CELL INJURY, REPAIR, AGING, AND APOPTOSIS

The Onset of Heat-Induced Testicular Calcification in Mice *Involvement of the Telomeric Locus on Chromosome 1*

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Spermatocytes of MRL/MpJ mice are more heat resistant than those of C57BL/6 mice in experimental cryptorchidism. This phenotype depends in part on the locus at the 81-cM region of MRL/MpJ-type chromosome 1 (Chr 1). To evaluate the function of this locus, we examined pathological changes in mouse testes resulting from transient scrotal heat stress. Immediately after scrotal heat stress, meiosis progression and blood–testis barrier integrity were preserved in MRL/MpJ but not in C57BL/6 mice, nor in a C57BL/6-based congenic strain carrying the MRL/MpJ-derived Chr 1 locus (B6.MRLc1). Testicular damage was severe in the weeks after scrotal heat stress in all three strains; however, testicular calcification was observed only in C57BL/6 and MRL/MpJ mice (initially as nanocrystals in mitochondria of degenerating germ cells). In testes, expression of gremlin 2, a bone morphogenetic protein antagonist encoded on Chr 1, was markedly higher in B6.MRLc1 than in C57BL/6 or MRL/MpJ mice. Furthermore, gremlin-2 and bone morphogenetic protein 2 mRNA levels in heated testes correlated negatively and positively, respectively, with calcification. Thus, although the MRL/MpJ-derived locus on Chr 1 may play a pivotal role in recovery from heat-induced testicular damage, especially via inhibition of calcification, MRL/MpJ mice have a precipitating factor for testicular calcification and heat shock–resistant factors that reside outside the 81-cM region of Chr 1. (*Am J Pathol* 2014; 184: 2480–2492; <http://dx.doi.org/10.1016/j.ajpath.2014.06.004>)

Male germ cells, or sperms, are produced in the cyclic and complex process of spermatogenesis, which occurs in seminiferous tubules in the testes. Mammalian spermatogenesis can be divided mainly into three phases: mitotic proliferation of stem spermatogonia, meiotic differentiation of spermatocytes, and transformation of spermatids into spermatozoa. The differentiation of germ cells is supported, nurtured, and supervised by somatic Sertoli cells, which line seminiferous tubules. Sertoli cells maintain the integrity of spermatogenesis by forming the blood–testis barrier (BTB), which divides the seminiferous epithelium into adluminal and basal compartments.^{1,2} Testes in most mammals are located in the scrotum, outside the main abdominal cavity. In addition, the temperature within the testes is regulated by a specialized blood supply that acts as a heat-exchange system. Thus, scrotal temperature is kept lower than the core body temperature.³ Disruption of this system has an adverse effect on spermatogenesis in several species, including humans.^{4,5}

Many studies have shown that the exposure of testes to a high-temperature environment induces serious damage to the seminiferous epithelium, resulting in germ-cell loss by apoptosis and alterations in Sertoli cell morphology and function.^{6–9} The cells most sensitive to testicular heat stress are spermatocytes (leptotene, zygotene, and pachytene) and round spermatids.^{10,11} Moreover, testicular heat stress impairs biosynthesis of protein, DNA, and RNA,¹² damages spermatocyte DNA, and disrupts chromatin packing in sperm.^{5,13} Testicular hyperthermia is also reported to disrupt the BTB.^{14,15} The molecular mechanisms responsible for the degenerative changes in heat-induced testicular dysfunction remain largely unknown.

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We have previously reported several unique characteristics of the MRL/MpJ (MRL) mouse testis, such as the appearance of testicular oocytes,¹⁶ smaller testis size than in other inbred strains,¹⁷ metaphase-specific apoptosis of meiotic spermatocytes,^{18–21} and heat shock-resistant spermatocytes.^{22,23} In addition, MRL mice exhibit regenerative wound healing, such as closure of ear punches and cardiomyocyte regeneration.^{24,25} These phenotypes depend on the MRL genetic background derived from C57BL/6 (B6), C3H/He, AKR/J, and LG/J mice.²⁶ Previously, we identified the locus associated with the metaphase-specific apoptosis of meiotic spermatocytes on the telomeric region of MRL-type chromosome 1 (Chr 1).¹⁸ Furthermore, we reported a mutation in *Exo1* (Chr 1, 81.90 cM), which encodes a protein important for DNA repair, as a potential cause of the metaphase-specific apoptosis of meiotic spermatocytes in MRL.^{17,18,27} Interestingly, using quantitative trait locus analysis of experimental cryptorchidism, we also found that the 81-cM region of MRL-type Chr 1 contains the locus responsible for heat shock-resistant spermatocytes.²⁸ Thus, the telomeric region of MRL-type Chr 1 has a significant effect on the phenotype of male gonads and is associated with the survival and death of germ cells.

In the present study, we investigated the roles of the telomeric region of MRL-type Chr 1 by comparing the pathological features of the testes at different time points after induction of transient scrotal heat stress (SHS) in B6 mice, MRL mice, and a B6-based congenic strain carrying the telomeric region of MRL-type Chr 1. We focused on the integrity of spermatogenesis and the BTB. Although the short-term testicular dysfunction induced by SHS was milder in MRL mice than in B6 mice or the congenic mice, the incidence of dystrophic calcification in testes at later time points after SHS induction was minimal in the congenic mice but high in B6 and MRL mice. These data suggest that the genes encoded on the telomeric region of murine Chr 1 regulate recovery from the heat-induced testicular damage that results in calcification. Furthermore, MRL/MpJ mice may have a precipitating factor for testicular calcification that resides outside the 81-cM region of Chr 1.

Materials and Methods

Animals

The B6-background congenic mouse strain B6.MRL-(D1Mit202–D1Mit403) carrying the telomeric region of MRL-type Chr 1 (67.97 to 81.63 cM) (B6.MRLc1) was generated in our laboratory.¹⁷ A/J, AKR/N, BALB/c, C3H/He, B6, ddY, ICR, MRL, WBB6F1-+/+, and WBB6F1-W/W^v mice were purchased (Japan SLC, Hamamatsu, Japan). Each mouse was maintained under specific pathogen-free conditions. Care and handling of experimental animals adhered to the Guide for the Care and Use of Laboratory Animals of Hokkaido University, Graduate School of Veterinary Medicine (approved by the Association for Assessment and Accreditation of Laboratory Animal Care International).

Heat Treatment

To examine the effects of heat stress on mouse testes, 12-week-old male mice were subjected to a single SHS of 43°C for 20 minutes. In brief, after anesthesia with intraperitoneal injection of 40 mg/kg sodium pentobarbital dissolved in 0.01 mol/L phosphate-buffered saline, the hindlimbs, tail, and scrotum containing the testes were immersed in a thermostatically controlled water bath (43°C). Untreated mice were used as controls. After 20 minutes, each animal was dried and returned to its cage. Mice were sacrificed at 24 to 72 hours or at 10 to 110 days after SHS induction to assess the short-term and long-term effects on testes.

Immunohistochemistry

Mouse testes were fixed with 4% paraformaldehyde in 0.1 mol/L phosphate buffer perfusion and embedded in paraffin. Sections (2 µm thick) were deparaffinized and hydrated. For immunohistochemistry, sections were incubated for 15 minutes at 105°C in buffered citrate (pH 6.0) for antigen retrieval of the following proteins: stimulated by retinoic acid gene 8 (STRA8), meiotic recombination protein DMC1/LIM15 homolog (DMC1), synaptonemal complex protein 3 (SCP-3, alias SYCP3; encoded by *Sycp3*) transcription factor, and gremlin-2 (GREM2). Samples were treated with methanol containing 0.3% H₂O₂ to eliminate endogenous peroxidase. After blocking with normal serum, sections were incubated with rabbit anti-STRA8 (dilution 1:2000; Abcam, Cambridge, UK), goat anti-DMC1 (dilution 1:500; Santa Cruz Biotechnology, Dallas, TX), rabbit anti-SCP-3 (dilution 1:800; Novus Biologicals, Littleton, CO), rabbit anti-single-stranded DNA (ssDNA) (dilution 1:200; IBL Immuno-Biological Laboratories, Fujioka, Japan), rabbit anti-SOX9 (dilution 1:2000; EMD Millipore, Billerica, MA), and anti-rabbit GREM2 (dilution 1:100; Proteintech Group, Chicago, IL) at 4°C overnight. Next, the sections were treated with biotin-conjugated goat anti-rabbit IgG antibodies (SABPO kit; Nichirei Biosciences, Tokyo, Japan) or with biotin-conjugated donkey anti-goat IgG antibodies (dilution 1:100; Santa Cruz Biotechnology) for 30 minutes at room temperature and then with streptavidin–biotin complex (SABPO kit; Nichirei Biosciences) for 30 minutes at room temperature. The sections were first incubated with 3,3'-diaminobenzidine tetrahydrochloride solution containing 0.006% H₂O₂ until the stain developed and then were counterstained with hematoxylin. For histometrical analyses, digital images of each section were acquired with a BZ-9000 microscope (Keyence, Osaka, Japan). Immunopositive cells were counted with BZ-II Analyzer software (Keyence).

BTB Integrity Assay

The permeability of the BTB was evaluated using Evans Blue dye as described previously.²⁹ In brief, Evans Blue dye (960.82 Da; Sigma-Aldrich, St. Louis, MO) was dissolved

in phosphate-buffered saline and intraperitoneally injected into mice at 25 mg/kg at 24 hours before sampling. Testes were fixed by 4% paraformaldehyde perfusion and embedded in paraffin. For immunofluorescence analysis, antigen retrieval was performed for 15 minutes at 105°C with Dako (Carpinteria, CA) target retrieval solution. Sections (5 μm thick) were incubated with normal donkey serum and then with rabbit anti-occludin (OCLN) (dilution 1:100; Life Technologies, Carlsbad, CA) at 4°C overnight. The sections were washed with phosphate-buffered saline and incubated with fluorescein isothiocyanate–conjugated donkey anti-rabbit IgG for 30 minutes at room temperature. Subsequently, the testis sections were observed under a confocal fluorescence microscope to assess the localization of Evans Blue dye and OCLN in seminiferous tubules. Leakage of Evans Blue dye to the adluminal compartment and mis-localization of OCLN indicated disruption of the BTB.

Histopathology

Paraffin sections were prepared from testes fixed in 4% paraformaldehyde, as described above, for periodic acid–Schiff–hematoxylin (PAS-H) or von Kossa staining. For von Kossa staining, 5-μm-thick sections were incubated with 5% silver nitrate solution under direct sunlight for 60 minutes. The slides were then washed in water, incubated with 5% sodium thiosulfate pentahydrate for 3 minutes, rinsed, and counterstained for 5 minutes with nuclear fast red.

RT-PCR and qPCR

Total RNA was extracted from whole mouse testes using TRIzol reagent (Life Technologies). Total RNA was then treated with Turbo DNase (Life Technologies) for DNA digestion, and cDNA was synthesized via RT-PCR using ReverTra Ace (Toyobo, Osaka, Japan) reverse transcriptase and oligo-dT (Life Technologies) primers. Quantitative real-time PCR (qPCR) analysis was performed using cDNA, gene-specific primers (Table 1), Brilliant III Ultra-Fast SYBR Green qPCR master mix (Agilent Technologies, Santa Clara, CA), and a real-time thermal cycler (MX 3000P; Agilent Technologies). The mRNA levels of the target genes were normalized to those of *Actb*.

Microarray Analysis

Total RNA extracted from testes of B6.MRLc1 (*n* = 3) and C57BL/6 (*n* = 3) mice at 10 days after SHS induction was purified with an RNeasy mini kit (Qiagen, Hilden, Germany; Valencia, CA) according to the manufacturer’s instructions. Using the purified RNA, the expression of 33,415 transcripts was profiled at an analysis service (DNA Chip Research, Yokohama, Japan). In brief, RNA samples were checked for RNA integrity with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and a Bioanalyzer 2100 system (Agilent Technologies), labeled with Cy3 dye,

Table 1 Primer Pairs Used

Gene symbol (accession no.*)	Primer position		Application
	Forward	Reverse	
<i>Ocln</i> (NM_008756)	1144–1163	1351–1332	qPCR
<i>Tjp1</i> (NM_009386)	5222–5241	5343–5324	qPCR
<i>Cldn3</i> (NM_009902)	802–821	1007–988	qPCR
<i>Grem2</i> (NM_011825)	197–216	380–360	qPCR
<i>Grem2</i> (NM_011825)	197–216	836–815	<i>In situ</i> hybridization
<i>Bmp2</i> (NM_007553)	38–57	164–143	qPCR
<i>Bmp4</i> (NM_007554)	660–680	865–845	qPCR
<i>Actb</i> (NM_007393)	1031–1050	1226–1207	qPCR

*<http://www.ncbi.nlm.nih.gov/gene>.

and hybridized to a SurePrint G3 Mouse GE 8×60K microarray (Agilent Technologies). Slides were scanned using an Agilent Technologies DNA microarray scanner, and the images were digitized with Feature Extraction software version 9.5.3 (Agilent Technologies). Data were normalized and expressed as fold change versus expression in C57BL/6 mice with GeneSpringGX11 (Agilent Technologies). Data from this study have been deposited with the NCBI Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>; accession number GSE53139). Gene ontology analysis was performed using GOTermFinder (<http://go.princeton.edu/cgi-bin/GOTermFinder>, last accessed September 30, 2013) to classify transcript data based on biological process, molecular function, and cellular component.

In Situ Hybridization Analysis

Complementary RNA probes for *Grem2* were synthesized in the presence of digoxigenin-labeled UTP using a digoxigenin RNA labeling kit (Roche Diagnostics, Mannheim, Germany; Indianapolis, IN). Primer pairs used for probe synthesis are listed in Table 1. Deparaffinized, proteinase K–digested sections (5 μm thick) were incubated with a prehybridization solution and then with hybridization buffer containing 50% formamide, 10 mmol/L Tris-HCl (pH 7.6), 200 mg/mL RNA, 16× Denhardt’s solution (0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, and 0.02% Ficoll PM400; Amersham–GE Healthcare, Chalfont St. Giles, UK; Uppsala, Sweden), 10% dextran sulfate, 600 mmol/L NaCl, 0.25% SDS, 1 mmol/L EDTA (pH 8.0), and a sense or antisense RNA probe (final concentration, 2 μg/mL) at 62°C overnight. Sections were then incubated with 0.2% polyclonal sheep anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (dilution 1:2000; nucleic acid detection kit; Roche Diagnostics) at 4°C overnight. The signal was detected by incubating the sections with a color substrate solution (Roche Diagnostics) containing nitro blue tetrazolium/X-phosphate in a solution composed of 100 mmol/L Tris-HCl (pH 9.5), 100 mmol/L NaCl, and 50 mmol/L MgCl₂ in a darkened room at room temperature overnight.

Energy Dispersive X-Ray Microanalysis

Paraffin-embedded samples were measured using energy-dispersive X-ray spectrometry (Falcon; AMETEK, Tokyo, Japan) with a scanning electron microscope (Super Scan SS-550; Shimadzu, Kyoto, Japan).

Electron Microscopy

Ultrastructural analysis with transmission electron microscope was performed as follows. After fixation with 3% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.2) at 4°C for 6 hours, small pieces of testis were fixed with 1% osmium tetroxide in 0.1 mol/L sodium cacodylate buffer at 4°C for 2 hours, dehydrated with graded alcohol, and embedded in epoxy resin (Quetol 812 mixture; Nissin EM, Tokyo, Japan). Ultrathin sections (70 nm thick) were double-stained with uranyl acetate and lead citrate. All samples were viewed under a transmission electron microscope (JEM-1210; JEOL, Tokyo, Japan).

Statistical Analysis

Statistical analyses were performed using PASW Statistics for Windows software version 18.0 (SPSS, Chicago, IL). The *U*-test was used to compare two groups, the Kruskal–Wallis test was used to compare three groups, and Scheffé's method was used for multiple comparisons when significant differences were observed. One-way analysis of variance followed by Dunnett's test was used to compare changes between a treatment group and the corresponding untreated group. Correlation between two parameters was analyzed using Spearman's correlation test (ρ). *P* values < 0.05 were considered statistically significant. Data are expressed as means $\pm$ SEM.

Results

Effect of Heat Stress on Spermatogenesis in Mice

Unlike tubules in untreated control testis (Figure 1A), seminiferous tubules in heat-exposed testes were injured 24

Figure 1 Histological abnormalities and apoptosis induction in mouse testes after SHS induction. **A–D:** PAS–H–stained cross sections of testes from mice exposed to heat shock. No histological abnormalities were observed in untreated control B6 testis (**A**); at 48 hours after SHS induction, seminiferous tubules of B6 (**B**) and B6.MRLc1 (**D**) mice contained vacuoles, pyknotic nuclei, and shrunken cells with extensive germ-cell loss, but germ cells were present in large numbers in most tubules of MRL mice (**C**). **Insets:** Enlargements show multinuclear giant cells (**arrows**) in tubules. **E–H:** Appearance of ssDNA⁺ cells in the seminiferous epithelium after SHS induction. ssDNA⁺ cells were infrequently detected in a testis from untreated B6 mice (**E**); at 24 hours after SHS induction, large portions of tubules from B6 (**F**) and B6.MRLc1 (**H**) mice contained numerous ssDNA⁺ cells, but testes of MRL mice contained fewer apoptotic cells (**G**). **I:** Number of apoptotic cells in B6 (black bars), MRL (white bars), and B6.MRLc1 (gray bars) mice after SHS induction. The total number of ssDNA⁺ cells was counted in 200 to 400 tubules per testis of control mice and mice exposed to heat shock. Data are expressed as means $\pm$ SEM. $3 \leq n \leq 9$ (**I**). **P* < 0.05, Kruskal–Wallis test followed by Scheffé's method. Scale bar = 50 μ m. d, day; h, hour.

to 72 hours after SHS induction (Figure 1, B–D). Although the appearance of multinuclear giant cells in some seminiferous tubules of B6, MRL, and B6.MRLc1 mice was similar (Figure 1, B–D), nuclear pyknosis and cellular shrinkage in parallel with germ-cell loss and epithelial vacuolization were more severe in B6 and B6.MRLc1 mice (Figure 1, B and D) than in MRL mice (Figure 1C) at 48 hours after SHS induction.

ssDNA⁺ apoptotic cells, mainly spermatocytes and round spermatids, were abundant in B6, MRL, and B6.MRLc1 mice at 24 hours after SHS induction, but were rare in the untreated B6 controls (Figure 1, E–H). In histometric analysis, the number of ssDNA⁺ cells peaked at 24 hours after SHS induction (Figure 1I). At 24 hours after SHS induction, the number of ssDNA⁺ cells was significantly lower in MRL mice than in B6 mice; at 48 hours, it was significantly higher in B6.MRLc1 mice than in B6 or MRL mice (Figure 1, F–I).

Changes in the number of spermatocytes at each developmental stage were examined by immunohistochemical analysis for STRA8, DMC1, and SYCP3 (Figure 2 and Supplemental Figure S1). Consistent with the induction of apoptotic germ cells (Figure 1), the number of SYCP3⁺ cells (leptotene to metaphase I spermatocytes) progressively decreased from 24 hours after SHS induction in all three strains (Figure 2C). By contrast, the number of primary spermatocytes, STRA8⁺ (preleptotene and early leptotene spermatocytes) and DMC1⁺ cells (leptotene and zygotene spermatocytes), remained the same in MRL mice until 48 hours (STRA8) or 72 hours (DMC1) after SHS induction (Figure 2, A and B). However, significant loss of spermatocytes positive for all three markers (ie, STRA8, DMC1, and SYCP3) was observed 10 to 20 days after SHS induction in all three strains (Figure 2).

Effects of Heat Stress on BTB Integrity in Mice

Expression in testes of *Ocln*, tight junction protein 1 (*Tjp1*, encoding ZO-1), and claudin 3 (*Cldn3*), genes that contribute to the BTB, was quantified by qPCR from 24 hours to 20 days after SHS induction (Figure 3, A–C). Consistent with a previous report,¹⁵ mRNA levels these genes decreased in testes of B6 and B6.MRLc1 mice from 24 to 48 hours after SHS induction (especially, *Tjp1* and *Cldn3* significantly decreased from 24 to 48 hours after SHS induction); however, in MRL mice, these mRNA levels remained the same until 48 hours after SHS induction (Figure 3, A–C).

BTB integrity after SHS induction was evaluated with a combination of Evans Blue dye and OCLN immunofluorescence (Figure 3, D–G). In testes of untreated B6 controls and in testes of MRL at 48 hours after SHS induction, the BTB, as visualized by OCLN staining, appeared as a continuous belt-like structure at the basal portion of seminiferous tubules; furthermore, Evans Blue dye was observed in the interstitial spaces and basal compartment, but not in

Figure 2 Changes in composition of spermatocytes in mouse testes after SHS induction. Numbers of STRA8⁺ (A), DMC1⁺ (B), and SYCP3⁺ (C) cells in testes of each mouse strain after SHS induction. Positive cells were counted in 200 to 400 tubules per testis. B6, black bars; MRL, white bars; B6.MRLc1, gray bars. Data are expressed as means $\pm$ SEM. $3 \leq n \leq 9$ (A–C). * $P < 0.05$ versus strain-matched control, one-way analysis of variance followed by Dunnett's test. † $P < 0.05$ versus another strain at the same time point, Kruskal–Wallis test followed by Scheffé's method.

the adluminal compartment (Figure 3, D and F). However, in testes of B6 and B6.MRLc1 mice at 48 hours after SHS induction, OCLN staining was diffuse in seminiferous tubules, and Evans Blue dye invaded the adluminal compartment (Figure 3, E and G). These results indicate that Sertoli cells in MRL mice blocked the movement of Evans Blue dye across the BTB even after SHS induction.

Long-Term Effects of Heat Stress on Mouse Testes

At 10 days after SHS induction, only a few spermatocytes were present in seminiferous tubules of the three strains; however, PAS-H–positive cellular debris and elongating spermatids were frequently observed in B6 and MRL mice, but not in B6.MRLc1 mice (Figure 4, A–C). Intriguingly, in long-term follow-up analyses after SHS induction, we found macroscopically visible disseminated white lesions in testes of B6 and MRL mice, but not in testes of B6.MRLc1 mice (Figure 4, D–F). Histopathologically, the lesions in B6 and MRL mice testes were von Kossa–positive mineral deposits in seminiferous tubules (Figure 4, G–I). At 60 days after SHS induction, most tubules contained von Kossa–positive materials, and only a few tubules had resumed spermatogenesis

Figure 3 BTB integrity in mouse testes after SHS induction. **A–C:** mRNA levels of *Ocln* (**A**), *Tjp1* (**B**), and *Cldn3* (**C**) in testes of each mouse strain after SHS induction. B6, black bars; MRL, white bars; B6.MRLc1, gray bars. **D–G:** Assay of BTB integrity by Evans Blue dye assay and OCLN immunofluorescence. In control B6 testis (**D**), Evans Blue dye (red) was sequestered in the testicular interstitium or the basal compartment of seminiferous tubules, and belt-like structures of OCLN (green) at the basal part of the tubules were observed. At 48 hours after SHS induction, Evans Blue dye was detected in the lumen (asterisks) of seminiferous tubules of B6 (**E**) and B6.MRLc1 (**G**) mice, and OCLN was weakly and diffusely stained in these testes. In testes of MRL mice (**F**), however, OCLN localized normally in tubules, and distribution of Evans Blue dye was limited to the basal compartment. Data are expressed as means $\pm$ SEM. $3 \leq n \leq 7$ (**A–C**). $*P < 0.05$ versus strain-matched control, one-way analysis of variance followed by Dunnett's test. Scale bars = 50 μ m.

in B6 and MRL mice (Figure 4, G and H). However, in B6.MRLc1 mice, von Kossa-positive mineral deposits were barely detectable, and partial recovery of spermatogenesis was observed (Figure 4I). Energy-dispersive X-ray

microanalysis revealed abnormal peaks of calcium, phosphorus, oxygen, and carbon, similar to those of hydroxyapatite [ie, $\text{Ca}_{10}(\text{PO}_4)_6\text{OH}_2$] in the B6 and MRL mouse testes after SHS induction (Figure 4J), indicative of intratubular calcification. This intratubular calcification initially appeared at 72 hours after SHS induction in B6 and MRL mice, and the incidence of calcification increased markedly at 10 to 20 days after SHS induction. By contrast, the onset of calcification was significantly inhibited in B6.MRLc1 mice throughout the observation period (Figure 4K).

To assess damage to the seminiferous epithelium induced by SHS, the number of Sertoli cells was evaluated by immunohistochemical analysis of SOX9, a Sertoli cell marker (Figure 5A and Supplemental Figure S1). The number of SOX9⁺ cells in B6 and MRL mice decreased at 20 days after SHS induction, coincident with the abrupt onset of calcification. By contrast, the number of Sertoli cells did not change significantly in the B6.MRLc1 mouse testis.

Representative transmission electron microscopy images of B6 and MRL mouse testes at 10 days after SHS induction are presented in Figure 5, B–J. The degenerating germ cells and their debris contained numerous electron-dense materials (Figure 5, B and G), which were apatite crystal-like needles in swollen mitochondria (Figure 5, D and I). Consistent with the light-microscopic observations (Figure 4, A and B), elongating spermatids remained in the damaged seminiferous epithelium, and these cells exhibited degenerative changes (Figure 5, C and H). Furthermore, we found increased numbers of mitochondrial granules (Figure 5, E and J), swollen mitochondria (Figure 5F), and abnormal multilamellar structures in the cytoplasm of Sertoli cells (Figure 5F). By contrast, although seminiferous tubules in time-matched B6.MRLc1 mice also contained swollen mitochondria in some Sertoli cells and germ cells, few multilamellar structures and needle-like crystals were detected (Figure 5K).

Strain-Specific Differences in the Onset of Heat-Induced Testicular Calcification

To validate genetic predisposition to heat-induced testicular calcification, we investigated testes in eight other strains (A/J, AKR/N, BALB/c, C3H/He, WBB6F1-+/+, WBB6F1-W/W^v, ddY, and ICR mice) at 20 days after SHS induction (Figure 6A). Although testicular calcification was detected in testes of A/J, AKR/N, BALB/c, C3H/He, ddY, ICR, and WBB6F1-+/+ mice, the incidence of calcified tubules in A/J, AKR/N, BALB/c, and C3H/He mice was significantly lower than in B6 mice and significantly higher than in B6.MRLc1 mice. These data indicate that B6 and B6.MRLc1 mice are susceptible and unsusceptible, respectively, to heat-induced testicular calcification. Interestingly, no testicular calcification was observed in WBB6F1-W/W^v mice, a strain rendered germ cell-deficient by a mutation in the *Kit* oncogene, whereas the corresponding wild-type WBB6F1-+/+ mice displayed severe testicular calcification (Figure 6, B and C). Despite the absence of calcified tubules, WBB6F1-

Figure 4 Long-term effects of SHS on mouse testes. **A–C:** PAS-H–stained cross sections of testes from B6 (**A**), MRL (**B**), and B6.MRLc1 (**C**) mice at 10 days after SHS induction. In all three strains, seminiferous tubules contained only a few spermatocytes. **Insets:** Enlargements show PAS-H–positive cellular debris and elongating spermatids. **D–F:** Gross appearance of testes at 60 days after SHS induction. Disseminated white lesions were present in testes of B6 (**D**) and MRL (**E**) mice, but not B6.MRLc1 (**F**) mice. **G–I:** Von Kossa–stained cross sections of testes from B6 (**G**), MRL (**H**), and B6.MRLc1 (**I**) mice at 60 days after SHS induction. Most tubules in B6 and MRL mice contained von Kossa–positive materials (black), but such materials were barely detectable in testes of B6.MRLc1 mice. **J:** Energy-dispersive X-ray microanalysis of testes with and without SHS. In testes of control B6 mice, oxygen and carbon peaks were observed. At 40 days after SHS induction, B6 and MRL testes exhibited intense calcium and phosphorus peaks, in addition to oxygen and carbon peaks, but calcium and phosphorus peaks were barely detectable in B6.MRLc1 testes. **K:** Incidence of von Kossa–positive seminiferous tubules in testes from each strain after SHS induction. B6, black bars; MRL, white bars; B6.MRLc1, gray bars. Seminiferous tubules containing von Kossa–positive mineral deposits were counted in 200 to 400 tubules per testis. Data are expressed as means $\pm$ SEM. $3 \leq n \leq 12$ (**K**). * $P < 0.05$ versus strain-matched controls, one-way analysis of variance followed by Dunnett's test; † $P < 0.05$ versus another strain at the same time point, Kruskal–Wallis test followed by Scheffé's method. Scale bar = 50 μ m. T, testis; E, epididymis.

W/W^v mouse testes at 20 days after SHS induction also exhibited widening of the intertubular space, compared with untreated control testes (**Figure 6, D and E**), with a decrease in Sertoli cell numbers similar to that observed in WBB6F1-+/+ mice (**Figure 6F**).

Gene Regulation Associated with the Onset of Heat Stress-Induced Testicular Calcification

Using microarray analysis, we compared the expression of 33,415 genes in B6 and B6.MRLc1 mice at 10 days after SHS induction (ie, at the early onset of testicular calcification in B6 mice). Expression of 231 genes was higher in B6 mice than in B6.MRLc1 mice, and the expression of 23 genes was higher in B6.MRLc1 mice than in B6 mice (**Supplemental Table S1**). Of the genes expressed at higher levels in B6.MRLc1 mouse testes after SHS induction, the greatest difference was detected in *Grem2* expression (4.30-fold greater than in B6 mice).

GREM2 Expression in Mouse Testes Exposed to Heat Stress

Based on the microarray analysis results, we focused on GREM2 as a potential mediator of the strain-specific differences observed in heat-exposed testes, particularly the differences in testicular calcification in B6, MRL, and B6.MRLc1 mice. qPCR analysis demonstrated that, although SHS increased GREM2 expression in B6, MRL, and B6.MRLc1 mice, GREM2 mRNA levels were higher in B6.MRLc1 mice than in the other two strains from 24 hours to 20 days after SHS induction (**Figure 7A**). Statistically significant differences in GREM2 expression were observed in B6.MRLc1 and B6 mice at 10 and 20 days after SHS induction and in B6.MRLc1 and MRL mice at 24 hours and at 10 days after SHS induction. *Grem2* encodes a cytokine that inhibits the activity of bone morphogenetic proteins 2 and 4 (BMP-2 and BMP-4).^{30,31} BMP-2 and BMP-4 mRNA levels in testes increased in a time-dependent manner after SHS induction in all three strains; however, there was a statistically significant difference between BMP-2 levels in B6 and B6.MRLc1 at 48 hours after SHS induction (**Figure 7, B and C**). Furthermore, in testes from various strains at 20 days after SHS induction, the incidence of intratubular calcification negatively and positively correlated with GREM2 and BMP-2 mRNA levels, respectively (**Figure 7, D and E**).

We investigated cell types in testes that expressed GREM2 after SHS induction by *in situ* hybridization and immunohistochemistry (**Figure 7, G–O**). At 10 days after SHS induction, the GREM2 transcript signal in B6.MRLc1 Sertoli cells was intense, whereas the signal in Sertoli cells from time-matched B6 and MRL mice was weak (**Figure 7, J–L**). GREM2 immunopositivity was weak in B6 and MRL Sertoli cells at 10 days after SHS induction (**Figure 7, M and N**). By contrast, more intense GREM2 immunostaining was observed, as

granular cytoplasmic aggregates, in B6.MRLc1 mouse Sertoli cells at 10 days after SHS induction (Figure 7O).

Discussion

Heat Shock—Resistant Spermatogenesis in MRL Mice

Accumulating evidence indicates that spermatogenesis, spermatocytes, and round spermatids in particular are sensitive to hyperthermia.^{10,11} Interestingly, spermatocytes in MRL mice were relatively resistant to heat stress, as previously demonstrated in experimental cryptorchidism.^{22,23,28}

In the present study, we investigated the time-dependent effect of SHS induction on spermatogenesis in B6, MRL, and B6.MRLc1 mice. Consistent with previous report,^{13,32} significant apoptotic germ-cell loss was observed in the days after SHS induction. Among the three strains, however, only MRL mice maintained a steady number of STRA8⁺ (preleptotene and early-leptotene spermatocytes) and DMC1⁺ cells (leptotene and zygotene spermatocytes) in the first days after SHS induction. These results suggest that, in addition to having heat shock—resistant spermatocytes, MRL mice partially maintain the progression of meiosis even after SHS induction.

Studies on irreversible BTB disruption have suggested that persistent spermatogenesis depends largely on the maintenance of BTB integrity.^{33–36} Because testicular hyperthermia disrupts the BTB,^{14,15} we speculated that heat shock—resistant spermatogenesis in MRL mice depends in part on the maintenance of BTB integrity. Predictably, only MRL mice

maintained BTB function immediately after SHS induction. Interestingly, an earlier study on gene regulation after SHS induction in AKR/N mice, whose spermatocytes are also relatively resistant to heat stress, suggested that both somatic cells and germ cells contribute to the heat resistance of AKR/N testes during the early response to hyperthermia.³⁷ Thus, contributions from spermatocytes and Sertoli cells, such as the maintenance of BTB integrity, might be necessary for heat shock—resistant spermatogenesis in MRL mice.

In the first days after SHS induction, neither heat shock—resistant spermatocytes nor retention of BTB function was observed in B6.MRLc1 mice. These results indicate that the MRL-type locus on Chr 1, responsible for heat shock—resistant spermatocytes, might be insufficient to maintain spermatogenesis in the first few days immediately after SHS induction. Indeed, MRL-type Chr 11 also contains a locus responsible for heat-shock resistance in spermatocytes in experimental cryptorchidism.²⁸ Thus, the cooperative effects of the MRL-derived loci on Chr 1 and Chr 11 need to be elucidated.

Onset of Testicular Calcification after Heat Stress

From 10 days after SHS induction, heat-induced testicular calcifications, composed of materials that resemble hydroxyapatite [Ca₁₀(PO₄)₆OH₂], were frequently observed in B6 and MRL mice, but not in B6.MRLc1 mice. At present, the etiology of testicular calcification is unknown. However, age-dependent testicular calcification has been reported in murine models with Sertoli cell dysfunction and progressive

Figure 6 Differences in the occurrence of testicular calcification after SHS induction in various mouse strains. **A:** Incidence of von Kossa–positive seminiferous tubules in each strain at 20 days after SHS induction. Seminiferous tubules containing von Kossa–positive mineral deposits were counted in 200 to 400 tubules per testis. **B** and **C:** von Kossa–stained cross sections of testes from WBB6F1-+/+ and WBB6F1-W/W^v mice at 20 days after SHS induction. Most tubules in testes of WBB6F1-+/+ mice (**B**) exhibit severe intratubular calcification (black deposition), but no calcified tubules were observed in the WBB6F1-W/W^v mouse testis (**C**). **D** and **E:** PAS–H–stained cross sections of testes from WBB6F1-W/W^v mice before (**D**) and 20 days after (**E**) SHS induction. At 20 days after SHS induction, widening of the intertubular space was observed (**E**). **F:** Number of SOX9⁺ cells in testes of WBB6F1-+/+ and WBB6F1-W/W^v mice before and after SHS induction. Positive cells were counted in 200 to 400 tubules, including calcified tubules, per testis. Data are expressed as means $\pm$ SEM. $3 \leq n \leq 5$ (**A**); $n = 4$ (**F**). * $P < 0.05$, ** $P < 0.01$ versus B6; † $P < 0.05$, †† $P < 0.01$ versus B6.MRLc1, one-way analysis of variance followed by Dunnett's test (**A**). * $P < 0.05$, U test (**F**). Scale bar = 100 μ m.

germ-cell degeneration, such as vitamin A–deficient mice and androgen-insensitive hypogonadal mice.^{38–41}

At the early onset of testicular calcification after SHS induction in B6 and MRL mice, we detected hydroxyapatite-like needle crystals in the swollen mitochondria of degenerating germ cells and their debris. Vera et al⁴² found that a mitochondrial pathway mediates heat-induced male germ-cell death. Importantly, we detected no calcified tubules in testes of germ cell–deficient WBB6F1-WW^v mice, whereas the corresponding wild-type WBB6F1-+/+ mice exhibited severe testicular calcification. Thus, our findings strongly suggest that massive germ-cell degeneration is involved in the onset of intratubular calcification and that pathogenesis is initiated by mitochondrial damage.

Furthermore, we observed an abrupt reduction in Sertoli cell numbers in B6 and MRL mice, but not in B6.MRLc1 mice, coincident with the abrupt onset of calcification. Intriguingly, WBB6F1-WW^v mice also exhibited a significant decrease in Sertoli cells after SHS induction, despite the absence of a calcified nidus. Furthermore, increased

numbers of mitochondrial granules, which are known to regulate Ca²⁺ transport, as well as swollen mitochondria, were observed in the Sertoli cells of heat-exposed B6 and MRL mouse testes. Thus, heat stress could directly and irreversibly damage Sertoli cells. Indeed, abnormal multi-lamellar structures appeared in B6 and MRL Sertoli cells during the early stages of calcification. An *in vitro* study of vascular smooth muscle cells revealed that calcification was initiated by the release of calcium-enriched membrane-bound matrix vesicles and/or apoptotic bodies that act as crystal nucleation points.⁴³ Sertoli cells eliminate apoptotic germ cells by phagocytosis.⁴⁴ Thus, Sertoli cells that engulf calcified germ-cell debris might promote further calcification in seminiferous tubules.

Predicted Mechanisms that Inhibit the Onset of Testicular Calcification

Differences in the incidence of testicular calcification among various strains of mice after SHS induction indicate a

Figure 7 Expression of candidate calcification regulatory molecules in mouse testes after SHS induction. **A–C:** qPCR results indicate changes in mRNA levels of GREM2 (**A**), BMP-2 (**B**), and BMP-4 (**C**) in testes of each mouse strain after SHS induction. **D–F:** Spearman's ρ correlation between incidence of von Kossa-positive tubules and mRNA levels of GREM2 (**D**), BMP-2 (**E**), and BMP-4 (**F**). Values were calculated from the data for testes from B6, MRL, B6.MRLc1, A/J, AKR/N, BALB/c, C3H/He, ddY, ICR, WBB6F1-+/+, and WBB6F1-W/W^v mice at 20 days after SHS induction. The average of the mRNA expression levels in the B6 mice was set to 1. No significant correlation was detected between Bmp4 mRNA levels and the incidence of intratubular calcification (Spearman's ρ value is -0.080). **G–O:** Localization of GREM2 transcripts and proteins in testes of B6 (**G**, **J**, **M**), MRL (**H**, **K**, **N**), and B6.MRLc1 (**I**, **L**, **O**) mice at 10 days after SHS induction. With *in situ* hybridization analysis, GREM2 transcript signals were more intense in the B6.MRLc1 mice (**L**) than in the B6 (**J**) and MRL (**K**) mice. This was especially true of Sertoli cells, as determined from the PAS-H-stained semiserial sections (**G–I**). In immunohistochemical analysis, GREM2 proteins were found as granular cytoplasmic aggregates in Sertoli cells of B6.MRLc1 mice (**O**), but immunopositivity of GREM2 was weak in B6 and MRL Sertoli cells (**M** and **N**). Data are expressed as means $\pm$ SEM or as individual data points. $3 \leq n \leq 7$ (**A–C**); $n = 43$ (**D–F**). $*P < 0.05$, one-way analysis of variance followed by Dunnett's test. $^{\dagger}P < 0.05$ versus another strain at the same time point, Kruskal–Wallis test followed by Scheffé's method (**A–C**). $**P < 0.01$, Spearman's correlation test (**D** and **E**). Scale bar = 10 μ m.

genetic basis for susceptibility to heat-induced testicular calcification. Indeed, a genetic predisposition has been suggested for human testicular microlithiasis (ie, microcalcification within seminiferous tubules).^{45,46} In clinical studies of humans, testicular microlithiasis has been detected in several pathological conditions, including infertility, cryptorchidism, testicular neoplasms, Klinefelter's syndrome, varicocele, and torsion of the testis,^{47,48} as well as in normal testes.^{45,49} Whether scrotal hyperthermia can induce irreversible calcification in human testes is unclear, but it has been reported that scrotal hyperthermia by exposure to sauna heat could have adverse effects on human spermatogenesis over 3 months.⁵ A genetic susceptibility to heat-induced testicular calcification in humans should be carefully investigated.

Using microarray analysis, we identified numerous transcripts that were differentially expressed in B6 and B6.MRLc1 testes after SHS induction. Of the 231 transcripts with higher expression in B6 mice than in B6.MRLc1 mice, 25 regulate spermatogenesis and/or sperm function. Gene ontology analysis revealed function of seven of the transcripts in acrosomal vesicles. Given that no significant difference was observed in the number of spermatocytes in B6 and B6.MRLc1 mice at 10 days after SHS induction (Figure 2), our microarray results may reflect a difference between the two strains in the presence of degenerated elongating spermatids (Figures 4 and 5). Although direct adverse effects of heat on elongating spermatids have not been established, B6.MRLc1 mice may possess the ability to remove degenerating germ cells.

Many recent studies have suggested that pathological calcification (eg, vascular calcification) is regulated through a mechanism similar to that of bone calcification.^{50,51} Molecules that play a key role in bone calcification, such as transforming growth factor- β family members, particularly BMP-2 and BMP-4, may be important modulators of vascular calcification.^{50,52} In the present study, BMP-2 and BMP-4 mRNA levels increased in a time-dependent manner after SHS induction in B6, MRL, and B6.MRLc1 mice. Furthermore, in various mouse strains, BMP-2 mRNA expression correlated with the incidence of calcification at 20 days after SHS induction. Microarray experiments identified eight transcripts with calcium ion-binding function that were expressed at higher levels in B6 mouse testes than in time-matched B6.MRLc1 mouse testes at the onset of calcification. These genes included cartilage oligomeric matrix protein (*Comp*) and matrix Gla protein (*Mgp*), which encode glycoproteins that bind BMP-2 and BMP-4.^{51,53,54} Interestingly, up-regulation and localization of COMP and MGP in cardiovascular calcification lesions have been reported.^{52,55–58} Thus, the heat-induced testicular calcification observed here might be similar to calcification in the cardiovascular system. Taken together, our results suggest that BMPs induce testicular calcification and that some factors inhibit BMP activity in B6.MRLc1 mice.

We focused on GREM2, whose expression in Sertoli cells after SHS induction was higher in B6.MRLc1 mice than in B6 and MRL mice. GREM2 is a cytokine that directly binds to BMP-2 and BMP-4 and blocks their promoter activity, thereby acting as an extracellular BMP antagonist.³⁰ Forced expression of *Grem2* in osteoblasts blocked osteoblastic differentiation, whereas suppression of *Grem2* promoted osteogenesis *in vitro*.⁵⁹ BMP-2 stimulation induced GREM2 mRNA expression in osteoblasts, suggesting that GREM2 plays an important role in protecting bone metabolism from excessive activation by BMPs during osteogenesis through a negative feedback system.³¹ Notably, endogenous BMP antagonists, such as noggin and chordin, may inhibit vascular calcification.^{51,60} Interestingly, transgenic mice overexpressing GREM1, a BMP antagonist with 42.9% amino acid sequence similarity to GREM2, exhibit severe osteopenia, ectopic calcification, and thinning of the dentin and enamel in the teeth.⁶¹ Thus, GREM2 could act similarly as an inhibitor of calcification by antagonizing BMP activity. Indeed, we observed a negative correlation between GREM2 mRNA expression and the incidence of calcification in testes from various strains of mice after SHS induction. Further studies on the phenotype of the testis in transgenic mice overexpressing *Grem2* would be needed to validate this hypothesis.

Grem2 localizes at 81.08 cM on murine Chr 1. Notably, GREM2 mRNA expression in MRL controls was 3.35-fold higher than in B6 controls. Interestingly, although only B6 and MRL mice, among several inbred mouse strains, spontaneously developed aortic cartilaginous metaplasia, a high-fat and high-cholesterol diet induced aortic calcification in B6 mice but not in MRL mice.^{62,63} Thus, it is possible that GREM2 expression in tissue correlates with the extent of calcification and that MRL-derived GREM2 acts as a novel calcification-inhibiting factor. However, after SHS induction, MRL mice exhibited severe calcification similar to that in B6 mice, and GREM2 mRNA expression was significantly lower in MRL mice than in B6.MRLc1 mice. We observed that GREM2 is expressed in Sertoli cells. Because the Sertoli cells of heat-exposed MRL mouse testes exhibited degenerative changes (Figure 5); the ability of MRL mice to secrete GREM2 might be impaired after SHS induction. Taken together, these data suggest that MRL mice have a potent precipitating factor for testicular calcification, as well as inhibitory mechanisms.

In summary, meiosis progression and BTB integrity was preserved in MRL mice in the first few days after heat exposure. However, transient scrotal hyperthermia induced testicular calcification in the weeks after heat exposure. Our data strongly suggest that genes located on the telomeric region of murine Chr 1 regulate the onset of calcification. The imbalance between the effects of BMPs and GREM2 may provide new insights into the etiology of testicular calcification.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2014.06.004>.

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