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Male infertility due to germ cell apoptosis in mice lacking the thiamin carrier, Tht1. A new insight into the critical role of thiamin in spermatogenesis

Kimihiko Oishi,[a] Marco Barchi,[b] Audrey C. Au,[a] Bruce D. Gelb,[a] and George A. Diaz[a,*]

[a] *Departments of Human Genetics and Pediatrics, Mount Sinai School of Medicine, New York, NY, USA*
[b] *Cell Biology Program, Memorial Sloan-Kettering Cancer Center, New York, NY, USA*

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Abstract

A mouse model of thiamin-responsive megaloblastic anemia (diabetes mellitus, deafness, megaloblastic anemia) lacking functional *Slc19a2* has been generated and unexpectedly found to have a male-specific sterility phenotype. We describe here the characterization of the testis-specific effects of absence of the high-affinity thiamin transporter, Tht1. Null males were found to have hypoplastic testes secondary to germ cell depletion. Morphologic and expression analysis revealed that under conditions of standard thiamin intake, tissues affected in the syndrome (pancreatic β-cell, hematopoietic cells, auditory nerve) maintained normal function but pachytene stage spermatocytes underwent apoptosis. Under conditions of thiamin challenge, the apoptotic cell loss extended to earlier stages of germ cells but spared Sertoli cells and Leydig cells. Injection of high-dose thiamin was effective in reversing the spermatogenic failure, suggesting that the absence of the thiamin carrier could be overcome by diffusion-mediated transport at supranormal thiamin concentrations. These observations demonstrated that male germ cells, particularly those with high thiamin transporter expression beyond the blood–testis barrier, were more susceptible to apoptosis triggered by intracellular thiamin deficiency than any other tissue type. The findings described here highlight an unexpected and critical role for thiamin transport and metabolism in spermatogenesis.

Keywords: Spermatogenesis; Male infertility; Germ cells; Apoptosis; Thiamin; Thiamin-responsive megaloblastic anemia syndrome; *Slc19a2*

Introduction

Spermatogenesis is a highly complex process involving several tightly regulated molecular interactions. The absence or dysregulation of genes involved in this process results in male infertility with arrest of spermatogenesis at wide variety of stages (Escalier, 2001). An additional important aspect for sperm production in mammals is the nutritional environment (Wong et al., 2000). Deficiencies in several vitamins have been found to impact on spermatogenesis through different mechanisms (Kodentsova et al., 1994). Vitamin A deficiency results in male infertility due to the degeneration of most germ cells (Kim and Wang, 1993). Supplementation with retinoic acid (RA) reverses the pathology (van Pelt and de Rooij, 1991). In mice, loss of the RA receptor (RARs) or retinoid receptor (RXRs) disrupts spermatogenesis (Kastner et al., 1996; Lufkin et al., 1993), implicating this signaling pathway in the regulation of germ call maturation. Interestingly, RXRβ is expressed principally in Sertoli rather than germ cells, illustrating that spermatogenic failure from nutritional deficiency may be mediated by supporting cells. α-Tocopherol (vitamin E) and ascorbic acid (vitamin C) also have critical roles in spermatogenesis, apparently as antioxidants that counteract oxidative damage occurring during sperm development (Ciereszko and Dabrowski, 1995; Kodentsova et al., 1994; Vezina et al., 1996) rather than through direct effects on differentiation or apoptosis.

Thiamin (vitamin B_1) is an essential coenzyme for several enzymes including transketolase, pyruvate dehydrogenase (PDH), α-ketoglutarate dehydrogenase, and branched-chain ketoacid dehydrogenase complexes. Dietary deficiency of

* Corresponding author. Departments of Human Genetics and Pediatrics, Mount Sinai School of Medicine, One Gustave Levy Place, PO Box 1498, New York, NY 10029. Fax: +1-212-849-2508.
E-mail address: george.diaz@mssm.edu (G.A. Diaz).

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thiamin causes beriberi and Wernicke–Korsakoff syndromes. Despite the metabolic demands involved in spermatogenesis and the importance of thiamin in oxidative metabolism, the role of this vitamin in spermatogenesis and male infertility has not been explored.

We and others identified mutations in *SLC19A2*, the gene encoding the high-affinity thiamin carrier, THT1, (Diaz et al., 1999; Fleming et al., 1999; Labay et al., 1999), as the cause of the thiamin-responsive megaloblastic anemia syndrome (TRMA; MIM249270), an autosomal recessive disorder with diabetes mellitus, sensorineural deafness, and megaloblastic anemia (Mandel et al., 1984; Porter et al., 1969; Viana and Carvalho, 1978). To further study the disease pathophysiology, we cloned the orthologous murine gene, *Slc19a2*, and generated a mouse model of TRMA (Oishi et al., 2001, 2002). *Slc19a2* null mice developed diabetes, deafness, and megaloblastosis when maintained on a thiamin-free diet (Oishi et al., 2002). Interestingly, it was additionally noted that while *Slc19a2*$^{-/-}$ females reproduced normally, the males were infertile even when fed with standard mouse chow. This observation suggested that thiamin and/or thiamin transport might play a critical role in spermatogenesis.

To verify this hypothesis and to investigate the molecular basis of male infertility in *Slc19a2*$^{-/-}$ mice, histological and molecular analyses of testes from *Slc19a2*$^{-/-}$ males were performed. These investigations revealed significantly decreased testicular mass and an absence of sperm in *Slc19a2*$^{-/-}$ males. We show here that spermatogenesis in those males was blocked in a stage-specific manner by the induction of apoptosis. This provides the first evidence that thiamin plays a critical role in spermatogenesis and that the male germ cell lineage is uniquely sensitive to impairment of thiamin-dependent metabolic pathways as a trigger for apoptotic cell death.

Materials and methods

Slc19a2 knockout mice

We created *Slc19a2* knockout mice using targeted gene disruption in embryonic stem cells (Oishi et al., 2002). These mice lacked the high-affinity component of thiamin transport as confirmed by radiolabeled thiamin uptake study in erythrocytes. Genotypes were screened by PCR using a primer pair (5′-CTCGTCCTGCAGTTCATTCA-3′ and 5′-AGACAATCGGCTGCTCTGAT-3′) to detect the knockout allele and another set (5′-TTACCTGCTGCTGCTGTTTC-3′ and 5′-GATGGTTAGCTGCTGGGGTA-3′) for the wild-type allele. Mice were maintained with Picolab Mouse Diet 20 (Lab Diet) with thiamin concentration of 16 mg/kg unless specified otherwise. *Slc19a2*$^{-/-}$ and *Slc19a2*$^{+/+}$ mice, aged 12–24 weeks, were used for analyses. All animal experiments followed approved protocols at Mount Sinai School of Medicine.

Histology

For histological examination, testes and epididymes were dissected and fixed with either 4% paraformaldehyde in PBS or Bouin's solution. Paraffin sections (5 μm) of tissues were deparaffinized, rehydrated and stained with periodic acid Schiff (PAS) and hematoxylin. PAS/hematoxylin images were used to identify cell types by appearance and cytological context, as previously described (Russel et al., 1990). For the detection of sperms in epididymes, sections were stained with DAPI (Vector Laboratories Inc.). Immunohistochemistry with an anti-phospho H3 antibody was performed on 4% paraformaldehyde fixed tissues using the ABC Vector Kit (Vector Laboratories). Sections (5 μm) were baked at 56°C overnight on positively charged slides (Fisher), deparaffinized, and refixed in ice-cold acetone for 10 min. Air-dried slides were then rehydrated in 0.1% BSA/PBS and quenched for endogenous peroxidase by treating with 0.1% H_2O_2 in PBS for 15 min at RT and washed in PBS. Antigen retrieval was performed by microwave-treating slides in 10-mM sodium citrate (pH 6.0). Slides were then washed in PBS and treated according to a standard protocol (Vector). Blocking was performed using 10% goat serum in 2% BSA/PBS. Phospho-H3 antibody (Upstate) was used at 5 μg/ml.

Testis-specific gene expression

Total testicular RNAs were extracted using TRIZOL reagent (Invitrogen). To assess gene expression, reverse transcription PCR (RT-PCR) was performed as described for the following genes: *H1t* (accession no. X72805), *Sprm* (L23864), *Ccna1* (X84311), *Cdc25c* (U15562), *Prm1* (K02926), and *β-actin* (X03672) (Honke et al., 2002). RT-PCR reactions were performed with Titan One Tube RT-PCR System (Roche) according to the manufacturer's instructions using 1 μg of total RNA as template. Reactions (30 cycles) were performed at annealing temperatures of 60 °C for *H1t*, *Ccna1*, *Prm1*, and β-*actin* and at 50°C for *Sprm* and *Cdc25c*.

Detection of apoptotic cell death

Terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end-labeling (TUNEL) assay was performed to detect apoptotic germ cells using the In Situ Cell Death Detection Kit (Roche) according to the manufacturer's instructions. In this assay, testes were fixed with paraformaldehyde at 4°C overnight. To identify the cell types that underwent apoptosis, serial sections were prepared and stained either by TUNEL or PAS for comparison.

Thiamin-free diet and high-dose thiamin injection challenge tests in Slc19a2$^{-/-}$ *mice*

Slc19a2$^{-/-}$ males were subjected to thiamin-free diet challenge or high-dose thiamin treatment. For the thiamin-

free diet challenge, mice were maintained for 16 days on the Thiamin Deficient Diet (0 mg/kg of thiamin; ICN Biomedicals, Inc.). For the high-dose thiamin rescue study, daily intraperitoneal injections of thiamin–HCl (1 mg/day) were administered for 10 and 20 days while the mice were fed standard chow. After completion of these challenges, testes were dissected and analyzed histologically.

In situ hybridization

Paraffin sections (5 μm) of adult wild-type testis were used for in situ hybridization. Mouse *Slc19a2* cDNA (nt 240–739 in exon 2) was PCR amplified using a primer pair (5′-TTACCTGCTGCTGCTGTTTC-3′ and 5′-GATGGTTAGCTGCTGGGGTA-3′) and then cloned into pCRII-TOPO vector (Invitrogen). Digoxigenin (DIG)-labeled probes were synthesized using DIG RNA Labeling Kit (SP6/T7) (Roche) according to the manufacturer's instruction. Hybridization was performed as described previously by (Braissant and Wahli, 1998). DIG signal was detected by DIG Nucleic Acid Detection Kit (Roche). To identify the cell types that showed *Slc19a2* expression, serial sections were prepared and were subjected to either in situ hybridization or PAS staining for comparison.

Statistics

Statistical significance was calculated using t test (significant threshold: $P < 0.05$). Values are given as mean ± SEM.

Results

Analysis of Slc19a2$^{-/-}$ mice

Using targeted gene disruption through homologous recombination in embryonic stem cells, we generated the *Slc19a2*$^{-/-}$ mice, as previously described (Oishi et al., 2002). These mice showed diabetes mellitus, sensorineural deafness, and megaloblastosis in their bone marrow when fed on thiamin-deficient diet. With standard chow, they did not present any TRMA symptoms and appeared to develop normally. While expanding the colony, it became apparent that *Slc19a2*$^{-/-}$ females reproduced normally but males were infertile. This infertility was present in males that were fed standard chow and observed over a period of 6 months. The sexual behavior of the *Slc19a2*$^{-/-}$ males was normal as assessed by the observation of normal mating behavior and copulatory plugs.

Despite normal sexual behavior, testes from *Slc19a2*$^{-/-}$ males were smaller than their wild-type littermates, suggesting an intrinsic defect in spermatogenesis. The average testis weight of the *Slc19a2*$^{-/-}$ males (36.3 ± 1.5 mg; n= 16) was approximately half that of wild type males at 12 weeks of age (72.5 ± 4.0 mg, n= 10; $P < 0.00001$) (Fig. 1). To

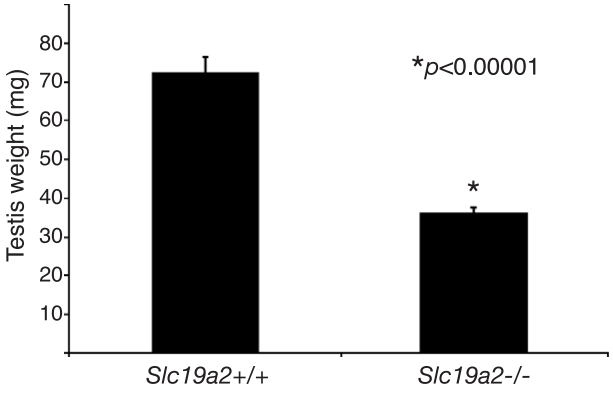

Fig. 1. Weights of *Slc19a2*$^{+/+}$ and *Slc19a2*$^{-/-}$ testes.

assess the basis of the infertility, seminal fluid was expressed from epididymes. In contrast to the wild-type males, no sperm was observed in seminal fluid obtained from *Slc19a2*$^{-/-}$ males (data not shown). Aspermia was confirmed by histological test of epididymes. As seen in Fig. 2, nuclear DAPI staining reveals abundant sperm heads in the epididymal lumen of *Slc19a2*$^{+/+}$ males, but luminal staining is absent from the *Slc19a2*$^{-/-}$ mice.

Histology of Slc19a2$^{-/-}$ testis

PAS-stained sections were used to characterize the progression of spermatogenesis in wild type and *Slc19a2*$^{-/-}$ testes. As shown in low- and high-power light micrographs of PAS-stained tissues, analysis of the *Slc19a2*$^{-/-}$ testes revealed a complete disruption of spermatogenesis (Fig. 2). Leydig cells and Sertoli cells in seminiferous tubules appeared normal in structure and number but there was a marked decrease in the cellularity of germ cells, particularly at the adluminal side of the tubules. No mature sperm was seen in tubules at any of the developmental stages. Specifically, cells at metaphase I (MI), metaphase II (MII), or spermatids were never observed in the *Slc19a2*$^{-/-}$ testes. The number of middle pachytene spermatocytes was dramatically decreased and diplotene spermatocytes were rarely seen, whereas the numbers of spermatogonia and early primary spermatocytes were normal. Thus, from a histological perspective, an arrest in spermatogenesis appeared to have occurred at the pachytene stage before the first meiotic division.

Testis stage-specific gene expression

To verify the stage of spermatogenesis arrest, we performed gene expression analysis using RT-PCR (Fig. 3). In this assay, we employed *H1t*, *Sprm1*, *Ccna1*, *Cdc25c*, and *Prm1*, which encode histone H1t, POU-domain transcription factor, cyclin A1, M-phase inducer phosphatase 3, and protamine 1, respectively (Andersen et al., 1993; Kleene et al., 1984; Kremer and Kistler, 1991; Ravnik and Wolgemuth, 1999; Wu and Wolgemuth, 1995). The window of

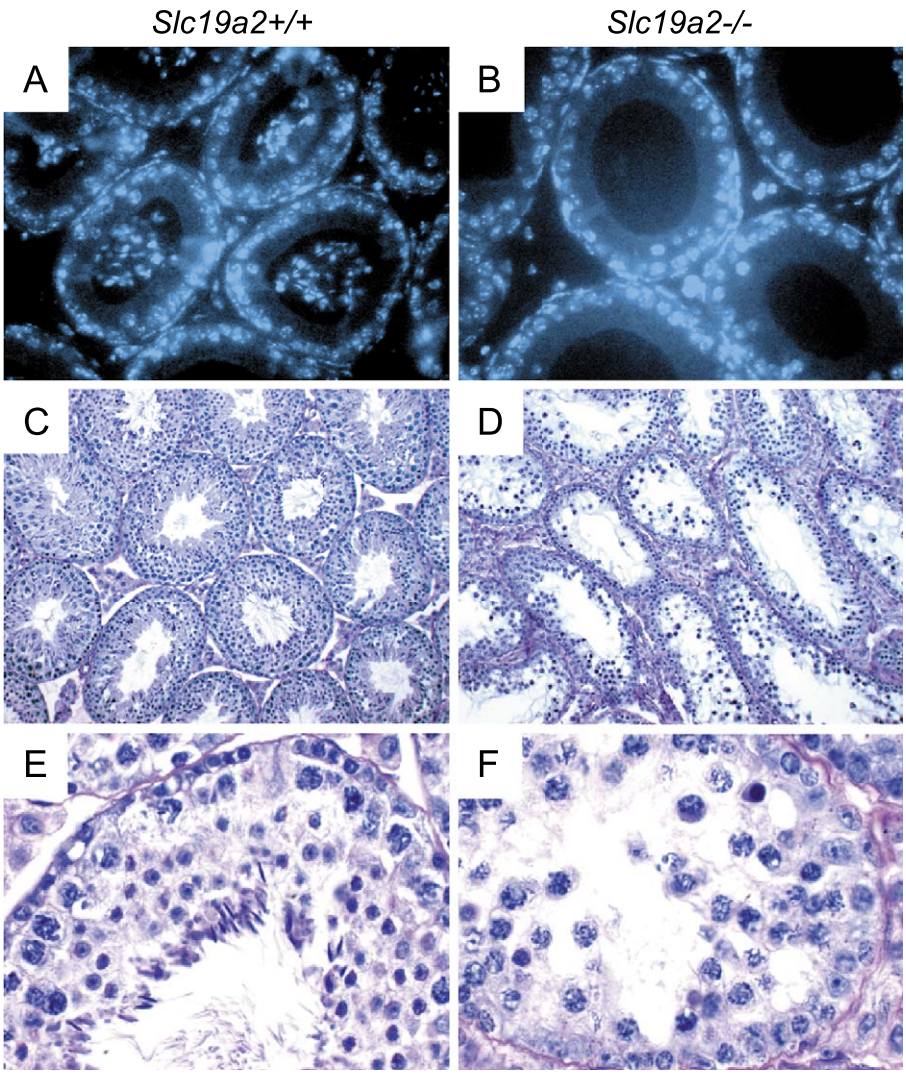

Fig. 2. Abnormal spermatogenesis in *Slc19a2*$^{-/-}$ mice. (A, B) Cross-sections of DAPI-stained epididymes from 12-week-old *Slc19a2*$^{-/-}$ and *Slc19a2*$^{+/+}$ mice revealed aspermia in *Slc19a2*$^{-/-}$ males (original magnification 600 ×). (C, D) PAS-stained testes exhibited arrest of spermatogenesis with profound loss of germ cells in the seminiferous tubules compared to controls (magnification 200 ×). (E, F) Post-meiotic haploid cells were never observed in the sections from *Slc19a2*$^{-/-}$ testes (magnification 600 ×).

expression for each of these genes during spermatogenesis has been well documented in previous studies. *H1t* is expressed in mid- to late-pachytene spermatocytes. *Sprm1* is transcribed transiently just before the first meiotic division from late pachytene to diplotene spermatocytes. *Ccna1* message is observed just before or during the first meiotic division. *Cdc25c* transcript, which is first expressed in late pachytene diplotene stages, is highest in round spermatids. *Prm1* is a haploid-specific gene whose expression is high in round spermatids. In the *Slc19a2*$^{-/-}$ testes, the expression levels of early stage-specific genes, *H1t* and *Sprm1*, were the same as observed in the wild type and *Slc19a2*$^{+/-}$ testes, while the expression of *Ccna1* and *Cdc25c*, which are expressed from the pachytene stage onwards, was detected at lower levels. Expression of the haploid-specific gene *Prm1* was not detectable in *Slc19a2*$^{-/-}$ testes. The results of the gene expression analysis were thus consistent with an arrest in spermatogenesis occurring during the late pachytene or diplotene stages.

Phospho-histone-H3 staining

To further characterize the stage of spermatogenesis arrest and to examine the proliferative activity of mitotic cells, we performed immunostaining with anti-phospho-H3 antibody. Phosphorylation of histone H3 on Serine 10 occurs in mitotic cells during chromosome condensation (Hendzel et al., 1997), in meiosis at the late pachytene-diplotene stage, when synapsed homologous chromosomes are separating, and at the MI stage (Cobb et al., 1999). Serial sections from wild type and *Slc19a2*$^{-/-}$ testes were stained with PAS and anti-phospho-H3 antibody and compared. In wild type mice, H3 phosphorylation was specifically observed in meiotic cells in stage XI diplotene spermatocytes

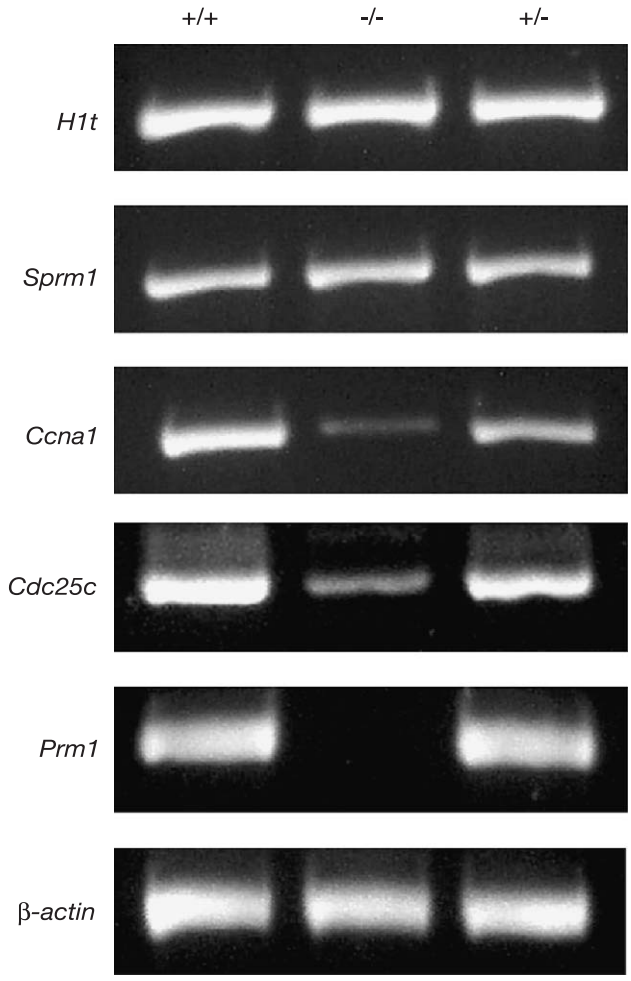

Fig. 3. Stage-specific gene expression in *Slc19a2*$^{-/-}$ testes. RT-PCR analyses of stage-specific genes. For genes expressed in early developmental stages, *H1t* and *Sprm1*, no difference was detected between *Slc19a2*$^{+/+}$, *Slc19a2*$^{+/-}$, and *Slc19a2*$^{-/-}$ testes, while *Ccna1* and *Cdc25c* expression, which are expressed from the pachytene stage onwards, was reduced in *Slc19a2*$^{-/-}$. Expression of the haploid-specific gene *Prm1* was not detectable in *Slc19a2*$^{-/-}$ testes. Experiments were repeated a minimum of three times.

(Fig. 4A) and in spermatogonia before stage XI (data not shown). In *Slc19a2*$^{-/-}$ testis, many H3 positive spermatogonia were observed (Fig. 4B), while rare H3-positive "diplotene-like" and no MI cells were found, demonstrating that the arrest in spermatogenesis occurred during the pachytene stage in *Slc19a2*$^{-/-}$ testis.

Mechanism of spermatogenic block

Since there were decreased numbers of germ cells in the testes of *Slc19a2*$^{-/-}$ males, we considered the possibility that loss of these cells might involve apoptosis. To test this hypothesis, we performed TUNEL assays on sections of *Slc19a2*$^{-/-}$ testes. In agreement with our hypothesis, there were many TUNEL positive cells in the *Slc19a2*$^{-/-}$ testes, while only a few positive spermatogonia were seen in the wild type control (Figs. 5A–D). In the *Slc19a2*$^{-/-}$ testes, apoptotic cells were observed only in the tubules, not in the interstitial area. Morphologically, the affected cells appeared to be germ cells. The distribution of apoptotic cells was not random, with clusters of positive cells observed in a subset of approximately 10–20% of the seminiferous tubules in the sections. To verify the identity of the cell types that were undergoing apoptosis, we examined serial sections stained with TUNEL and PAS (Figs. 5E, F). This analysis revealed that spermatogonia and early meiotic cells at the base of the tubules (preleptotene to zygotene stages) were not TUNEL positive, whereas early- and mid-pachytene spermatocytes were dying by apoptosis. These results suggested that the apoptosis observed in the *Slc19a2*$^{-/-}$ testes occurred in a stage-specific fashion.

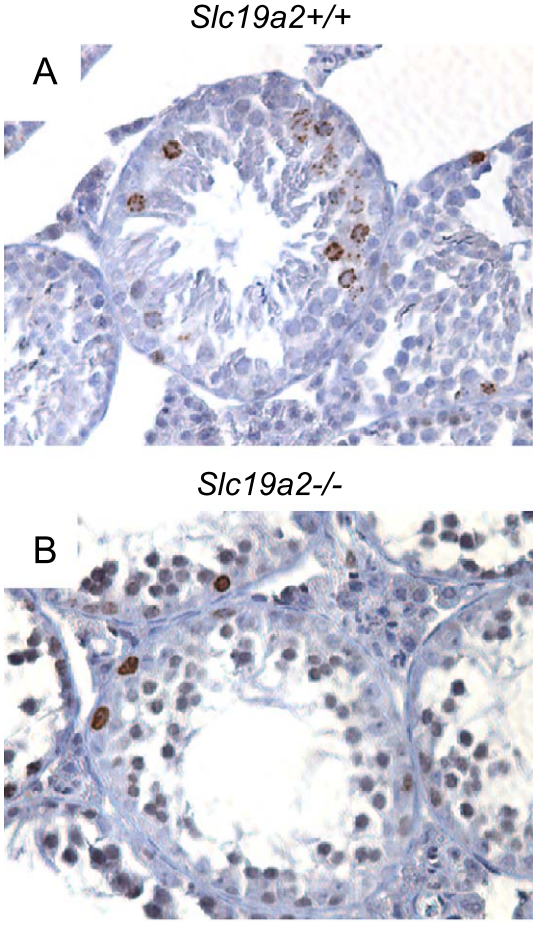

Fig. 4. Immunostaining with anti-phospho-H3 antibody. Immunostaining against phospho-H3 was performed in testis sections from *Slc19a2*$^{+/+}$ and *Slc19a2*$^{-/-}$ mice (magnification 400×). (A) Section of stage XI tubule from *Slc19a2*$^{+/+}$ testis showed diplotene (Di) spermatocytes with the characteristic "speckled" staining at the centromeric heterochromatin located near the nuclear envelope. (B) Testis section from *Slc19a2*$^{-/-}$ mouse showed normal proliferating spermatogonia with H3 phosphorylation, which were observed in the basal area of the seminiferous tubule, but no proliferating diplotene-stage cells were present.

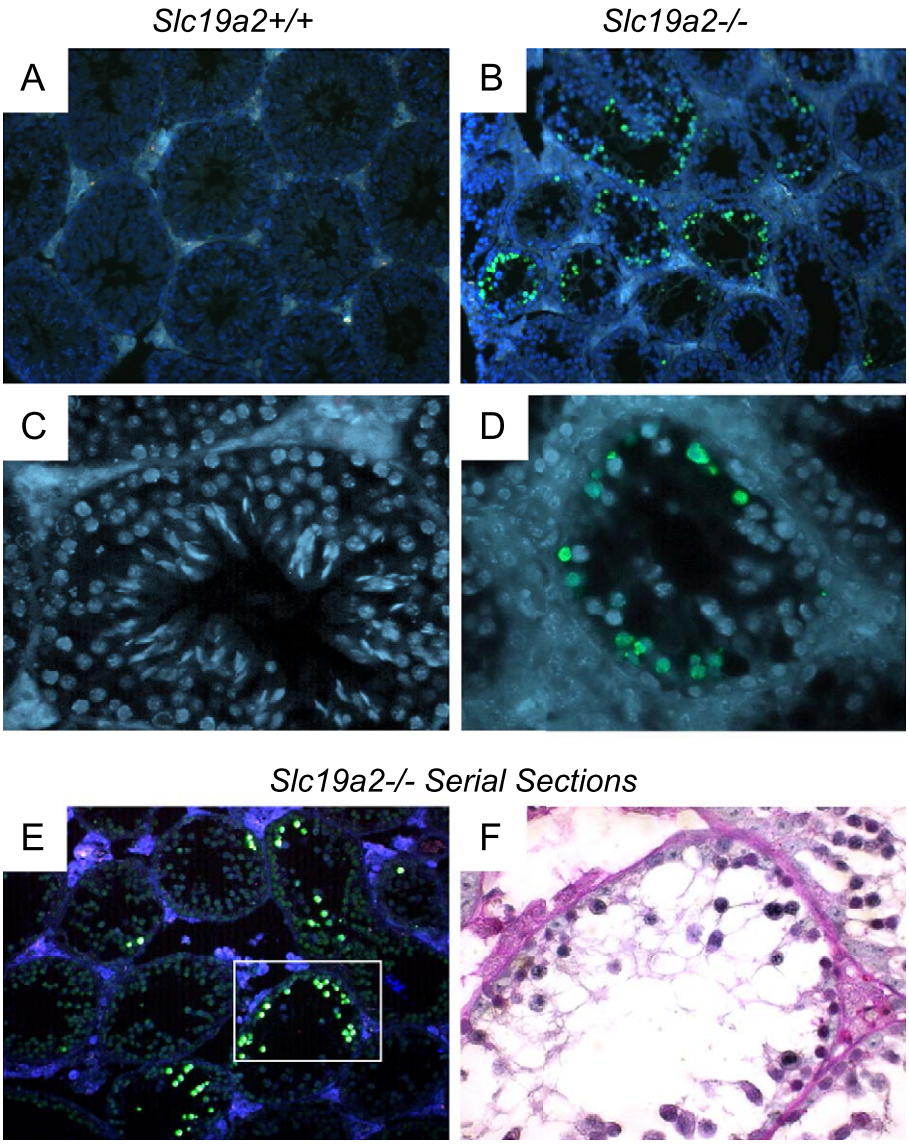

Fig. 5. Loss of high-affinity thiamin transport induces apoptotic cell death in spermatocytes. In situ TUNEL staining of *Slc19a2*$^{+/+}$ and *Slc19a2*$^{-/-}$ testes. TUNEL-positive cells are stained in green. (A, B) Occasional TUNEL-positive spermatogonia were seen in the wild type testes, but abundant positive cells were observed in the *Slc19a2*$^{-/-}$ testes (magnification 200 ×). (C, D) Apoptotic cells were observed only in the tubules of *Slc19a2*$^{-/-}$ testes towards the adluminal surface, not in the interstitial area (magnification 600 ×). (E, F) Serial sections from *Slc19a2*$^{-/-}$ testis with TUNEL and PAS staining showed that the distribution of apoptotic cells was restricted to pachytene cells (magnification E: 400 × , F: 600 ×). E inset highlights the area shown as PAS stained serial section in F.

Effects of thiamin deficiency and high-dose thiamin injection

We challenged *Slc19a2*$^{-/-}$ mice with thiamin-free diet for 16 days and assessed the effect on the germ cell population. At the end of the challenge period, plasma thiamin concentration was significantly decreased and the TRMA phenotype appeared, as previously described (Oishi et al., 2002). The histology of testes from wild type animals maintained on thiamin-deficient diet revealed no apparent effects on the cellularity of seminiferous tubules (data not shown). In contrast, testes from thiamin-depleted *Slc19a2*$^{-/-}$ males demonstrated massive destruction of germ cells (Fig. 6B). In addition to the loss of late spermatocytes, the spermatogonia and early spermatocytes that were unaffected under normal dietary conditions were almost completely absent under thiamin-deficient condition. Interestingly, Leydig cells and Sertoli cells in null animals were resistant to thiamin deficiency and the integrity of the seminiferous tubule basement membranes appeared to be maintained.

These results suggested that the male germ cell lineage was particularly dependent on thiamin status for survival, but that cells beyond the BTB were particularly sensitive. To test the hypothesis that higher plasma thiamin levels might increase intracellular levels by diffusion-mediated transport and rescue spermatogenesis, we administered daily intraper-

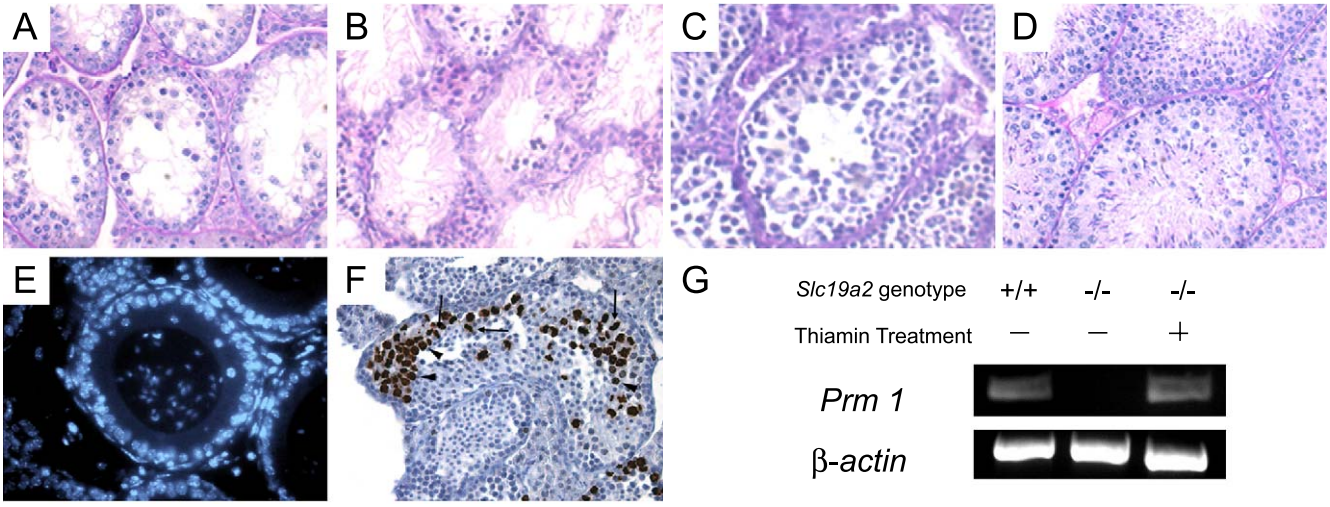

Fig. 6. Effects of thiamin deficiency and high-dose thiamin injection on spermatogenesis in *Slc19a2*$^{-/-}$ mice. (A–D) PAS-stained sections from *Slc19a2*$^{-/-}$ testes at 200 × original magnification. (A) Testis section from mouse maintained on standard chow. (B) Testis section from mouse challenged with thiamin-free diet for 16 days. Thiamin depletion causes massive loss of germ cells in *Slc19a2*$^{-/-}$, including spermatogonia and early spermatocytes. (C) The block in spermatogenesis observed under standard diet is reversed with the administration of intraperitoneal (i.p.) high-dose thiamin (1 mg/day). After 10 days of i.p. thiamin, the tubule histology normalized significantly, with increased number of germ cells in seminiferous tubules and differentiation of primary spermatocytes into secondary spermatocytes and round spermatids. (D) After 20 days of i.p. thiamin, spermatogenesis has recovered completely, with the appearance of all developmental cell types. (E) DAPI staining of *Slc19a2*$^{-/-}$ epididymis sections confirmed the presence of mature sperm in the lumen (magnification 600 ×). (F) Anti-phospho-H3 immunostaining in sections from mice treated with thiamin for ten days showed many diplotene (arrowheads) and MI (arrows) cells (magnification 400 ×). (G) RT-PCR analysis revealed equivalent *Prm1* expression in testes from wild type and *Slc19a2*$^{-/-}$ mice treated for ten days, whereas untreated *Slc19a2*$^{-/-}$ males showed no expression.

itoneal injection of thiamin–HCl (1 mg/day) to 12-week old *Slc19a2*$^{-/-}$ males. After 10 days of treatment, significant normalization of tubule histology was appreciated with increased numbers of germ cells observed in seminiferous tubules (Figs. 6C, F). In addition to increased cellularity, differentiation of primary spermatocytes into secondary

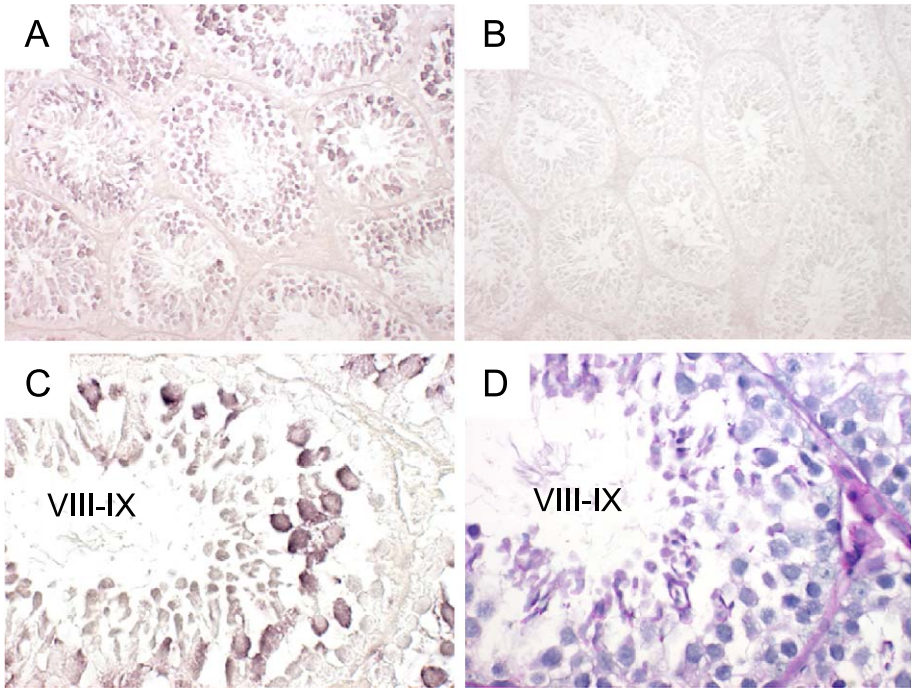

Fig. 7. Localization of mRNA for Slc19a2 in of adult wild type testes by in situ hybridization. (A, C) High levels of signal were observed in spermatogenic cells from the mid-pachytene stage to the round spermatid stage, with the highest expression in late pachytene spermatocytes in stage VIII–XI tubules. (C, D) Serial sections of stage VIII–IX tubule with in situ hybridization and PAS showed the highest signal in late pachytene spermatocytes. (B) Hybridization with sense probe exhibited no signal.

spermatocytes and round spermatids (stages VII–VIII) and staining of acrosomes by PAS was observed, but mature sperm was not seen in any tubules. After 20 days of treatment, the testicular mass of treated *Slc19a2*$^{-/-}$ mice was significantly greater than that of untreated mice and equivalent to wild type (81.7 ± 7.2 mg; n= 6; $P < 0.002$). Although histologic remnants of the preexisting pathology were observed in occasional tubules, the block in spermatogenesis was overcome, with all developmental cell types present (Fig. 6D). Mature sperm production was confirmed both in epididymis and testis (Fig. 6E). As expected from the histological observation, *Prm1* expression in testes from mice treated with thiamin for 10 days was identical to wild type controls (Fig. 6G). The reestablishment of spermatogenesis was accompanied by the reduction of apoptosis to levels equivalent to those observed in control testes as assessed by TUNEL staining (data not shown).

To investigate whether fertility of the thiamin-treated *Slc19a2*$^{-/-}$ males was restored, we mated two *Slc19a2*$^{-/-}$ males after 20 days of high-dose thiamin injection with non-thiamin-treated *Slc19a2*$^{-/-}$ females. Over a period of 3 months of observation, females experienced multiple pregnancies and deliveries from each mated male, confirming that sperm from the treated males was completely functional.

Slc19a2 gene expression in testis

To detect and localize the *Slc19a2* mRNA message in mouse testis, we performed in situ hybridization using adult wild type testis sections. Overall, high expression levels were detected in a subpopulation of cells in the seminiferous tubules, while the level of mRNA expression in interstitial cells was low (Fig. 7). PAS staining of serial sections confirmed that the expression of *Slc19a2* is developmentally regulated during spermatogenesis. The upregulation of *Slc19a2* gene was initiated at the mid-pachytene stage and persisted to the round spermatids stage, with the highest expression observed in late pachytene spermatocytes in stage VIII–XI tubules. The signal was low in Sertoli cells as well as spermatogonia, preloptotene, leptotene, zygotene, pachytene spermatocytes present in stage I–IV tubules, and in elongated spermatids.

Discussion

In this report, we describe male-specific infertility in mice lacking the high-affinity thiamin transporter, Tht1, due to the induction of apoptosis in pachytene-stage spermatogenic precursors. In contrast to the germ cells, the supporting Sertoli and Leydig cells appeared to be resistant to thiamin deficiency. The dependence of spermatogenic precursors on Tht1 for survival is striking in light of the fact that the tissues classically affected in TRMA showed no phenotype under these dietary thiamin conditions. Plasma thiamin concentration in animals fed standard mouse chow reaches > 600 nM (Oishi et al., 2002) and is adequate for intracellular thiamin in most cell types through non-saturable diffusion. We hypothesize that the sensitivity of spermatogenic cells under conditions of normal thiamin intake derives from several interacting factors including the metabolic demands of spermatogenic precursors, the presence of the blood–testis barrier (BTB), and the relative balance of pro- and anti-apoptotic factors in male germ cells.

There are several developmental changes associated with spermatogenesis that are potentially relevant to the Tht1 requirement observed in germ cells. Spermatogonia close to the seminiferous tubule basement membrane divide and migrate towards the tubule lumen along the surface of supporting Sertoli cells as they differentiate. Upon traversing the BTB at the preleptotene to leptotene stage, spermatocytes are completely dependent on cell–cell communication with Sertoli cells for nutritional support and molecular signaling. Germ cells beyond the BTB no longer have free access to glucose and the transition to this environment is accompanied by changes in germ cell carbohydrate metabolism (Bajpai et al., 1998). Unlike spermatogonia and spermatids, which utilize glucose or glucose/fructose as a principal source of energy, spermatocytes preferentially utilize lactate/pyruvate (Jutte et al., 1981; Mita and Hall, 1982; Nakamura et al., 1984a,b). The change in energy substrates coincides with the transcriptional inactivation of the *X*–*Y* chromosome pair and the expression of autosomal isoforms of proteins involved in energy metabolism, including phosphoglucokinase, cytochrome *c*, lactate dehydrogenase subunit C, and the E1α subunit of pyruvate dehydrogenase (PDH) (Goldberg et al., 1977; Iannello and Dahl, 1992; McCarrey et al., 1992; Wheat et al., 1977). Changes related to the expression pattern of PDH are of particular significance, as this enzyme requires thiamin pyrophosphate as a cofactor.

The upregulation of the testis-specific autosomal gene encoding E1α2, *Pdha*-2, is initiated at the leptotene stage and high expression levels are observed at the pachytene stage (Iannello and Dahl, 1992). The switch in expression pattern occurs in the context of a general increase in PDH protein levels at the pachytene stage, corresponding to the point at which apoptosis is initiated in *Slc19a2*$^{-/-}$ germ cells. Interestingly, the upregulation of *Slc19a2* in late pachytene spermatocytes mirrors the expression pattern of *Pdha*-2. E1α binds to thiamin pyrophosphate at two catalytic sites in the holoenzyme (Ciszak et al., 2003). The requirement for high-affinity thiamin transport might therefore reflect increased cellular requirement reflecting the increased abundance of the apoenzyme or decreased affinity of the *Pdha*-2-encoded subunit for thiamin pyrophosphate secondary to variations in the coding sequence. This latter possibility is not supported by studies of kinetic parameters for thiamin pyrophosphate interactions with the two PDH isoforms (Patel, personal communication). The change in energy substrate makes the proper function of PDH critical

for the production of acetyl-CoA and efficient energy production by the TCA cycle. Lack of adequate intracellular thiamin secondary to loss of Slc19a2 would thus be expected to impair PDH activity and cellular energy metabolism, conceivably triggering spermatogenesis arrest at this stage. Interestingly, antisense inhibition of the E1α subunit in the anther tapetum of the sugar beet caused male-specific sterility (Yui et al., 2003), highlighting the role of PDH in male germ cell development in highly divergent species.

In addition to its effect on the energy substrates available to developing germ cells, the BTB is likely to have an effect on the availability of thiamin. Little is known about thiamin exchange across the BTB but a significant barrier to diffusion has been demonstrated in the case of methotrexate, a small molecule using a transporter homologous to Tht1 (Goldman and Matherly, 1985; Riccardi et al., 1982). The induction of *Slc19a2* expression in spermatogenic cells that have crossed the BTB raises the possibility that expression of the transporter is required to compensate for low thiamin concentration in the intraluminal fluid. Thus, it seems reasonable to speculate that inefficient thiamin diffusion across the BTB may contribute to the spermatogenic failure of *Slc19a2*$^{-/-}$ mice. Interestingly, the central nervous system is not affected to the same extent as spermatogenic cells, despite a high metabolic requirement and evidence that thiamin transport across the blood–brain barrier (BBB) is also carrier-mediated (Greenwood et al., 1982; Spector, 1976). This difference may reflect the involvement of alternative thiamin transporters in the BBB or a different intrinsic susceptibility to thiamin deficiency-induced apoptosis.

We propose a model of male infertility in the *Slc19a2*$^{-/-}$ mice that centers on the relationship between the thiamin metabolic demand of cells and the role of the BTB in excluding thiamin from germ cells in the adluminal compartment of the tubule. In the *Slc19a2*$^{-/-}$ testis, germ cells up to leptotene-stage spermatocytes have access to thiamin available by interstitial-capillary diffusion, although they have low expression of high-affinity thiamin transporter and can maintain adequate energy homeostasis at plasma thiamin concentrations maintained with standard mouse chow. More differentiated germ cells beyond the BTB without Tht1 are dependent on the thiamin diffused through BTB and Sertoli cells and are thus susceptible to decreased adluminal thiamin concentration, a situation compounded by the reliance of pachytene spermatocytes on thiamin-dependent pyruvate dehydrogenase for ATP synthesis. Injection of high-dose thiamin serves to increase the adluminal thiamin concentration by passive diffusion, allowing resumption of spermatogenesis.

The precise trigger for apoptosis in the susceptible germ cells is not yet known. In contrast to the spermatogenic failure observed with vitamin A deficiency, the loss of high-affinity thiamin transport is unlikely to perturb differentiation signals but rather to expose the susceptibility of germ cells to mitochondrial insult. While we cannot rule out a mechanism whereby Sertoli cells initiate apoptosis in germ cells in response to metabolic stress, current models suggest that maintenance of germ cell population size through apoptosis is operative at the level of spermatogonia (Rodriguez et al., 1997), a population relatively resistant to apoptosis under standard thiamin intake. In addition to mitochondrial dysfunction resulting from the block in oxidative phosphorylation, impairment of transketolase function may lead to dysfunction of the pentose phosphate pathway resulting in oxidative stress (Calingasan et al., 1999; Langlais et al., 1997) and impaired DNA synthesis secondary to inefficient ribose production (Boros et al., 1998). Both of these processes are candidates in the pathway leading to spermatogenic failure in *Slc19a2*$^{-/-}$ mice. Although our studies focused on mutant animals, extended (30 days) dietary thiamin deficiency has been reported to lead to aspermia and decreased numbers of spermatocytes and spermatogonia in wild-type rats (Onodera et al., 1980), suggesting that this mechanism is not restricted to organisms lacking high-affinity thiamin transport.

One of the most intriguing questions arising from this study is whether humans with TRMA or dietary thiamin deficiency conditions have impaired spermatogenesis. Up to now, male infertility has not been reported in TRMA. In an adolescent male with TRMA, testicular size and development was age-appropriate (Shalata, personal communication), but the significance of this observation is uncertain as patients are maintained on high-dose thiamin from diagnosis, which is generally in early childhood. Nonetheless, mice have a higher daily thiamin requirement than humans due to a higher energetic demand increase per unit weight, raising the possibility that murine germ cells may be intrinsically more sensitive to thiamin. To our knowledge, the effect of thiamin deficiency on spermatogenesis in humans has not been reported. Should human germ cells prove as sensitive as murine germ cells, it may be reasonable to assess the contribution of subclinical dietary thiamin deficiency to male infertility.

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