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Protamine 2 Deficiency Leads to Sperm DNA Damage and Embryo Death in Mice[1]

Chunghee Cho,[2] Haesook Jung-Ha,[3] William D. Willis,[3] Eugenia H. Goulding,[3] Paula Stein,[4] Zhe Xu,[4] Richard M. Schultz,[4] Norman B. Hecht,[5] and Edward M. Eddy[3,6]

Department of Life Science,[2] Kwangju Institute of Science and Technology (K-JIST), Kwangju 500-712, Republic of Korea
Gamete Biology Section,[3] Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709
Department of Biology[4] and Center for Research on Reproduction and Women's Health and Department of Obstetrics and Gynecology,[5] University of Pennsylvania, Philadelphia, Pennsylvania 19104

ABSTRACT

Cytokinesis is incomplete in spermatogenic cells, and the descendants of each stem cell form a clonal syncytium. As a result, a heterozygous mutation in a gene expressed postmeiotically affects all of the haploid spermatids within a syncytium. Previously, we have found that disruption of one copy of the gene for either protamine 1 (PRM1) or protamine 2 (PRM2) in the mouse results in a reduction in the amount of the respective protein, abnormal processing of PRM2, and inability of male chimeras to transmit either the mutant or wild-type allele derived from the 129-genotype embryonic stem cells to the next generation. Although it is believed that protamines are essential for compaction of the sperm nucleus and to protect the DNA from damage, this has not been proven experimentally. To test the hypothesis that failure of chimeras to transmit the 129 genotype to offspring was due to alterations in the organization and integrity of sperm DNA, we used the single-cell DNA electrophoresis (comet) assay, ultrastructural analysis, and the intracytoplasmic sperm injection (ICSI) procedure. Comet assay demonstrated a direct correlation between the fraction of sperm with haploinsufficiency of PRM2 and the frequency of sperm with damaged DNA. Ultrastructural analysis revealed reduced compaction of the chromatin. ICSI with PRM2-deficient sperm resulted in activation of most metaphase II-arrested mouse eggs, but few were able to develop to the blastocyst stage. These findings suggest that development fails because of damage to paternal DNA and that PRM2 is crucial for maintaining the integrity of sperm chromatin.

early development, gamete biology, gametogenesis, sperm, spermatogenesis

INTRODUCTION

During the postmeiotic phase of male germ cell development in mammals, the chromatin is profoundly remodeled and becomes highly condensed. Transition proteins replace the histones, and they are in turn replaced by protamines, small basic proteins rich in arginine. The evolutionary advantage of replacing histones with novel proteins found only in spermatids and sperm has engendered considerable discussion, but few conclusions have been drawn. This process has been proposed to serve a role in shaping the sperm head, to provide a means to inactivate temporally transcription from the haploid male genome, and/or to afford protection and stabilization of sperm DNA.

A single protamine gene is expressed in most mammals, but a second protamine gene is expressed in a few mammals, including mice and humans [1]. The protein encoded by the second gene (*Prm2*) constitutes about half of the protamine in human sperm and two thirds of the protamine in mouse sperm. To determine if sperm need both protamines, we disrupted the coding sequence of the *Prm1* or *Prm2* gene in embryonic stem (ES) cells from 129-strain mice and injected the ES cells into blastocysts from C57BL/6-strain mice to produce chimeras. The male chimeras produced 129-genotype sperm, C57BL/6-genotype sperm, and C57BL/6-genotype offspring but no 129-genotype-bearing offspring. Disruption of one allele of either *Prm1* or *Prm2* resulted in relatively modest reductions in the amount of PRM1 (by ~16% of total protamine) or PRM2 (by ~33% of total protamine), respectively. In addition, the amount of PRM1 was reduced (less than 16% of total protamine) in PRM2-deficient sperm. These findings strongly suggest that sperm require a full complement of both protamines to convey the male genome to the next generation [2]. Although sperm are haploid, all 129-genotype sperm were affected because spermatids develop in syncytial clones, share mRNA and protein through cytoplasmic bridges, and are phenotypically diploid [3, 4]. Thus, inactivation of one copy of either *Prm1* or *Prm2* caused a protein deficit (haploinsufficiency) in all of the sperm produced by a clone.

We had observed that the nuclei of sperm with reduced amounts of protamine were less resistant to chemical disruption than the nuclei of wild-type sperm [2]. In addition, earlier studies reported that PRM2 deficiency correlates with infertility in men [5, 6]. Furthermore, the frequency of sperm with DNA damage is predictive of failure of development of human embryos after intracytoplasmic sperm injection (ICSI) [7]. The present study examines why a re-

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[6]Correspondence: E. M. Eddy, Gamete Biology Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709. FAX: 919 541 3800; e-mail: eddy@niehs.nih.gov

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duction in the amount of either protamine results in failure to transmit the male genome to the next generation. We find that the chimeras are unable to produce 129-genotype-bearing offspring because PRM2 haploinsufficiency results in alterations in the organization and integrity of sperm DNA.

MATERIALS AND METHODS

Genotyping Sperm of Prm2 *Chimeric Mice*

Chimeric mice were produced as described previously [2]. In brief, the *Prm2* gene was disrupted by homologous recombination in ES cells from 129-strain mice, and the ES cells were injected into blastocysts from C57BL/6-strain mice. Each male chimera was paired with one or two C57BL/6 females for 2–4 mo to test fertility. To obtain sperm without killing the chimeras, they were mated with C57BL/6 female mice primed with pregnant mare's serum gonadotropin and human chorionic gonadotropin. The mice were placed together at ~0700 h, and the females were euthanized 1–5 h after a copulation plug was found, the uteri were removed, and sperm were flushed from them with PBS (pH 7.4). These sperm were subjected to two separate polymerase chain reaction (PCR) assays to distinguish 129-genotype sperm from C57BL/6-genotype sperm and to determine the relative amounts of 129-genotype sperm with a copy of the intact allele (129-*Prm2*$^+$) and the mutant allele (129-*Prm2*$^-$) in the sample [2]. Animal care was in accordance with U.S. Public Health Service guidelines for use of animals, and the Institutional Animal Care and Use Committee, National Institute of Environmental Health Sciences, reviewed and approved all procedures before use.

Comet Assay for DNA Damage

DNA damage was assessed with the single-cell gel electrophoresis (comet) assay [8]. Sperm from chimeric and wild-type mice were suspended in 0.7% low-temperature-melting agarose, placed on a frosted slide, and chilled on ice. The slides were submerged in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, pH 10, and 1% Triton X-100) at 4°C for 1 h, in lysis buffer with 10 mM dithiothreitol (DTT) at 4°C for 30 min, and finally in lysis buffer with 4 mM lithium diiodosalicylate at room temperature for 90 min. The slides were subjected to electrophoresis in 300 mM NaOH and 1 mM EDTA at 25 V for 10 min, neutralized with 0.4 M Tris-Cl (pH 7.5), and stained with ethidium bromide. The experiments were repeated two times, and approximately 600 sperm from each mouse were examined. The slides were observed using a Leica TCS laser scanning confocal microscope (Leica Microsystems, Mannheim, Germany).

Electron Microscopy

Sperm for scanning electron microscopy were isolated from the epididymis and vas deferens and fixed in 2.5% glutaraldehyde and 2% formaldehyde in 0.15 M sodium phosphate buffer overnight at 4°C. They were washed in buffer, collected on Nuclepore filters, subjected to critical point drying, coated with gold/palladium, and examined in a Cambridge S200 scanning electron microscope at 20 KV. For transmission electron microscopy, cauda epididymides were fixed under the same conditions, postfixed in 2% osmium tetroxide in cacodylate buffer, and embedded in Lowicryl resin. Sections were stained with uranyl acetate and lead citrate and examined in a LEO910 transmission electron microscope at 80 KV.

ICSI and Embryo Genotyping

Sperm from the epididymis and vas deferens were suspended in M16 medium containing 0.01% polyvinyl alcohol, filtered through Kimwipes, and resuspended in NIM medium (123 mM KCl, 2.6 mM NaCl, 7.8 mM NaH_2PO_4, 1.4 mM KH_2PO_4, 3 mM EDTA, pH 7.2) containing 1% polyvinyl alcohol and 50% glycerol [9]. Sperm from *Prm2* chimeric mice and wild-type C57BL/6 mice were sonicated mildly to remove tails, and randomly selected heads were injected in less than 1 pl of medium into metaphase II-arrested eggs from B6D2 mice (progeny derived from matings of C57BL/6 and DBA mice). Injected eggs were transferred into CZB medium [10] and cultured at 37°C under 5% CO_2 in air for 3–5 days. The cultured embryos were observed under a dissecting microscope to determine developmental stages and immediately frozen. For subsequent PCR analysis, each embryo was suspended in 10 μl of embryo lysis buffer (1% Tween-20, 1% Triton X-100, 5 mM EDTA, 10 mM Tris-Cl, pH 7.5, 2 mM DTT, and 4 U/ml of proteinase K) and incubated at 65°C for 10 min and subsequently at 95°C for 10 min. A total of 5 μl of lysate was used in PCR reactions as template. To distinguish embryos produced by injecting 129- or C57BL/6-genotype sperm, PCR was performed (35 cycles) using primers (sense, 5′-GATCTTCCTTTATACACAAGTCATAGC-3′; antisense, 5′-GTGGTACAGAACTTAGGTGTTTAATTG-3′) that amplify a microsatellite in the *D10Mit180* locus of mouse chromosome 10. The PCR products were 134 base pair (bp) for the C57BL/6 genotype, 206 bp for the 129 genotype, and 156 bp for the DBA genotype.

RESULTS

Genotypes of Sperm and Progeny of Chimeras

We generated male chimeric mice and selected chimeras with zero or a relatively high percentage of 129-genotype sperm for experimental analyses (2 chimeras with 0% 129-genotype sperm as control and the other 2 chimeras with 70% or 90% of 129-genotype sperm as test). None of the chimeras produced progeny with 129-genotype-derived coat color (agouti). The chimeras with 70% or 90% 129-genotype sperm produced more morphologically abnormal sperm (30% and 37%, respectively) than did the chimeras with 0% 129-genotype sperm (average, 8.1%). In addition, staining of sperm nuclei with acridine orange showed that the percentage of sperm with abnormal staining patterns was much higher in the chimeras with 70% or 90% 129-genotype sperm (70% and 79%, respectively) than the chimeras with 0% 120-genotype sperm (0%). Consistent with a previous study [2], these results indicate alteration of sperm structure and nuclear integrity in 129-genotype sperm from the chimeras.

The DNA of Sperm from Chimeras Is Damaged

The comet assay was used to assess the integrity of DNA in individual sperm from chimeras. This assay has been widely used to measure DNA damage in many different cell types, including sperm from laboratory animals and humans [8, 11]. The assay was performed under alkaline electrophoresis conditions to measure single-stranded DNA breaks and alkali-labile sites of DNA. Approximately 80% of the sperm from a chimera that produced 70% PRM2-deficient sperm had a comet tail, whereas 10% of the sperm from a wild-type mouse had a comet tail (Fig. 1). It is unknown how much of the 10% background level was due to prior DNA damage or was caused by preparative procedures. Although we did not measure comet dimensions, comet tail lengths were variable, suggesting that the degree of DNA damage differed between individual sperm. This is consistent with the differences in sperm head morphology noted herein and the differences in acridine orange staining reported previously [2]. There were no apparent differences between intact and sonicated sperm in the frequency and quality of comet tails, suggesting that sonication did not cause additional DNA damage (Fig. 1).

Frequency of Abnormal Sperm Correlates with 129 Genotype

Scanning and transmission electron microscopy were used to characterize the differences between PRM2-deficient and wild-type sperm. The shape and external appearance of sperm were examined by scanning electron microscopy. Sperm from a *Prm2* chimera producing 90% 129-genotype sperm were compared with sperm from wild-type mice (Fig. 2A). The most common differences seen were a decrease in lateral width and a reduced ventral flexure of

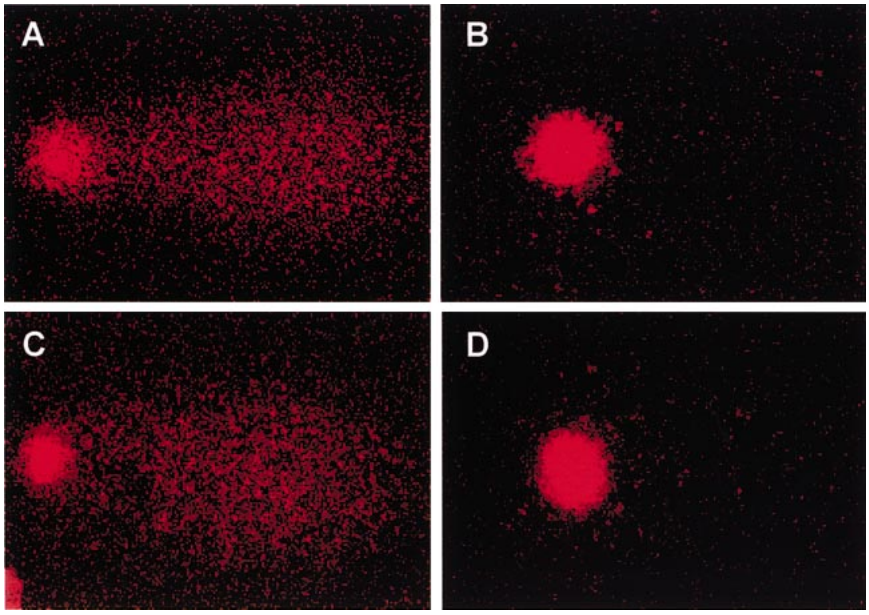

FIG. 1. Comet assay of DNA integrity in nuclei of sperm. Sperm were from a chimera producing 100% wild-type sperm (C57BL/6 genotype) (**B**, **D**) and a chimera producing 70% PRM2-deficient sperm (129 genotype) (**A**, **C**). The presence of a comet tail in **A** and **C** indicates that fragmented DNA was present in these cells. **A**, **B**) Intact sperm. **C**, **D**) Sonicated sperm. Original magnification ×200.

the head of most sperm from the chimera (Fig. 2B and C). What appeared initially to be globular sperm heads was found on closer inspection to be the result of the heads being folded back onto and fused with the flagellum (Fig. 2D and E).

Transmission electron microscopy was used to determine the appearance of the chromatin, the association between the acrosome and the nucleus, and the organization and placement of flagellar components. The chromatin in most sperm from a chimera producing 90% of 129-genotype sperm appeared to be loosely packed and heterogeneous in density (Fig. 3B-D). This appearance is more similar to the

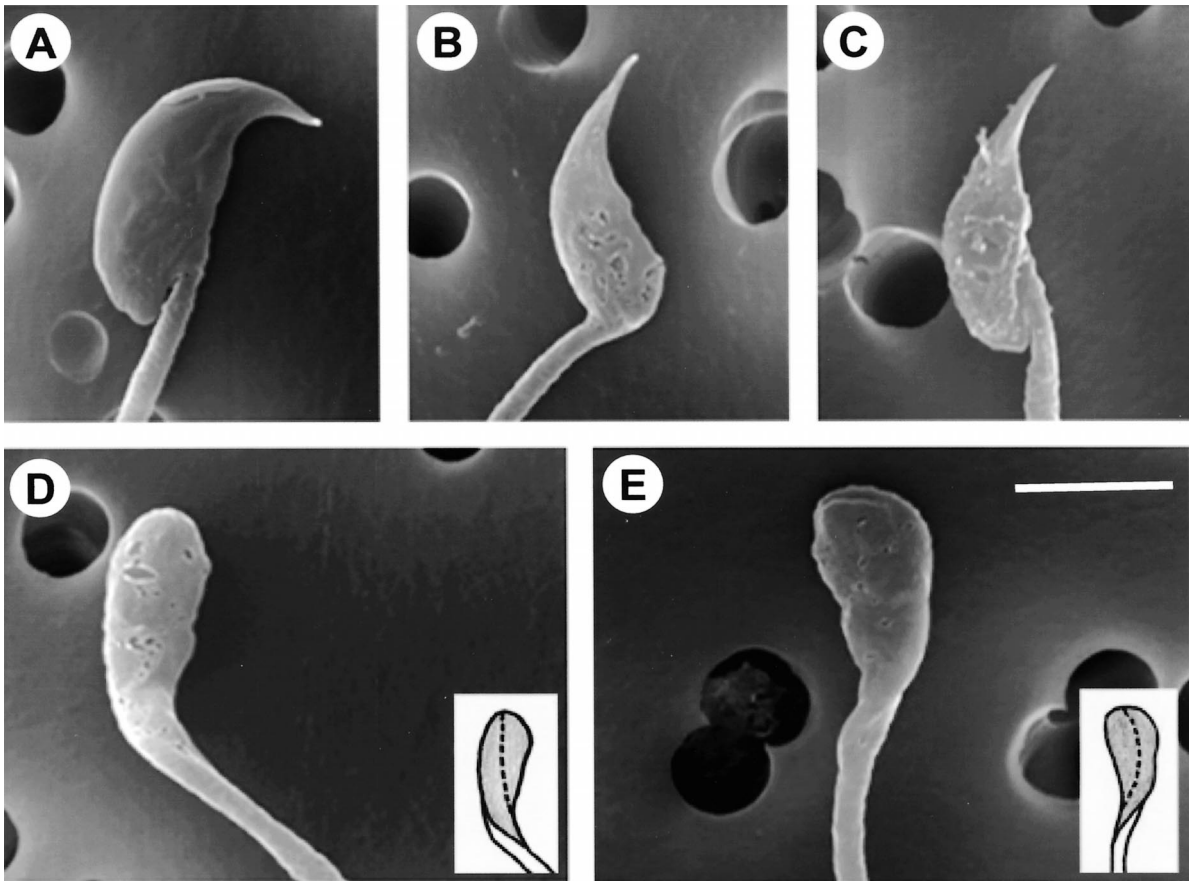

FIG. 2. Scanning electron micrographs of sperm from wild-type and *Prm2* chimera mice. Sperm shown are from a wild-type mouse (**A**) and a chimera producing 90% PRM2-deficient sperm (**B**–**E**). The heads of PRM2-deficient sperm frequently are reduced in size and have a less pronounced ventral flexure (**B**, **C**) than sperm of wild-type mice. In other PRM2-deficient sperm, the head is folded back onto the flagellum (**D**, **E**). Insets are drawings that indicate the locations of the head (shaded) and of the flagellum (dotted lines) (**D**, **E**). Bar = 4 μm.

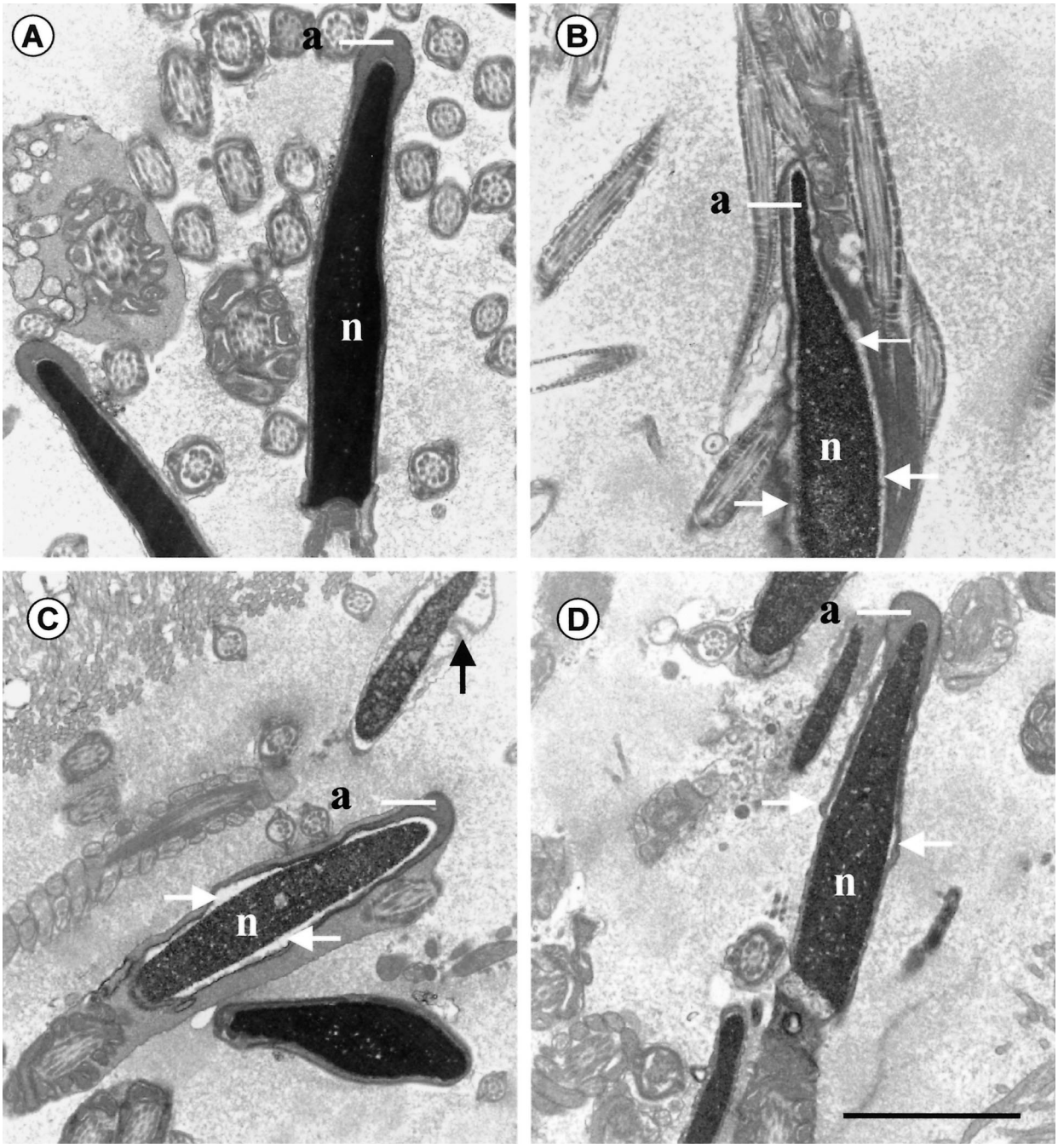

FIG. 3. Transmission electron micrographs of sperm from wild-type and *Prm2* chimera mice. Sperm shown are in the cauda epididymis of a wild-type mouse (**A**) and a chimera that produced 90% PRM2-deficient sperm (**B**–**D**). The chromatin of PRM2-deficient sperm is heterogeneous in density and loosely dispersed in the outer portion of the nucleus (n), and spaces (arrow) are present between the nucleus and acrosome (a) (**B**–**D**). A sperm with its head folded back onto the flagellum is shown (**B**). Bar = 2 μm.

incompletely packed chromatin in condensing spermatids (data not shown) than to the tightly packed and homogeneously dense chromatin seen in sperm from wild-type mice (Fig. 3A). In addition, the chromatin of sperm from chimeras was often loosely dispersed in the outer portion of the nucleus, the acrosomes frequently were irregular in contour and appeared to be attached loosely to the nucleus, and flagellar components were seen to be abnormally located in close apposition to the acrosome and underlying nucleus (Fig. 3B-D). These results indicate that a reduction in amount of PRM2 causes distinct changes in chromatin packing, in the shape and organization of the acrosome, and in the overall architecture of the sperm.

129-Genotype Sperm Fail to Produce Viable Embryos

Embryos in the early stages of development were analyzed to determine if there was a relationship between the frequency of DNA damage and the failure of chimeras to transmit the mutant or intact allele to their progeny. The ICSI procedure was used to minimize the number of variables that might be contributing to the failure. Sperm from a chimera or a wild-type mouse were microinjected into metaphase II-arrested eggs, and the ensuing preimplantation development was monitored in vitro to determine if the embryos developed to the blastocyst stage. The chimera produced a mixed population of C57BL/6-genotype

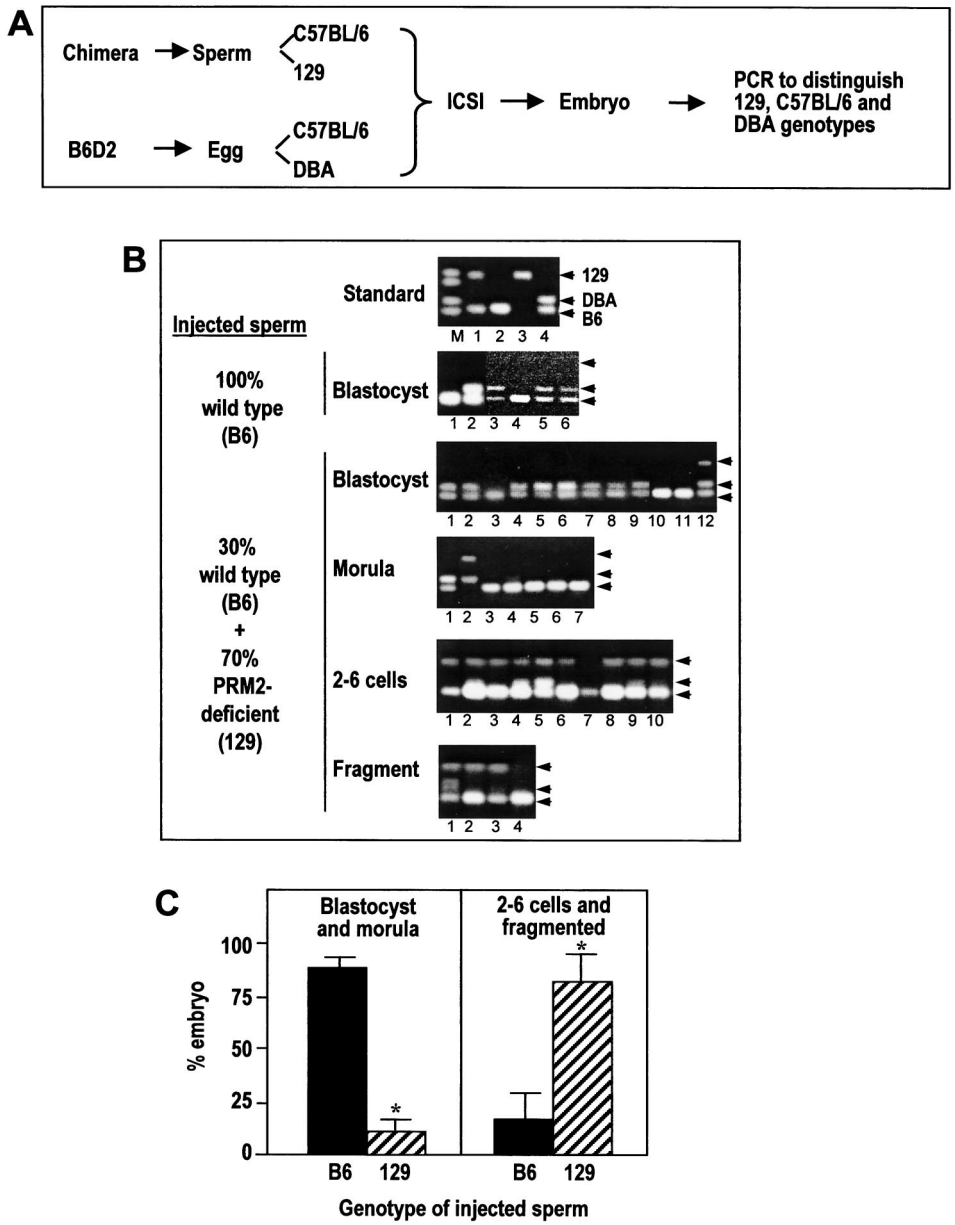

FIG. 4. ICSI with sperm from *Prm2* chimera mice. **A**) Experimental procedure. Eggs from B6D2-strain mice were injected with sperm from a *Prm2* chimera and allowed to develop in culture. The embryos that resulted were subjected to PCR genotyping to determine whether C57BL/6- or 129-genotype sperm had been injected. **B**) A PCR primer pair amplifying a microsatellite in each embryo was used to distinguish 129, C57BL/6 (B6), and DBA genotypes. Standards were produced by amplifying DNA from 129- plus C57BL/6-genotype (lane 1), C57BL/6-genotype (lane 2), 129-genotype (lane 3), and DBA- plus C57BL/6-genotype (lane 4) mice. It is likely that the three different genotypes in one of the blastocysts (lane 12) result from contamination during the PCR experiments. More than two genotypes with different band intensity in some of the two- to six-cell stage and fragmented embryos appear to be due to egg polar bodies that remained in the embryos. **C**) Summary of ICSI analysis with sperm from the chimera with a population of 30% C57BL/6-genotype and 70% 129-genotype sperm. Most well-developed embryos (89%) (blastocyst and morula) were derived from C57BL/6-genotype sperm. In contrast, most two- to six-cell stage and fragmented embryos (86%) were derived from 129-genotype sperm. The data are expressed as the mean percentage of embryos ± SD. $^*P <$ 0.05 in relation to the percentage of embryos derived from C57BL/6-genotype sperm.

(~30%) and 129-genotype (~70%) sperm, and oocytes were from B6D2 mice (progeny derived from matings of C57BL/6 and DBA mice). Cultured embryos were analyzed by PCR to identify those developing from eggs injected with 129-genotype sperm or with wild-type sperm from the chimera (Fig. 4A). Sperm from a wild-type mouse were injected as controls, and all six embryos that survived injection developed to the blastocyst stage. A total of 33 eggs survived injection with sperm from the chimera, and 12 developed into blastocysts, 7 developed into morula, 10 developed into two-cell to six-cell stage embryos, and 4 underwent fragmentation. PCR analysis determined that of the 19 embryos that developed to the morula or blastocyst stage, only two (11%) developed from eggs injected with 129-genotype sperm. In addition, 12 (86%) of the 14 embryos that developed only to the two-cell to six-cell stage or underwent fragmentation were derived from 129-genotype sperm (Fig. 4B). The frequency of failures in development that occurred for eggs injected with sperm from the chimera and the genotypes of the failed embryos was comparable to the ratio of 129-genotype to C57BL/6-genotype sperm produced by the chimera. The comet analysis was performed on sperm from the same chimera.

DISCUSSION

We found previously that sperm haploinsufficient for PRM1 or PRM2 failed to give rise to offspring. DNA was released from nuclei of PRM1- or PRM2-deficient sperm in SDS lysis buffer in the absence of a reducing agent, whereas disulfide-bond cleavage was necessary to release DNA from nuclei of wild-type sperm [2]. It is believed that protamines stabilize sperm chromatin by their assembly in the minor groove of DNA into densely packed arrays linked by intermolecular and intramolecular disulfide bonds. In addition, 1) protamine deficiencies are associated with infertility in men [5, 6, 12], 2) the frequency of human sperm with DNA damage correlates with failure of embryonic development after ICSI [7], 3) damage to sperm DNA induced by paternal exposure to cyclophosphamide resulted in greater than 80% peri-implantation progeny loss in rats [13], and 4) the percentage of blastocysts that developed

from mouse sperm exposed to increasing amounts of gamma radiation declined as the degree of DNA damage increased [14]. This observation led us to hypothesize that the failure of chimeras to transmit the 129 genotype to offspring was because haploinsufficiency of protamines results in alteration in the organization and integrity of sperm DNA. The findings in the present study support this hypothesis.

Observations at the light microscope level suggested that some protamine-deficient sperm had abnormally shaped heads [2]. By using scanning electron microscopy, we found the frequency of sperm with small or abnormally shaped heads that were loosely enclosed by the plasma membrane correlated with the percentage of PRM2-deficient sperm. Transmission electron microscopy revealed that the chromatin in PRM2-deficient sperm was less compact than chromatin in sperm from wild-type mice and similar to condensing chromatin in late elongating spermatids. These differences might be due to structural changes that result from the reduced level of PRM2, because the positively charged arginine groups on protamines are believed to neutralize the negatively charged phosphate groups on DNA [15, 16]. Therefore, a reduction in the amount of protamine would change not only the stoichiometry of the major components of the chromatin but also the net charge in the sperm nucleus, thereby affecting chromatin condensation and stability.

Considered along with other studies, our results provide insights into the relationship between PRM2 deficiency and developmental failure after ICSI. To date, the minimal requirement for a functional paternal genome to participate in embryogenesis is the sperm nucleus with a structurally intact nuclear matrix. Sperm nuclei stripped of cytoplasmic components and used for ICSI produced embryos that developed to live offspring [17]. However, treatment with a protease or a disulfide-bond-reducing agent before ICSI resulted in developmental failure [18]. We have found previously that PRM2-deficient sperm are immotile [2]. Impaired sperm motility could be due to loss of sperm viability, leading to chromatin deterioration. In the present study, sperm were mildly sonicated to separate heads and tails before ICSI. It is possible that sonication causes additional damage to altered chromatin integrity of PRM2-deficient sperm. Thus, the developmental failure of some of the eggs injected with PRM2-deficient sperm might be an indirect effect due to damages present in dead sperm and/or induced by sonication. Nonetheless, the aberrant nuclear shape and reduced chromatin density of PRM2-deficient sperm strongly suggest that the structural integrity of these nuclei has been compromised, leading to the failure of embryo development.

Incomplete processing of PRM2 was seen in sperm of mice with heterozygous mutations in *Prm1* and *Prm2* [2] and is frequently seen where there are changes in the level of PRM1 or PRM2 or of other nuclear components. This was observed in sperm of mice with null mutations in *Tp1* [19], *Tp2* [20], and *Camk4* [21, 22]; with suppression of *Prm1* translation in MSY4 gain-of-function transgenic mice [23]; with premature translation of PRM1 in transgenic mice [24]; and in sperm of infertile men [6, 25]. Although infertility occurred in some of these studies [23, 24], it was not seen in others [19–21], suggesting that incomplete processing of PRM-2 is not a major cause of developmental failure that occurs after ICSI with PRM2-deficient sperm.

The defects in condensation of sperm chromatin seen in mice with heterozygous mutations in *Prm1* and *Prm2* [2] also occurred when a transgene-expressing avian protamine was expressed in spermatids [26]; in null mutations of *Tp1* [19], *Tp2* [20], and *Csnk2a2* [27]; with suppression of *Prm1* translation in MSY4 gain-of-function transgenic mice [23]; and in sperm of some infertile men [28]. Since infertility was seen in only some of the studies that observed incomplete DNA condensation [2, 23], defective chromatin condensation alone might not be sufficient to prevent participation of the male genome in development. However, more severe defects in chromatin condensation that include disruption of the nuclear matrix might have an effect on DNA integrity.

PRM2 constitutes from 0% to nearly 80% of the total protamine in sperm from different mammalian species, indicating that PRM1, PRM2, and DNA interact to form complexes in different species with considerably dissimilar stoichiometries [1]. Although the DNA of mammalian sperm is packaged in toroidal subunits substantially larger than nucleosomes [29], protamines do not interact with each other in a consistent manner, and their binding to DNA is sequence independent [1]. However, for a given species, the assembly of protamine and DNA into complexes is likely to have precise stoichiometries. Although spermatids in mice can compensate for overexpression of PRM1 [30] and expression of high levels of avian protamine [26], they fail to compensate for reduction in the amount of PRM1 or PRM2 [2, 21, 23]. This suggests that the set points for expression of PRM1 and PRM2 are near the minimum required for complex formation and DNA protection.

PRM2-deficient sperm have a reduced amount of PRM1, indicating that PRM1 requires the normal amount of PRM2 to become incorporated into sperm chromatin. Although the mechanism responsible for the DNA damage in PRM1/PRM2-deficient sperm remains to be determined, we believe a likely scenario is that haploinsufficiency of PRM2 perturbs the stoichiometries of complex formation by major components of the chromatin. This results in incomplete chromatin condensation, compromising the structural integrity of the nuclear matrix. This condition is exacerbated by a decrease in disulfide bond formation due to the reduced amount of PRM1 and PRM2. As a result of these changes, the DNA is highly susceptible to damage in the reproductive tract by conditions that can be tolerated by sperm with a full complement of nuclear components but not by PRM1/PRM2-deficient sperm.

In summary, we found that 1) the percentage of sperm with damaged DNA seen with the comet assay, 2) the frequency of sperm with abnormally shaped heads seen by electron microscopy, 3) and the percentage of metaphase II-arrested mouse eggs that failed to develop to the blastocyst stage after ICSI were comparable to the percentage of PRM2-deficient sperm in the samples analyzed. Although similar results have been seen individually in other studies, we believe this is the first study to use an animal model to demonstrate that they occur together. These findings strongly suggest that a moderate reduction in protamine levels in sperm results in damage to DNA incompatible with transmission of the male genome to the next generation.

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REFERENCES

1. Corzett M, Mazrimas J, Balhorn R. Protamine 1: protamine 2 stoichiometry in the sperm of eutherian mammals. Mol Reprod Dev 2002; 61:519–527.
2. Cho C, Willis WD, Goulding EH, Jung-Ha H, Choi YC, Hecht NB, Eddy EM. Haploinsufficiency of protamine-1 or -2 causes infertility in mice. Nat Genet 2001; 28:82–86.
3. Braun RE, Behringer RR, Peschon JJ, Brinster RL, Palmiter RD. Genetically haploid spermatids are phenotypically diploid. Nature 1989; 337:373–376.
4. Caldwell KA, Handel MA. Protamine transcript sharing among postmeiotic spermatids. Proc Nat Acad Sci U S A 1991; 88:2407–2411.
5. Balhorn R, Reed S, Tanphaichitr N. Aberrant protamine 1/protamine 2 ratios in sperm of infertile human males. Experientia 1988; 44:52–55.
6. de Yebra L, Ballesca JL, Vanrell JA, Bassas L, Oliva R. Complete selective absence of protamine P2 in humans. J Biol Chem 1993; 268: 10553–10557.
7. Morris ID, Ilott S, Dixon L, Brison DR. The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (comet assay) and its relationship to fertilization and embryo development. Hum Reprod 2002; 17:990–998.
8. Steele EK, McClure N, Maxwell RJ, Lewis SE. A comparison of DNA damage in testicular and proximal epididymal spermatozoa in obstructive azoospermia. Mol Hum Reprod 1999; 5:831–835.
9. Kuretake S, Kimura Y, Hoshi K, Yanagimachi R. Fertilization and development of mouse oocytes injected with isolated sperm heads. Biol Reprod 1996; 55:789–795.
10. Chatot CL, Ziomek CA, Bavister BD, Lewis JL, Torres I. An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. J Reprod Fertil 1989; 86:679–688.
11. Haines G, Marples B, Daniel P, Morris I. DNA damage in human and mouse spermatozoa after in vitro-irradiation assessed by the comet assay. Adv Exp Med Biol 1998; 444:79–93.
12. Belokopytova IA, Kostyleva EI, Tomilin AN, Vorob'ev VI. Human male infertility may be due to a decrease of the protamine P2 content in sperm chromatin. Mol Reprod Dev 1993; 34:53–57.
13. Harrouk W, Codrington A, Vinson R, Robaire B, Hales BF. Paternal exposure to cyclophosphamide induces DNA damage and alters the expression of DNA repair genes in the rat preimplantation embryo. Mutat Res 2000; 461:229–241.
14. Ahmadi A, Ng SC. Fertilizing ability of DNA-damaged spermatozoa. J Exp Zool 1999; 284:696–704.
15. Balhorn R. A model for the structure of chromatin in mammalian sperm. J Cell Biol 1982; 93:298–305.
16. Ward WS, Coffey DS. DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. Biol Reprod 1991; 44:569–574.
17. Ward WS, Kimura Y, Yanagimachi R. An intact sperm nuclear matrix may be necessary for the mouse paternal genome to participate in embryonic development. Biol Reprod 1999; 60:702–706.
18. Ward WS, Kishikawa H, Akutsu H, Yanagimachi H, Yanagimachi R. Further evidence that sperm nuclear proteins are necessary for embryogenesis. Zygote 2000; 8:51–56.
19. Yu YE, Zhang Y, Unni E, Shirley CR, Deng JM, Russell LD, Weil MM, Behringer RR, Meistrich ML. Abnormal spermatogenesis and reduced fertility in transition nuclear protein 1-deficient mice. Proc Natl Acad Sci U S A 2000; 97:4683–4688.
20. Zhao M, Shirley CR, Yu YE, Mohapatra B, Zhang Y, Unni E, Deng JM, Arango NA, Terry NH, Weil MM, Russell LD, Behringer RR, Meistrich ML. Targeted disruption of the transition protein 2 gene affects sperm chromatin structure and reduces fertility in mice. Mol Cell Biol 2001; 21:7243–7255.
21. Wu JY, Ribar TJ, Cummings DE, Burton KA, McKnight GS, Means AR. Spermiogenesis and exchange of basic nuclear proteins are impaired in male germ cells lacking Camk4. Nat Genet 2000; 25:448–452.
22. Wu JY, Means AR. Ca(2+)/calmodulin-dependent protein kinase IV is expressed in spermatids and targeted to chromatin and the nuclear matrix. J Biol Chem 2000; 275:7994–7999.
23. Giorgini F, Davies HG, Braun RE. Translational repression by MSY4 inhibits spermatid differentiation in mice. Development 2002; 129: 3669–3679.
24. Lee K, Haugen HS, Clegg CH, Braun RE. Premature translation of protamine 1 mRNA causes precocious nuclear condensation and arrests spermatid differentiation in mice. Proc Natl Acad Sci U S A 1995; 92:12451–12455.
25. Bench G, Corzett MH, de Yebra L, Oliva R, Balhorn R. Protein and DNA contents in sperm from an infertile human male possessing protamine defects that vary over time. Mol Reprod Dev 1998; 50:345–353.
26. Rhim JA, Connor W, Dixon GH, Harendza CJ, Evenson DP, Palmiter RD, Brinster RL. Expression of an avian protamine in transgenic mice disrupts chromatin structure in spermatozoa. Biol Reprod 1995; 52: 20–32.
27. Xu X, Toselli PA, Russell LD, Seldin DC. Globozoospermia in mice lacking the casein kinase II alpha-catalytic subunit. Nat Genet 1999; 23:118–121.
28. Evenson DP, Larson KL, Jost LK. Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. J Androl 2002; 23:25–43.
29. Balhorn R, Cosman M, Thjornton K, Krishnan VV, Corzett M, Bench G, Kramer C, Lee IV J, Hud NV, Allen M, Preito M, Meyer-Ilse W, Brown JT, Kirz J, Zhang X, Bradbury EM, Maki G, Braun RE, Breed W. Protamine mediated condensation of DNA in mammalian sperm. In: Gagnon C (ed.), The Male Gamete: From Basic Knowledge to Clinical Applications. Vienna, IL: Cache River Press; 1999:55–70.
30. Peschon JJ, Behringer RR, Brinster RL, Palmiter RD. Spermatid-specific expression of protamine 1 in transgenic mice. Proc Natl Acad Sci U S A 1987; 84:5316–5319.