level on day 70. There was no signal seen in the intertubular regions of epididymis (Figure 2).

Unlike testis and epididymis, Itm2b signal was strongly detected in the epithelium of vas deferens from neonates, and became slightly higher in every age up to day 70. There was no signal seen in the stromal cells or muscular layers of vas deferens (Figure 2).

3.2 Itm2b expression under castration and TP treatment

Castration caused a significant difference in Itm2b expression after 1 day only in caput epididymis. Caput epididymis that received vehicle for 1 day expressed a basal level of Itm2b signal, whereas testosterone replacement for 1 day stimulated Itm2b expression. In corpus, cauda, and vas deferens, a strong level of Itm2b expression was maintained in both the control and TP treatment groups for 1 day (Figures 3, 4).

In the control group after 5 days, Itm2b was detected at a basal level in caput, corpus, and vas deferens,

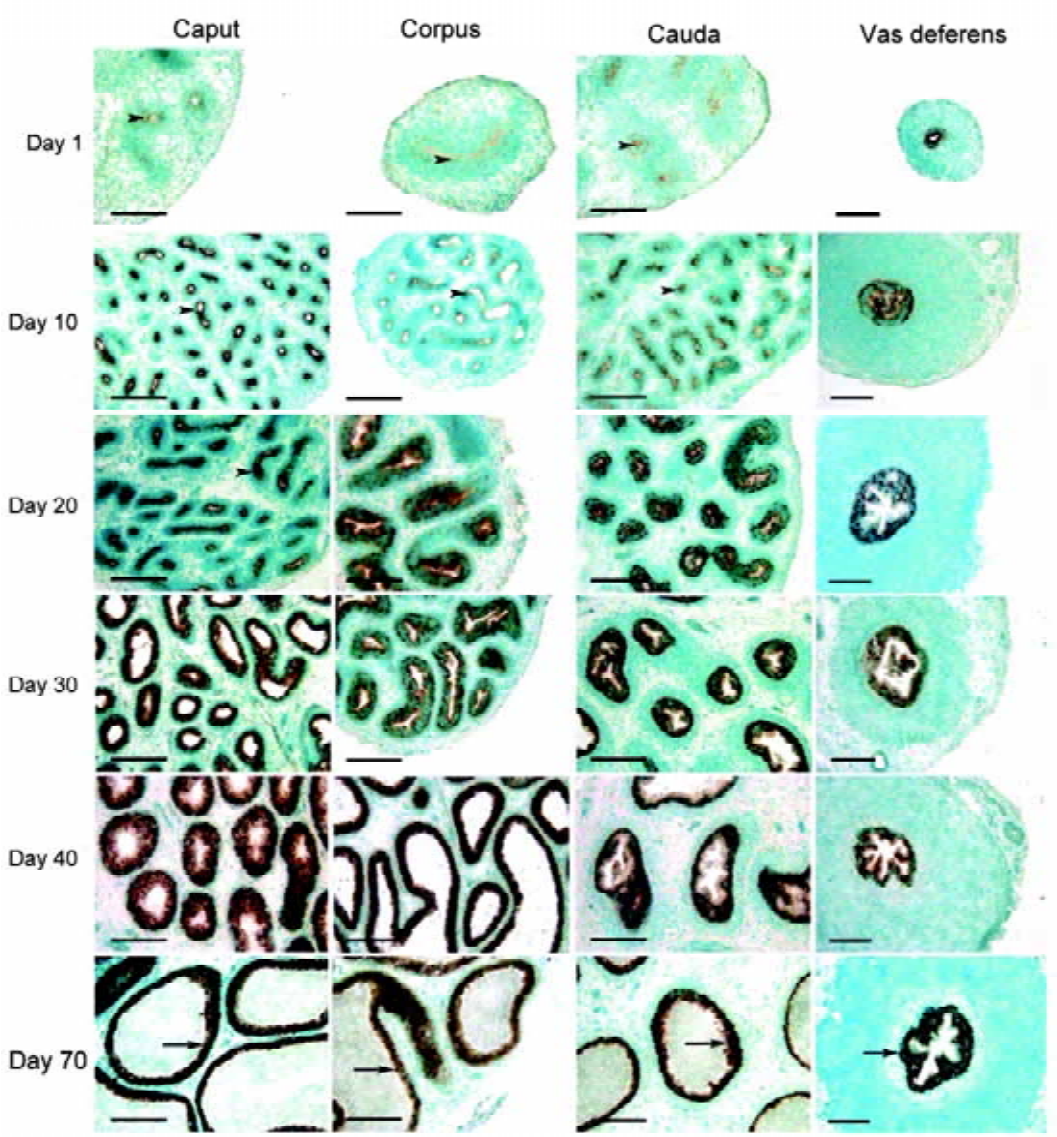

Figure 2. Integral membrane protein 2b (Itm2b) expression in rat caput, corpus, cauda epididymides and vas deferens during sexual maturation on postnatal days 1, 10, 20, 30, 40 and 70. Itm2b signal was detected in the apical region of tubular epithelium until day 20 in caput, and until day 10 in corpus and cauda (arrow heads). Then the signal was highly concentrated in all epithelial cells (arrows indicate the strong signal found throughout the epithelium on day 70). Bars = 30 μm.

and at a low level in cauda epididymis. When the rats were treated with testosterone for 5 days, the signal was significantly higher in all the regions of epididymis and vas deferens compared to controls (Figures 3, 4).

The Itm2b signal was drastically downregulated in all the regions of epididymis and vas deferens in castrated rats that received vehicle for 12 days. Testosterone replacement for 12 days slightly induced Itm2b expression in all tissues, and particularly in cauda, showing a higher level of Itm2b signal (Figure 3). Furthermore, the decrease of epididymis and vas deferens size due to castration was more prominent in treated groups, and

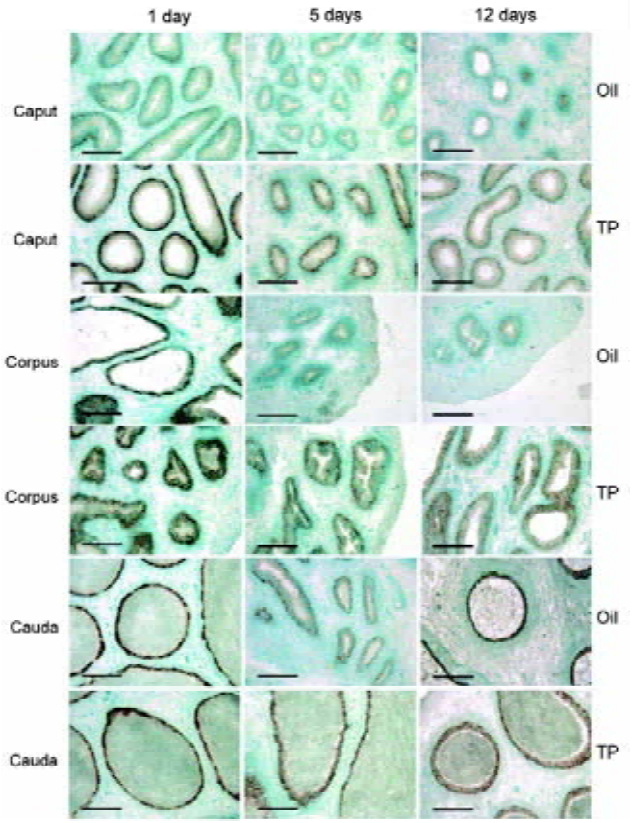

Figure 3. Integral membrane protein 2b (Itm2b) expression in caput, corpus, and cauda epididymides in castrated rats treated with sesame oil as vehicle (0.2 mL/kg/day) and testosterone propionate (TP; 3 mg/kg/day) dissolved in sesame oil for 1, 5, and 12 consecutive days. Bars = 30 μm.

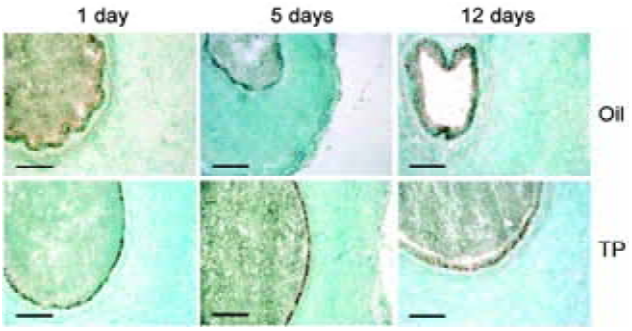

Figure 4. Integral membrane protein 2b (Itm2b) expression in vas deferens in castrated rats treated with sesame oil as vehicle (0.2 mL/kg/day) and testosterone propionate (TP; 3 mg/kg/day) dissolved in sesame oil for 1, 5 and 12 consecutive days. Bars = 30 μm.

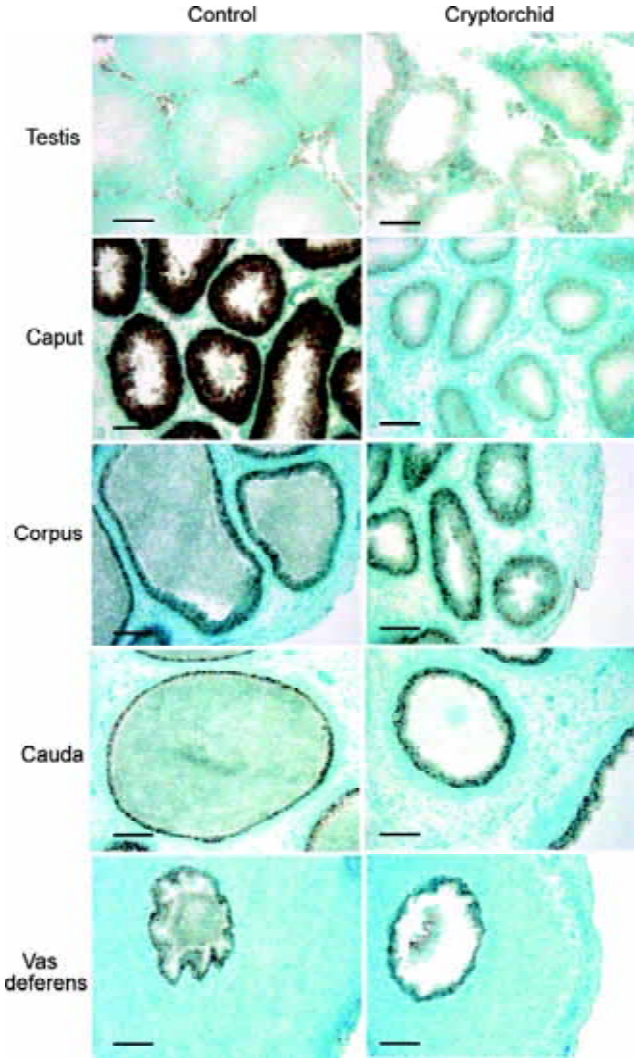

Figure 5. Integral membrane protein 2b (Itm2b) expression in rat testis, caput, corpus, cauda and vas deferens under unilateral induced cryptorchidism. Tissues shown in the left-hand panels were from the euthermic control where the testis was not disturbed. Tissues shown in the right-hand panels were from the cryptorchid side where the testis was displaced to the abdomen for 30 days. Bars = 30 μm.

testosterone could partially restore the castration effects. Testosterone replacement for 5 and 12 days maintained the local sperm storage in cauda epididymides compared to the respective controls (Figures 3, 4).

3.3 Itm2b expression under unilateral induced cryptorchidism

In this experiment, the right testis was displaced into the abdomen to arrest the testicular functions by exposure to body temperature, and the left testis remained within the scrotum. Thirty days after surgery, testis, epididymis, and vas deferens from the right side (so-called cryptorchid) and left side (so-called euthermic control) were subjected to Itm2b detection. Itm2b expression was severely affected only in testis and caput epididymis. In the control, the testis and caput region showed a strong level of Itm2b signal. In contrast, there was a basal level of the signal detected in cryptorchid testis and caput epididymis. The other tissues such as corpus, cauda, and vas deferens from both control and cryptorchid groups did not show any obvious difference in Itm2b signal (Figure 5). Tissue damage was markedly seen in cryptorchid testis and epididymis. Particularly, cryptorchid testis lost testicular mass, including seminiferous tubules and Leydig cells. Due to loss of testicular factors, there were no sperm seen in any region of epididymis or vas defer-

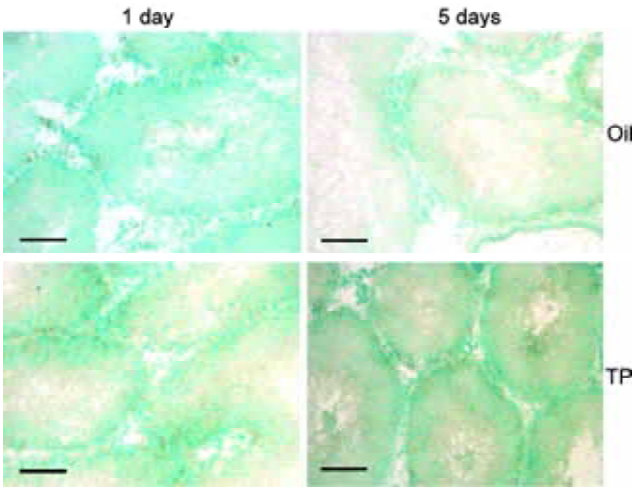

Figure 6. Integral membrane protein 2b (Itm2b) expression in testis of rats treated with a single injection of ethylene dimethane sulfonate (75 mg/kg). Seven days later, the rats were treated with sesame oil as vehicle (0.2 mL/kg/day) or testosterone propionate (TP; 3 mg/kg/day) dissolved in sesame oil for 1 and 5 days, respectively. Bars = 30 μm.

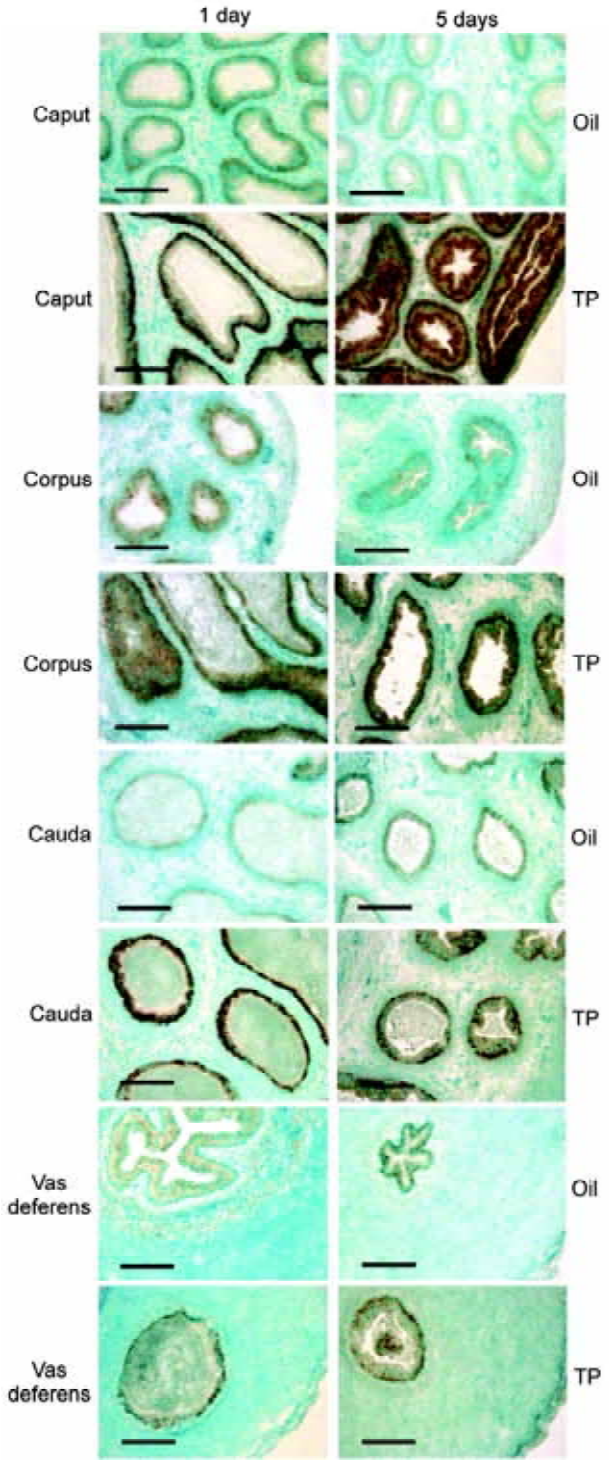

Figure 7. Integral membrane protein 2b (Itm2b) expression in rat epididymis and vas deferens treated with a single injection of ethylene dimethane sulfonate (75 mg/kg). Seven days later, the rats were treated with sesame oil as vehicle (0.2 mL/kg/day) or testosterone propionate (TP; 3 mg/kg/day) dissolved in sesame oil for 1 and 5 days, respectively. Bars = 50 μm.

ens on the cryptorchid side (Figure 5).

3.4 Expression of Itm2b under EDS treatment and testosterone replacement

EDS treatment markedly regulated the expression of Itm2b in testis, epididymis and vas deferens of adult rats. Leydig cells of testis were completely depleted by EDS treatment. Due to the loss of Leydig cells, Itm2b failed to express in the testis of rats injected with vehicle or testosterone for 1 and 5 days (Figure 6).

Seven days after EDS treatment, a single injection (1 day) of testosterone effectively elevated the Itm2b signal in caput and cauda epididymis compared to that of the vehicle group, but in corpus epididymis the signal was detected at a low level in both vehicle and testosterone groups. However, testosterone treatment for 5 days highly stimulated Itm2b expression in all the regions of epididymis compared to that of the vehicle groups. Furthermore, caput epididymis that received testosterone for 5 days showed the highest level of Itm2b compared to all other tissues that received testosterone for both 1 and 5 days (Figure 7).

Seven days after EDS treatment, vehicle or testosterone injection for 1 day had no obvious effects on Itm2b expression in vas deferens. In contrast, testosterone injection for 5 days significantly elevated the Itm2b signal in vas deferens compared to the vehicle group (Figure 7).

3.5 Itm2b expression under busulfan treatment

Busulfan caused structural damage in testis only by depletion of spermatogonia in many of the tubules. Under busulfan treatment, Itm2b expression was not changed compared to the control. Itm2b expression was moderately located in the Leydig cells of control and treated testis. Interestingly, Itm2b expression was also detected in the peritubular cells. There was a strong level of Itm2b signal detected in all three regions of epididymis and vas deferens in both control and busulfan-treated groups (data not shown).

4 Discussion

In mouse and human, a strong level of Itm2b expression was detected in different tissues, indicating its broader functions [1, 2, 4]. However, Itm2b expression in rat tissues was unknown. In the present study, Itm2b expression was moderate in the testicular tubules on days 1–10, then decreased to a basal level up to day 40. In addition, the Itm2b signal appeared at a low level in Leydig cells on day 20 and gradually increased to a high level up to day 40. This transition of Itm2b signal from tubules to Leydig cells suggested the role of Itm2b in adult-type Leydig cells and testosterone production during sexual development. By reverse transcription–polymerase chain reaction analysis, the presence of Itm2b in mouse testis was detected [2]. In epididymis and vas deferens, Itm2b was detected in all age groups and the signal gradually increased up to adult stage. The epithelium of epididymis and vas deferens is involved in conversion of testosterone, secretion of fluids, and detoxification processes for maturation, storage, and transport of spermatozoa [18, 19]. The expression of Itm2b in the epithelium of epididymis and vas deferens suggested that Itm2b plays a critical role in the pseudostratified layer of these tissues.

A strong level of Itm2b in adult Leydig cells, epididymis, and vas deferens indicated that this gene might be under the control of testosterone. Surgical removal of both testes, as the main source of testosterone, eliminated all the endogenous testosterone and dramatically affected the structure, functions, and gene expression of accessory sex organs including epididymis and vas deferens [20–23]. Injection of exogenous testosterone can partially restore the castration effects shown in the accessory sex organs [21, 23]. Both orchidectomy and induced cryptorchidism will result in loss of circulating as well as luminal testosterone, and testicular factors secreted into the rete testis fluid [21, 24]. Our results confirmed that Itm2b expression in accessory organs was under the control of testosterone. Due to its nearest position, caput epididymis showed the highest difference in both castration and cryptorchidism treatments. Abdominal testis functionally arrests spermatogenesis [24]. We found that loss of spermatogenesis or testicular fluids had no effect on Itm2b expression in corpus, cauda, or vas deferens on the cryptorchid side.

Leydig cells are the major components in the testicular interstitium, and germ cells are the major components in the tubules [25, 26]. Complete depletion of Leydig cells after EDS treatment was observed by using Leydig cell-specific 3β-hydroxysteroid dehydrogenase staining [27]. To examine the regulation of Leydig cells and germ cells on Itm2b expression, adult rats were treated with EDS or busulfan to deplete Leydig cells or germ cells, respectively. Depletion of Leydig cells by EDS treatment or depletion of germ cells by busulfan will regulate

the functions of each other cell type and the effects should be reflected in the accessory sex organs including epididymis and seminal vesicle [28–30]. EDS treatment completely depleted the Leydig cells and led to a significant decrease of Itm2b expression in epididymis and vas deferens. The upregulation of Itm2b expression in epididymis and vas deferens by injecting exogenous testosterone into EDS-treated rats suggested that Itm2b expression was under the control of testosterone. When compared to the sexual maturation of adult testis in the present study, Itm2b expression was moderate in the DMSO-treated control and busulfan-treated testis. This correlated with previous reports that depletion of germ cells influences the functions of Leydig cells [29, 30]. Busulfan had no obvious effects on Itm2b expression in epididymis or vas deferens.

The mechanism of Itm2b action in rat male reproduction is still unknown. The expression of Itm2b in different tissues, organs, and cell lines was reported previously, however, the expression of Itm2b in any of the rat male reproductive tissues was not reported.

Due to the insufficient literature on molecular mechanism of Itm2b in male reproduction, our knowledge to support the present investigation is limited. The present study was a novel idea to examine the expression and regulation of Itm2b in rat testis, epididymis, and vas deferens under sexual maturation, castration, induced cryptorchidism, EDS, and busulfan treatment. Itm2b was strongly detected in rat testis, epididymis and vas deferens in accordance with age groups. Itm2b expression in epididymis and vas deferens was downregulated by castration, cryptorchidism, and EDS treatment. Our data suggested that Itm2b expression is significantly upregulated by testosterone and might have an important role in rat male reproduction.

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