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ORIGINAL PAPER

Expression and regulation of type II integral membrane protein family members in mouse male reproductive tissues

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Abstract Type II Integral membrane protein (Itm2) family consists of three members, Itm2a, Itm2b and Itm2c. ITM2B has been shown to be closely related to human male reproduction. The expression and regulation of Itm2 family members in male reproductive tissues are still unknown. The aim of the present study was to examine the expression pattern and regulation of Itm2 family members in male mouse reproductive tissues during sexual maturation, castration, and busulfan treatment by in situ hybridization. During sexual maturation, a low level of Itm2a was detected in testicular interstitium on days 30–70. Itm2b expression was basally detected in the epithelium of seminiferous tubules on days 1, 5, and 10, and then the signal was transited into Leydig cells and gradually increased up to day 70. Itm2c was detected at a basal to low level in the testis during sexual maturation. Both Itm2a and Itm2c were not detected in the epididymis and vas deferens during sexual maturation. In contrast, Itm2b expression was detected in the epithelium of caput, corpus, cauda epididymis, and vas deferens from neonate to adult mice. In the caput, Itm2b expression reached the highest level on day 15 and maintained this level up to day 70. However, in corpus and cauda epididymis, the signals gradually reached a high level from days 15 to 70. In vas deferens, Itm2b gradually increased to a high level from days 25 to 70. In the castrated mice, Itm2b expression was upregulated in epididymis and vas deferens by testosterone treatments. When busulfan was used to specifically destroy the germ cells in the testis, there were no observable effects on Itm2b expression in the male reproductive organs. Our results suggested that Itm2b mRNA was differentially expressed in mouse male reproductive tissues, during sexual maturation and up-regulated by testosterone.

Keywords Itm2b · Testis · Epididymis · Vas deferens · Mouse

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Introduction

Testis, epididymis, and vas deferens play pivotal roles in mammalian reproduction. Testicular factors of germ cell origin and Leydig cell origin are conveyed to caput epididymis through seminiferous tubules for sperm maturation along the epididymis and for maintaining the structure and functions of accessory sex organs [1, 2]. Immotile and maturing spermatozoa could be seen in the lumen of caput and corpus epididymis, whereas complete mature spermatozoa are stored in cauda epididymis until ejaculation [3, 4]. The lumen of epididymal tubules are filled with fluids that were secreted by epididymal epithelial cells for sperm maturation and survival [1]. Except for the transport function, vas deferens contributes to absorption of minerals and steroid synthesis from the epithelial cells [5].

There are three members in the family of type II integral membrane protein (Itm2), including Itm2a, Itm2b, and Itm2c [6–8]. The human homolog of ITM2A, ITM2B and ITM2C were identified as BRI1, BRI2, and BRI3, respectively [7]. The amino acid sequences between mouse and human are identical up to 94% for Itm2a, 93% for Itm2b and 91% for Itm2c, whereas the different family members share 38–49% homology at the amino acid level within the

same species [6, 8, 9]. Itm2a was considered as chondro-osteogenic specific molecule [6, 10]. Itm2b was ubiquitously expressed in different neonatal and adult tissues [7, 8, 11]. Itm2c was mainly expressed in the brain [8].

Testicular amyloid deposition is considered as a cause of secondary azoospermia [12]. During the spermatogenesis, amyloid beta-protein precursor (APP) was localized only in the acrosome and tail of spermatids in the seminiferous tubules [13, 14]. Amyloid is mainly composed of amyloid-beta peptides which are derive from processing of APP. It has been shown that ITM2B can regulate APP processing to reduce the level of amyloid beta peptides [15]. ITM2B and APP were colocalized with amyloid beta peptide in sporadic Alzheimer disease. The presence of ITM2B had a modulatory effect on APP processing [16]. It is possible that Itm2b may be important for spermatogenesis. The expression pattern and regulation of integral membrane protein family in mammalian male reproductive tissues were still unknown. The aim of the present study was to examine the expression pattern and regulation of Itm2 family members in mouse testis, epididymis and vas deferens during sexual maturation, castration and busulfan treatment.

Results

Expression of Itm2 members in mouse testis during sexual maturation

The expression of Itm2a, Itm2b and Itm2c in mouse testis during sexual maturation was examined by in situ hybridization (Fig. 1). In the testis, there was no detectable Itm2a signal from days 1 to 25 after birth. A low level of Itm2a expression was seen in Leydig cells from days 30 to 70 after birth.

There was a basal level of Itm2b expression in the epithelium of seminiferous tubules on days 1, 5 and 10 after birth. However, Itm2b expression was detected in the Leydig cells from day 15 after birth and gradually increased to a high level on day 70 after birth.

A basal level of Itm2c expression was detected in the seminiferous tubules on days 1, 5, and 10 after birth. There was no detectable Itm2c expression from days 15 to 25 after birth. A low level of Itm2c expression appeared in the Leydig cells from day 30 and maintained this low level through day 70.

Expression of Itm2 members in mouse epididymis during sexual maturation

The expression of Itm2a, Itm2b, and Itm2c in mouse epididymis during sexual maturation was examined by in situ hybridization. There was no detectable signal for both Itm2a and Itm2c in mouse epididymis during sexual maturation (data not shown). Itm2b expression was detected in the epithelium of caput, corpus and cauda from neonate to adult mice (Fig. 2).

In the caput epididymis, a low level of Itm2b expression was detected in the epithelium of the neonatal mice. Itm2b expression gradually increased from day 5, reached the highest level on day 15 and maintained at the high level through day 70. In the corpus epididymis, Itm2b expression was at a basal level on days 1 and 5 after birth, at a low level on day 10 and reached a high level from days 15 to 70 after birth. In the cauda epididymis, there was a basal level of Itm2b on days 1 and 5, a low level on day 10, and a high level from days 15 to 70 after birth.

Itm2b expression in mouse epididymis by castration treatment and testosterone replacement

Since there was no detectable signal for Itm2a and Itm2c in adult epididymis, their expression patterns were not examined in the castrated mice. In order to see whether Itm2b expression in epididymis was under the control of testicular testosterone, Itm2b expression was examined in the castrated mice. To further examine the effects of testosterone on Itm2b expression, castrated mice were treated with testosterone propionate for 1, 5, and 12 days, respectively.

In the control, there was a low level of Itm2b expression in the caput, corpus and cauda epididymis after the castrated mice were treated with sesame oil for 1, 5 and 12 days, respectively (Fig. 3). Compared to controls, Itm2b expression was significantly upregulated by testosterone propionate treatment for 1, 5, and 12 days, respectively. Furthermore, Itm2b expression following testosterone propionate treatment for 12 days was significantly stronger than that treated for 1 or 5 days (Fig. 3).

Expression of Itm2 members in vas deferens during sexual maturation

The expression of Itm2a, Itm2b, and Itm2c in mouse vas deferens during sexual maturation was examined by in situ hybridization. There were no detectable signals for both Itm2a and Itm2c in the vas deferens from neonate to adult mice (data not shown). A basal level of Itm2b expression was detected in the vas deferens on days 1 and 5 after birth. Itm2b expression was at a low level from days 10 to 20 after birth and was strongly detected in the epithelial cells of vas deferens from days 25 to 70 (Fig. 4).

Itm2b expression in mouse vas deferens by castration treatment and testosterone replacement

The Itm2a and Itm2c expression was not examined in the castrated mice as their expression was not detected in adult vas deferens. In order to see whether Itm2b expression in

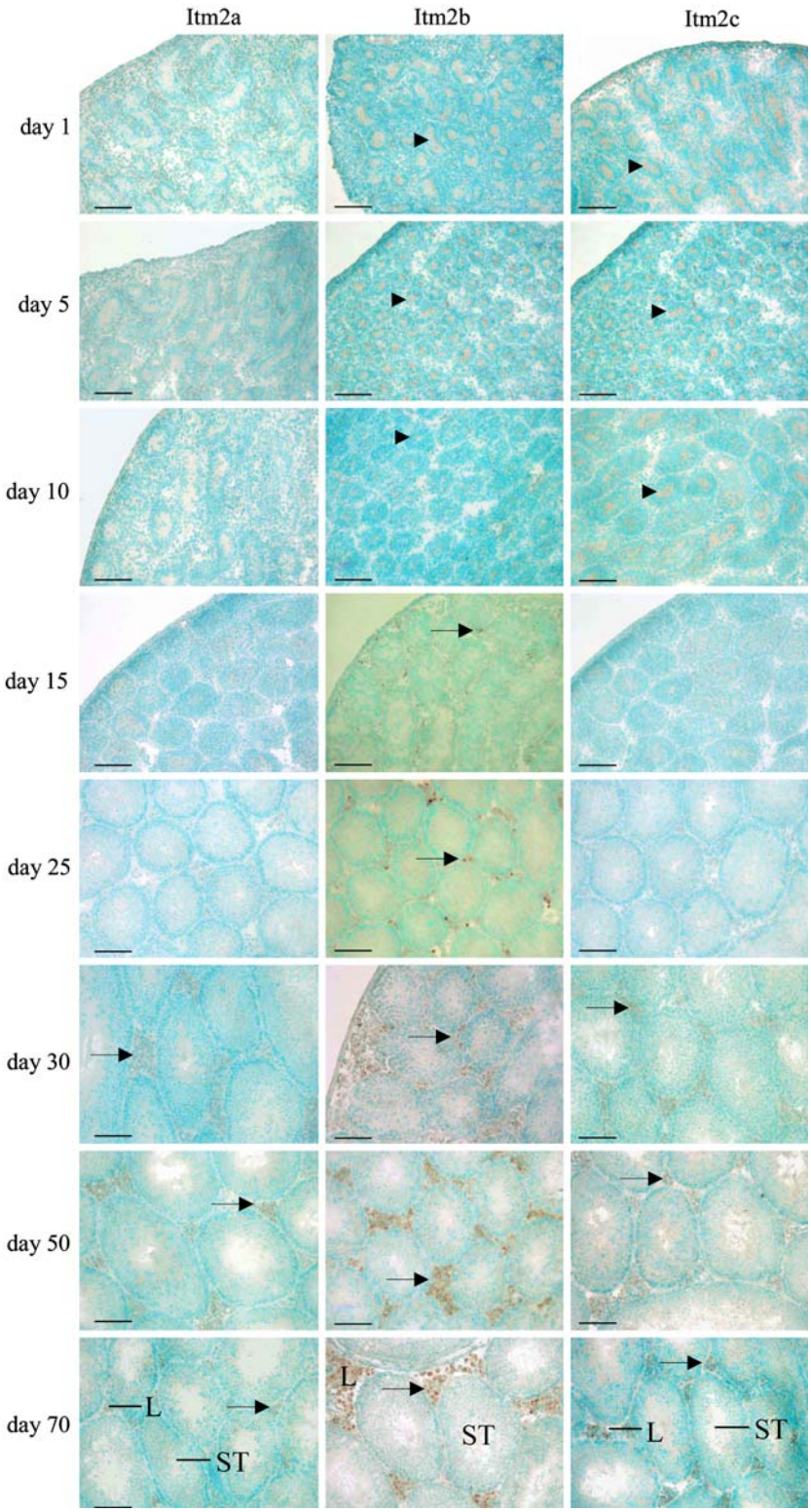

Fig. 1 Differential expression of Itm2 members in mouse testis during sexual maturation on postnatal days 1, 5, 10, 15, 25, 30, 50, and 70, respectively. L: Leydig cells; ST: seminiferous tubule; Arrow head: signal within the tubules; Arrow: signal in the Leydig cells; Bar = 100 μm

the vas deferens was under the control of testicular testosterone, Itm2b expression was examined in castrated male mice. To further examine the effects of testosterone on Itm2b expression, castrated mice were treated with testosterone propionate for 1, 5, and 12 days, respectively.

In the castrated mice, there was a basal level of Itm2b expression in the lumen of vas deferens following 1 day treatment with oil. Itm2b expression was slightly upregulated by testosterone propionate treatment for 1 day. In the control groups treated with oil for 5 and 12 days, there was

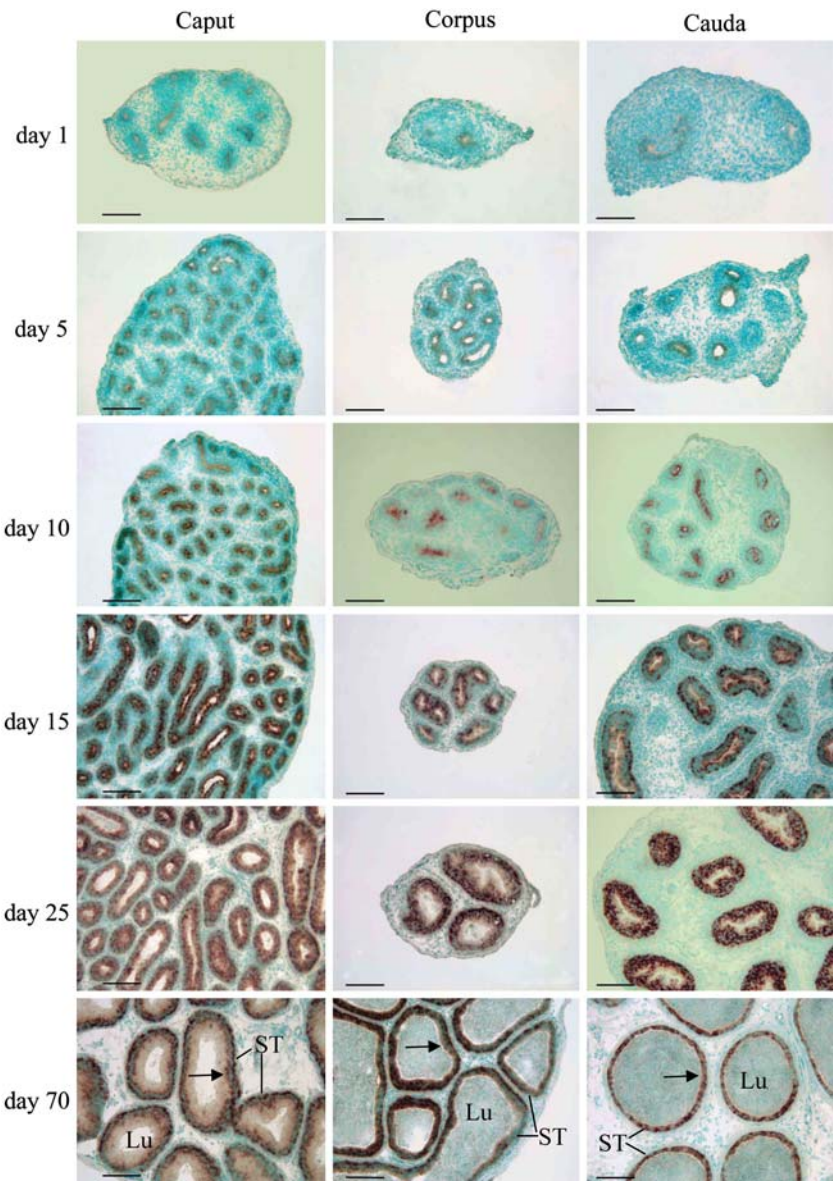

Fig. 2 Differential expression of Itm2b in mouse caput, corpus and cauda epididymis during sexual maturation on postnatal days 1, 5, 10, 15, 25, and 70, respectively. Lu: Lumen; ST: Seminiferous tubules; Arrow: signal in the tubular epithelium; Bar = 100 μm

a low level of Itm2b expression in the epithelium of vas deferens, respectively. Itm2b expression in the epithelium of vas deferens was also slightly up-regulated by testosterone propionate treatment for 5 and 12 days, respectively. Compared to 5 days, Itm2b expression was stronger following testosterone propionate treatment for 12 days (Fig. 5).

Regulation of Itm2b by busulfan treatment

Germ cells are the main cells in seminiferous tubules of adult testis and can also regulate the functions of peritubular Leydig cells. To examine whether Itm2b expression in the testis, epididymis and vas deferens is regulated by germ cells, busulfan was used to specifically destroy the germ cells and arrest the spermatogenesis [17]. In the control groups, Itm2b expression was detected in the tubular epithelium of testis, epididymis, and vas deferens. Busulfan had no obvious effects on Itm2b expression in testis, epididymis, and vas deferens up to 30 days after busulfan treatment (Fig. 6).

Discussion

In this study, a low level of Itm2a expression was detected in the Leydig cells from days 30 to 70, but no signal was seen in the epididymal regions and vas deferens. In a previous study, Itm2a expression was also barely detected in mouse testis [8]. Itm2a is considered as a useful marker for chondro-osteogenic differentiation [6, 10]. Additionally, Itm2a was specifically expressed during the differentiation of T-cells

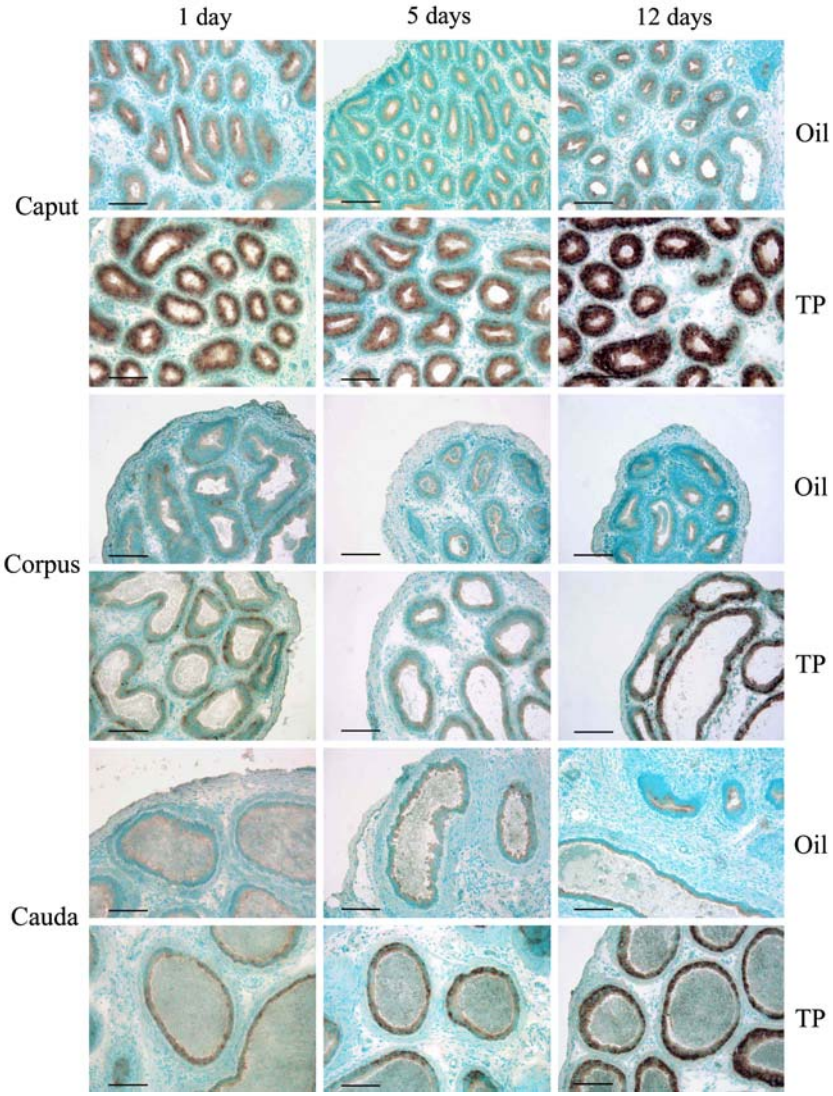

Fig. 3 Itm2b expression in the caput, corpus and cauda epididymis of castrated mice following treatments with oil (0.1 ml/mouse/day) or testosterone propionate (0.1 mg/mouse/day) for 1, 5 and 12 consecutive days, respectively. Bar = 100 μm

and myogenic tissues [9, 18]. A basal level of Itm2c expression was first detected in the seminiferous tubules from days 1 to 10, and then mainly seen in the Leydig cells from days 30 to 70. A low level of Itm2c expression was also detected in adult mouse testis by RT-PCR [8]. It should be interesting to know the significance for the transition of Itm2c expression from seminiferous tubules to Leydig cells.

In contrast to Itm2a and Itm2c, Itm2b was strongly expressed in the adult testis, epididymis, and vas deferens. Itm2b expression was also strongly detected in adult mouse testis by RT-PCR [8]. Since Itm2b expression became stronger in adult male reproductive organ, it seems that Itm2b expression might be under the control of testosterone. This hypothesis was also supported by our data from castration and testosterone replacement. Since germ cells are the main components in the seminiferous tubules, it is possible that Itm2b expression is also under the regulation from germ cell-derived factors. Busulfan was used to test the hypothesis by destroying germ cells in adult testis. Busulfan treatment led to germ cell depletion [17, 19, 20]. In this study, busulfan depleted germ cells in seminiferous tubules. However, busulfan had no obvious effects on Itm2b expression in testis, epididymis, and vas deferens, suggesting that Itm2b expression may be solely regulated by testosterone.

The function of Itm2b in male reproduction is still unknown. Testicular amyloid deposition is considered as a cause of secondary azoospermia [12]. During sperm formation, amyloid beta-protein precursor (APP) was localized only in acrosome and tail of spermatids in the seminiferous tubules [13, 14]. ITM2B expression regulates APP processing resulting in a reduced amyloid-beta peptide level [15]. ITM2B and APP were colocalized with amyloid-beta peptide in sporadic Alzheimer disease. The presence of ITM2B had a modulatory effect on APP processing [16]. The strong expression of Itm2b in the testis

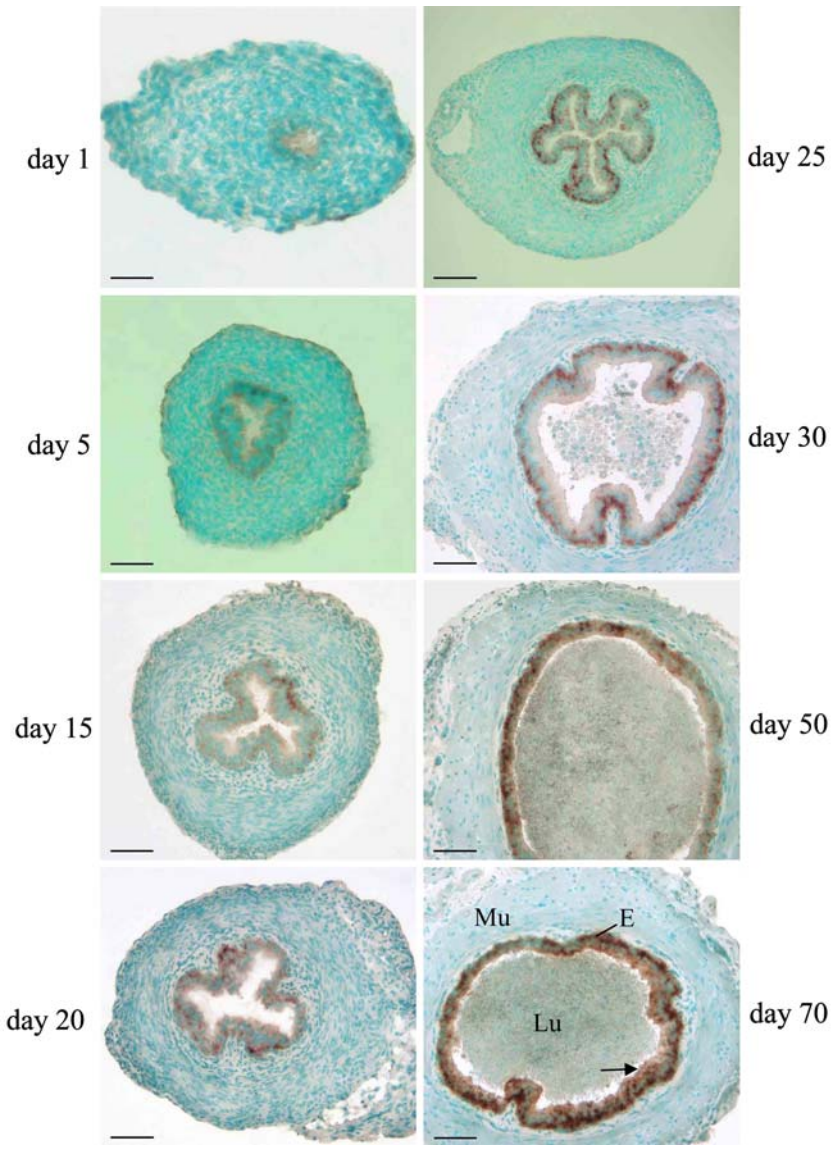

Fig. 4 Itm2b expression in mouse vas deferens during sexual maturation on postnatal days 1, 5, 15, 20, 25, 30, 50, and 70, respectively. Lu: Lumen; Mu: Muscular layer; E: Epithelium; Arrow: signal in the tubular epithelium; Bar = 100 μm

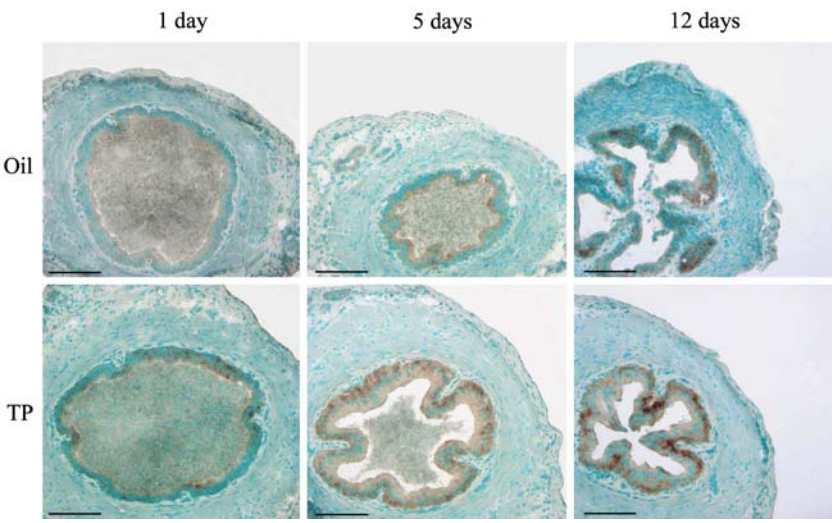

Fig. 5 Itm2b expression in vas deferens of castrated mice following treatments with oil (0.1 ml/mouse/day) or testosterone propionate (0.1 mg/mouse/day) for 1, 5 and 12 consecutive days, respectively. Bar = 100 μm

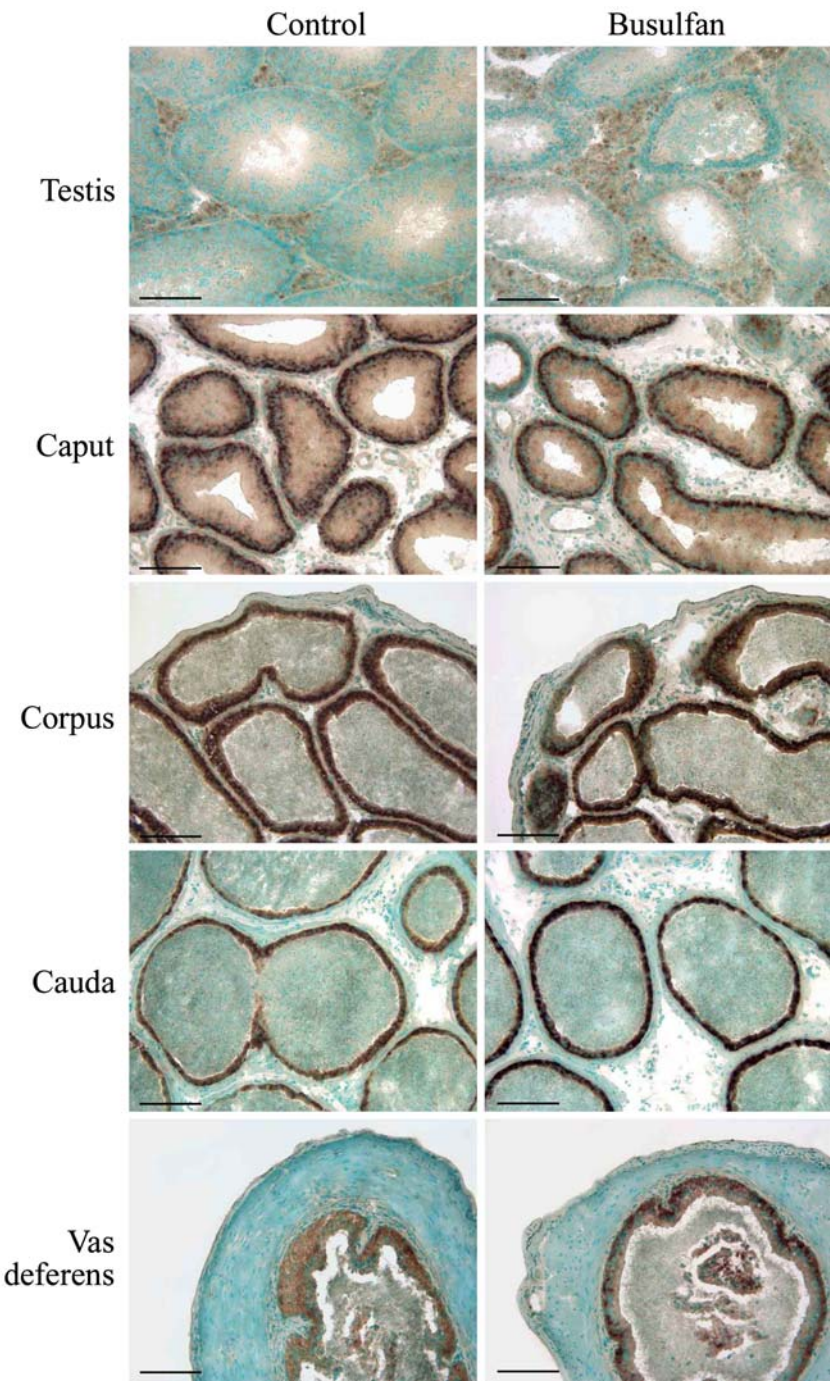

Fig. 6 Itm2b expression in mouse testis, caput, corpus, cauda, and vas deferens 30 days after the mice were treated with 0.1 ml of 50% DMSO (for control) or 20 mg/kg busulfan in 0.1 ml of 50% DMSO. Bar = 100 μm

may suggest a key role in cellular differentiation or morphologic change in the testis.

Epididymis is important for secretion, absorption, detoxification, and transportation during sperm storage and maturation [1, 4, 21]. In this study, Itm2b did not show any region-specific or cell-specific expression in the epididymis, suggesting that Itm2b is involved in the diverse function in the epididymis. Since sperm survival in the cauda epididymis is maintained by the normal functioning of the androgen-stimulated epithelium, castration may initiate a mechanism dealing with the degradation and disposition of dead spermatozoa by dissolution [22]. The vas deferens, a steroid-responsive organ, is lined with a columnar epithelium that is thought to modulate the ionic composition of the luminal environment through which sperm must pass [23]. In our study, Itm2b expression gradually increased in the epididymis and vas deferens during sexual maturation and was also regulated by testosterone. Itm2b may have a role in mouse epididymis and vas deferens.

In conclusion, our results suggested that Itm2b mRNA was differentially expressed in mouse male reproductive tissues during sexual maturation and up-regulated by testosterone.

Materials and methods

Sexual maturation

Male Kunming White, outbred mice were maintained in a controlled environment with 14:10 h Light/Dark cycle, and provided with standard pellet diet and clean water *ad libitum*. Young mice (stated below) were free access to breast feeding in the same litter until use. All the animal procedures were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University. Every experiment was repeated at least three times. In order to examine the expression patterns of Itm2 family members during sex development, testis, epididymis and vas deferens were collected on postnatal days 1, 5, 10, 15, 20, 25, 30, 50 and 70, respectively (the day of birth was designated as postnatal day 1). Epididymis was divided into caput, corpus and cauda. All the tissues were immediately frozen in liquid nitrogen and preserved at –70°C until cryosectioning. The tissues on days 1, 5 and 10 were first embedded in Tissue-tek OCT freezing medium and frozen in liquid nitrogen.

Castration and treatments with testosterone propionate

In order to examine the effects of castration and testosterone on Itm2 member expression, adult male mice (about 35 g body weights) were bilaterally orchidectomized and then epididymis was carefully returned into scrotum under anesthesia. Castrated mice were rested for three days to eliminate endogenous testosterone. Then the castrated mice were injected with testosterone propionate (TP) (0.1 mg/mouse/day, s.c., Sigma, St. Louis, MO, USA) in 0.1 ml of sesame oil (Sigma) for 1, 5, and 12 consecutive days, respectively. For control, castrated mice were sham operated and injected with 0.1 ml of sesame oil. The epididymis and vas deference were collected 24 h after last injection, frozen in liquid nitrogen and preserved at –70°C until cryosectioning.

Busulfan treatment

In order to examine Itm2 family expression after testicular germ cell ablation, adult male mice (about 35 g body weights) were treated with a single i.p. injection of 20 mg/kg busulfan in 0.1 ml of 50% DMSO [24]. Control mice received 0.1 ml of 50% DMSO. Thirty days after the injection, the testis, epididymis and vas deferens were collected, frozen in liquid nitrogen and preserved at –70°C until cryosectioning.

Hybridization probes

Total RNAs were extracted with Trizol reagent from mouse skeletal muscle for Itm2a, uterus for Itm2b and lung for Itm2c. After total RNAs were reverse transcribed, the cDNAs were amplified with appropriate forward and reverse primers. All the primers were shown in Table 1. The amplified fragment of each gene with correct size was recovered from the agarose gel and cloned into pGEM-T plasmid. The cloned fragment was further 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 sense or antisense cRNA probes was transcribed in vitro using a DIG RNA labeling kit (Roche Diagnostics GmbH, Mannheim, Germany).

In situ hybridization

Frozen sections (10 μm) were mounted on 3-aminopropyltriethoxy-silane (Sigma) coated slides, dried on a 50°C hot plate and fixed in freshly prepared 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS, pH7.4) for 1 h. The sections were washed in PBS twice,

Table 1 Primers for mouse Itm2a, Itm2b and Itm2c

Gene	Primer sequences[a]	Accession Number	Size
Itm2a	5′-AGACCTTCTGCTGGGTTTCA	NM_008409	834–1234 bp
	5′-TCAGGATGGTAAGATGACAC		
Itm2b	5′-GTGGCGGTGGATTGCAAGGA	U76253	245–676 bp
	5′-GGGCGGCATAACGATGGAAG		
Itm2c	5′-CTGTACCAGGGCTGTGATCT	NM_022417	1055–1362 bp
	5′-TCCACACCCAGGCCTAAGAA		

[a] Sequences for Itm2a and Itm2c were from Choi et al. [8].

treated in 1% (v/v) Triton-100 for 20 min, and washed again in PBS three times. Following prehybridization in 50% formamide and 5X SSC (pH7.0) at room temperature for 15 min, the sections were hybridized in the hybridization buffer (5 × SSC, 50% formamide, 0.02% BSA, 250 μg/ml yeast tRNA, 10% dextran sulfate sodium salt) with denatured DIG-labeled antisense RNA probes at 55°C for 16 h. The sections were washed for stringency in 50% (v/v) formamide/5 × SSC at 55°C for 15 min, 50% (v/v) formamide/2 × SSC at 55°C for 30 min, 50% (v/v) formamide/0.2 × SSC at 55°C twice for 30 min each, and 0.2 × SSC at room temperature for 5 min. After nonspecific binding was blocked in 1% (w/v) block mix (Roche Diagnostics GmbH) for 1 h at room temperature, the sections were incubated with sheep anti-DIG antibody conjugated to alkaline phosphatase (1:5000, Roche Diagnostics GmbH) in 1% block mix overnight at 4°C. The signal was visualized with 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate and 0.4 mM nitroblue tetrazolium in the buffer containing 100 mM Tris-HCl (pH 9.5), 100 mM NaCl and 50 mM $MgCl_2$. Endogenous alkaline phosphatase activity was inhibited with 2 mM levamisole (Sigma). Sections were counterstained with 1% (w/v) methyl green in 0.12 M glacial acetic acid and 0.08 M sodium acetate for 30 min. The positive signal was visualized as a dark brown color.

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References

1. B. Robaire, R.S. Viger, Biol. Reprod. **52**, 226–236 (1995)
2. S. Fouchecourt, J.J. Lareyre, P. Chaurand, B.B. Dague, K. Suzuki, D.E. Ong, G.E. Olson, R.J. Matusik, R.M. Caprioli, M.C. Orgebin-Crist, Endocrinology **144**, 887–900 (2003)
3. B.P. Setchell, L.G. Sanchez-Partida, A. Chairussyuhur, Reprod. Fertil. Dev. **5**, 601–612 (1993)
4. T.R. Chauvin, M.D. Griswold, Biol. Reprod. **71**, 560–569 (2004)
5. C.J. Flickinger, Anat. Rec. **176**, 205–211 (1973)
6. W. Deleersnijder, G. Hong, R. Cortvrindt, C. Poirier, P. Tylzanowski, K. Pittois, E. Van Marck, J. Merregaert, J. Biol. Chem. **271**, 19475–19482 (1996)
7. K. Pittois, W. Deleersnijder, J. Merregaert, Gene **217**, 141–149 (1998)
8. S.C. Choi, J. Kim, T.H. Kim, S.Y. Cho, S.S. Park, K.D. Kim, S.H. Lee, Mol. Cells. **12**, 391–397 (2001)
9. J. Kirchner, M.J. Bevan, J. Exp. Med. **190**, 217–228 (1999)
10. J.P. Tuckermann, K. Pittois, N.C. Partridge, J. Merregaert, P. Angel, J. Bone Miner. Res. **15**, 1257–1265 (2000)
11. F. Pickford, L. Onstead, C. Camacho-Prihar, J. Hardy, E. McGowan, Neurosci. Lett. **338**, 95–98 (2003)
12. C.G. Schrepferman, D.R. Lester, J.I. Sandlow, Urology **55**, 145 (2000)
13. M. Shoji, T. Kawarabayashi, Y. Harigaya, H. Yamaguchi, S. Hirai, T. Kamimura, T. Sugiyama, Am. J. Pathol. **137**, 1027–1032 (1990)
14. M. Fardilha, S.I. Vieira, A. Barros, M. Sousa, O.A. Da Cruz E Silva, E.F. Da Cruz E Silva, Ann. N. Y. Acad. Sci. **1096**, 196–206 (2007)
15. S. Matsuda, L. Giliberto, Y. Matsuda, P. Davies, E. McGowan, F. Pickford, J. Ghiso, B. Frangione, L. D'Adamio, J. Biol. Chem. **280**, 28912–28916 (2005)
16. A. Fotinopoulou, M. Tsachaki, M. Vlavaki, A. Poulopoulos, A. Rostagno, B. Frangione, J. Ghiso, S. Efthimiopoulos, J. Biol. Chem. **280**, 30768–30772 (2005)
17. H. Jackson, M. Partington, B.W. Fox, Nature **194**, 1184–1185 (1962)
18. D. Van den Plas, J. Merregaert, Cell. Biol. Int. **28**, 199–207 (2004)
19. M.T. Hochereau-de-Reviers, M.C. Viguier-Martinez, C. Perreau, Andrologia **14**, 297–305 (1982)
20. I.D. Morris, C.W. Bardin, N.A. Musto, R.B. Thau, G.L. Gunsalus, Int. J. Androl. **10**, 691–700 (1987)
21. N. Hsia, G.A. Cornwall, Biol. Reprod. **70**, 448–457 (2004)
22. R. Jones, Biol. Reprod. **71**, 1405–1411 (2004)
23. M.L. Phillips, B.D. Schultz, Biol. Reprod. **66**, 1016–1023 (2002)
24. A.E. Moisan, R.A. Foster, K.J. Betteridge, A.C. Hahnel, Reproduction **126**, 205–216 (2003)