

The Effects of Chemotherapeutic Agents, Bleomycin, Etoposide, and Cisplatin, on Chromatin Remodeling in Male Rat Germ Cells¹

Negar Bagheri-Sereshki,³ Barbara F. Hales,³ and Bernard Robaire^{2,3,4}

³Department of Pharmacology and Therapeutics, McGill University, Montréal, Québec, Canada

⁴Department of Obstetrics and Gynecology, McGill University, Montréal, Québec, Canada

ABSTRACT

The coadministration of bleomycin, etoposide, and cisplatin (BEP) has increased the survival rate of testicular cancer patients to over 90%. Previous studies have demonstrated that BEP induces germ cell damage during the final stages of spermatogenesis, when major chromatin remodeling occurs. Chromatin remodeling permits histone-protamine exchange, resulting in sperm head chromatin compaction. This process involves different epigenetic modifications of the core histones. The objective of these studies was to investigate the effects of BEP on epigenetic modifications to histones involved in chromatin remodeling. Brown Norway rats were treated with BEP, and their testes were removed to isolate pachytene spermatocytes and round spermatids by unit gravity sedimentation. Western blot analyses were conducted on extracted proteins to detect the expression of key modified histones. In a second cohort testes were prepared for immunohistochemical analysis. The stage-specific expression of each modified histone mark in rat spermatogenesis suggests the involvement of these modifications in chromatin remodeling. BEP treatment significantly increased expression of H3K9m and decreased that of H2B (or Hist1h2ba) in pachytene spermatocytes, suggesting that nucleosomes were not destabilized to allow for transcription of genes involved in chromatin remodeling. Moreover, BEP treatment altered the expression of H4K8ac in round and elongating spermatids, suggesting that histone eviction was compromised, leading to a looser chromatin structure in mature spermatozoa. Less-compacted sperm chromatin, with alterations to the sperm epigenome, may have an adverse effect on male fertility.

epigenetics, histones, male fertility, spermatogenesis, testicular cancer

INTRODUCTION

Testicular cancer, the most common cancer affecting men of reproductive age, is rising globally [1]; however, because of early diagnosis and advancements made in treatment protocols [2, 3], mortality rates have declined remarkably. Coadministration of the chemotherapeutic cocktail bleomycin, etoposide,

and cisplatin (BEP) has increased the 5-yr survival rate of testis cancer patients to over 90% [4–6]. We have shown previously that BEP has a persistent effect on sperm chromatin quality, as sperm of patients treated with BEP had significant DNA damage and reduced chromatin compaction for up to 24 mo posttreatment [7]. More recently, in animal studies, we have shown that BEP has persistent effects on proteins in the sperm head involved in chromatin remodeling; spermatozoa of BEP-treated Brown Norway rats had significant elevations in several somatic histones and a concomitant decrease in the expression of protamine 1 (PRM1), the only known protamine (PRM) in rats [8, 9].

In rodents, chromatin remodeling is responsible for the replacement of more than 95% of somatic histones with PRMs, small basic proteins. By forming disulfide bonds, PRMs allow for sperm chromatin compaction, a process indispensable for reproductive success [10]. This dynamic process is initiated in early postmeiotic germ cells, the round spermatids, where the core somatic histones (with the exception of H4) are replaced by their testis-specific variants, including testis-specific H2B (H2B or Hist1h2ba) and H1 histone family member N, testis specific (H1T2 or HIFNT) [11]. In elongating spermatids, the testis-specific variants are replaced by small basic transition proteins (TPs) and finally by PRMs [10]. There are four known TPs and two known PRMs, namely TPs 1 through 4 and PRMs 1 and 2 [12–14]. The TP and PRM genes are transcribed in the premeiotic germ cells, the pachytene spermatocytes, and translated when they are ready to be used in elongating spermatids [14].

Epigenetic regulation is fundamental to the chromatin remodeling process. In premeiotic germ cells, covalent modifications of histones serve to regulate nucleosome stability to control gene transcription [15]. In postmeiotic cells, the same marks serve to direct histone-PRM exchange [16]. Histone acetylation on lysine residues is of particular interest as it directs histone removal by neutralizing the positive charge of histones. In addition, hyperacetylation of histone H4 (recognized as H4 acetylated on lysines 5 and 8) serves as a signal platform for the recruitment of testis-specific bromodomain (BRDT) protein that, through an unknown mechanism, directs histone removal [17]. The role of histone methylation and ubiquitination in transcriptional regulation is well defined [18–21]; in contrast, their role in chromatin remodeling is less clear. However, previous studies have illustrated the stage-specific expression of histone H3 monomethylated on lysine 9 (H3K9m) and histone H2A monoubiquitinated on lysine 119 (H2AK119u) in mouse spermiogenesis, serving as an indication of their involvement in chromatin remodeling [22, 23].

Previously, we proposed that histone eviction was compromised in germ cells of rats treated with BEP, as mature spermatozoa had elevations in the amounts of several histones and a concomitant decrease in PRM1 expression [8]. In addition, the expression of a testis-specific histone variant remained elevated in spermatozoa of BEP-sired offspring,

¹Supported by the Canadian Institutes of Health Research (CIHR) grant (MOP-119275). B.F.H. and B.R. are James McGill Professors.

²Correspondence: Bernard Robaire, Department of Pharmacology & Therapeutics, McGill University, 3655 Promenade Sir-William-Osler, Montréal, Québec, Canada, H3G 1Y6.
E-mail: bernard.robair@mcgill.ca

Received: 11 December 2015.
First decision: 3 February 2016.
Accepted: 11 February 2016.

© 2016 by the Society for the Study of Reproduction, Inc. This article is available under a Creative Commons License 4.0 (Attribution-Non-Commercial), as described at <http://creativecommons.org/licenses/by-nc/4.0>
eISSN: 1529-7268 <http://www.biolreprod.org>
ISSN: 0006-3363

TABLE 1. List of histones that were studied, and details of the antibodies for different experiments.

Depicted protein	Catalog no.	Source	Dilution for IHC (DAB; fluorescence)*	Dilution for Western blotting*
H4K8ac	D0012 (Assay Biotech)	Rabbit polyclonal	1:100; 1:100	n/a
H4K8ac	07-328 (Millipore)	Rabbit polyclonal	n/a	1:1000
H4K5ac	ab51997 (Abcam)	Rabbit monoclonal	1:200; 1:200	1:10 000
H4K12ac	04-112 (Millipore)	Rabbit polyclonal	1:1000; 1:250	1:5000
H3K9m	ab9045 (Abcam)	Rabbit polyclonal	n/a	1:1000
H3K9m	07-450 (Millipore)	Rabbit polyclonal	1:200; 1:50	n/a
H2AK119u	#8240 (Cell Signaling)	Rabbit monoclonal	1:1000	1:2000
H1T2	sc-136702 (Santa Cruz)	Rabbit polyclonal	n/a	1:100
tH2B	07-680 (Millipore)	Rabbit polyclonal	n/a	1:1000
H4	ab177840 (Abcam)	Rabbit monoclonal	n/a	1:1000
H3	ab1791 (Abcam)	Rabbit polyclonal	n/a	1:5000

* n/a, not applicable.

highlighting the possibility that there was a reprogramming of the male germ cell epigenome [9].

The aim of this study was to determine the effects of BEP treatment on histone modifications at all phases of male germ cell chromatin remodeling in order to elucidate a potential mechanism by which BEP treatment alters normal sperm chromatin compaction. We mapped histone modifications associated with chromatin remodeling throughout rat spermatogenesis using immunohistochemical techniques, and determined the effects of BEP on these epigenetic marks during germ cell chromatin remodeling.

MATERIALS AND METHODS

Animals and Treatment

Male Brown Norway rats (4 mo of age) were purchased from Harlan and housed under controlled light conditions (12L:12D) in the Animal Resources Centre of McGill University. Animals were provided with food and water ad libitum and all experiments were conducted in accordance with the principles and procedures outlined in *A Guide to the Care and Use of Experimental Animals* prepared by the Canadian Council on Animal Care (McGill Animal Research Centre protocol 5619).

Rats were treated ($n = 7$ per group) as previously described [8], with a few modifications. Briefly, drug-treated animals received one cycle of 3 wk of the BEP regimen at a therapeutically relevant dose of $0.6\times$, with a $1\times$ dose being equivalent to a human treatment regimen, as adjusted for weight and surface area. Animals were gavaged with 1.8 mg/kg *cis*-platinum (Mayne Pharma) and 9.0 mg/kg etoposide (Novapharm) in 1 ml 0.9% saline on Days 1 through 5 of each week. On Day 2 of each week, rats were given an intraperitoneal injection of 0.9 mg/kg bleomycin (Bristol-Meyers) dissolved in 0.9% saline. All drugs were purchased from the Royal Victoria Hospital pharmacy (Montreal, QC, Canada). The control animals were gavaged with 1 ml of 0.9% saline on Days 1 through 5 of each week. On Day 2 of each week, control rats were intraperitoneally injected with 1 ml of 0.9% saline.

Tissue Collection and Preparation

At the end of the 3-wk treatment period, rats ($n = 7$ per group) were anesthetized with a continuous flow of isoflurane. The abdominal wall was surgically removed and an incision was made in the diaphragm to expose the heart. A perfusion needle attached to a container of 0.9% saline was inserted through the ventricle to reach the aorta. Animals were then cleared for 15 min or until the blood vessels on both testes were cleared. The perfusion needle was connected to a container of modified Davidson fixative and animals were perfused through the abdominal aorta for 20 min, or until both testes were tactilely solid [24]. Then both testes were excised and postfixed for an additional 24 h in the same fixative. Testis sections were prepared by the Histology Facility (Goodman Cancer Research Centre, McGill University) for immunohistochemistry (IHC) and immunofluorescence analyses. Briefly, fixed testes were dehydrated through a series of ethanol changes and xylene and embedded in paraffin; testis sections ($5\ \mu\text{m}$) were mounted on microscope slides. A second cohort of animals ($n = 5$ per group) was euthanized by CO_2 asphyxiation and decapitation; their testes were removed for isolation of spermatogenic cells.

Germ Cell Separation

Pachytene spermatocytes and round spermatids were obtained through cell separation using the STA-PUT velocity sedimentation technique, as described by Bellve et al. [25] and modified by Aguilar-Mahecha et al. [26]. Briefly, both testes were decapsulated to remove the tunica albuginea and large blood vessels. Testes were digested to obtain single-cell suspensions, first by enzymatic treatment with 0.5 mg/ml collagenase (Sigma-Aldrich) under continuous agitation for 16 min at 34°C followed by sedimentation and washing, and then with 0.5 mg/ml trypsin (Type 1, T8003; Sigma) and DNase I (Type 1, DN-25; Sigma) for 16 min. After dissociation with flamed glass Pasteur pipettes, tubules were filtered through a $70\text{-}\mu\text{m}$ nylon mesh in the presence of DNase I and washed with RPMI (RPMI medium 160; Invitrogen) containing 0.5% bovine serum albumin (BSA). Following centrifugation and filtration through a $56\text{-}\mu\text{m}$ nylon mesh, 5.6×10^8 cells in 25 ml of RPMI containing 0.5% BSA were loaded into the velocity sedimentation apparatus (STA-PUT; Proscience) and separated on a 2%–4% BSA gradient in RPMI for sedimentation at unit gravity. Fractions of pachytene spermatocytes and round spermatids (steps 1–9) were identified by phase-contrast microscopy. Fractions with greater than 80% purity were pooled, aliquoted, pelleted at $13\,400 \times g$, and stored at -80°C until further analysis.

Immunohistochemistry

For IHC analyses, tissue sections were deparaffinized with xylene and rehydrated through a graded series of ethanol changes (100%, 95%, 70%, 50%, 30%) and finally PBS for 5 min each. Antigen retrieval was done by incubating tissue sections in sodium citrate buffer (10 mM, pH 6.0) for 15 min at 95°C , followed by cooling for 20 min at room temperature (method adapted from Wakayama et al. [27]). Tissue sections were then washed three times in PBS for 5 min. All sections were blocked with PBS containing 1.5% normal goat, rabbit, or mouse serum for 30 min at room temperature to prevent nonspecific antibody binding. They were then incubated with the corresponding primary antibody for 2 h at room temperature (Table 1). After washing three times with PBS for 5 min, the slides were incubated successively with the corresponding biotinylated secondary antibody (1:200 in blocking serum; Vector Laboratories) for 30 min, avidin-peroxidase buffered aqueous solution (Vector Laboratories) for 30 min, and ImmPact DAB (Vector Laboratories). As a negative control, slides were incubated with the corresponding normal serum instead of the primary antibody. IHC with fluorescence detection was done using the Tecan GenePaint System (GenePaint). Briefly, the slides were subjected to the procedure described above up to incubation with primary antibody, then washed 10 times with PBS for 2 min and incubated with an Alexa Fluor 488 secondary antibody (ThermoFisher; 1:200 in PBS) for 2 h at room temperature. Slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI; 1:1000 in PBS) to stain nuclear DNA and mounted with Permafluor antifade mounting medium (Thermo Scientific). Staining was visualized on a Leica SP8MP confocal microscope using a 40/1.30 oil-immersion objective. Fluorescence intensity of proteins in representative cell types was quantified using Imaris software (Bitplane; $n = 4$ per group).

Protein Extraction and Western Blotting

Total protein was extracted from pachytene spermatocytes and round spermatids from control and BEP-treated rats using a radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with proteinase inhibitor cocktail (Sigma) and phosphatase inhibitor (Active Motif) at a concentration of $10\ \mu\text{l/ml}$

of RIPA. Samples were homogenized in RIPA (100 μ l \times 10⁶ cells) by sonication at power 20 (Vibra-Cell; Sonics and Materials) for 3–5 sec on ice. Homogenates were spun down for 10 min at 15 700 \times g at 4°C. The supernatant containing total protein was transferred into a new tube and protein concentrations were determined using the BioRad DC Protein Assay Kit II (Cat. no. 500-0112) according to the manufacturer's instructions (BioRad). Samples were resolved in SDS polyacrylamide (w/v) gradient (4%–12%) Bis-Tris gels (Invitrogen) at 200 V for 35 min using MES (2-[N-morpholino] ethanesulfonic acid) SDS running buffer (Invitrogen). Gels were transferred onto polyvinylidene fluoride membranes (GE Healthcare Life Bio-Sciences Inc.) using NuPAGE transfer buffer (Invitrogen) at 100 V for 1 h. Membranes were washed briefly with Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and blocked with 5% nonfat milk in TBS-T. Proteins were detected using antibodies specific for H4K5ac, H4K8ac, H4K12ac, H3K9m, H2AK119u, HIT2, and tH2B, all diluted in 5% nonfat milk in TBS-T (Table 1). Blots stained for acetylated H4 and methylated H3 were stripped with Western Blot Stripping Buffer (ZmTech Scientifique) for 15 min (as suggested by company protocol), and restained with H4 and H3 antibodies, respectively.

Statistical Analyses

Statistical analyses were done using Prism 6.0 (GraphPad Software, Inc.). Data, presented as means and standard errors of the means, were analyzed using the Student *t*-test.

RESULTS

BEP Treatment Prevents Chromatin Remodeling in Pachytene Spermatocytes and Round Spermatids

Using Western blot analysis, we determined the expression levels of activation and repressive histone marks in pachytene spermatocytes and round spermatids from control and BEP-treated rats (Fig. 1). Specifically, we determined whether BEP treatment affected protein expression of histones, and the amounts of covalent modification on these histones. In pachytene spermatocytes, histone H4 acetylated on lysine 5 (H4K5ac), lysine 8 (H4K8ac), and lysine 12 (H4K12ac) expression levels remained unaltered after BEP treatment (Fig. 1A and Supplemental Table S1; Supplemental Data are available online at www.biolreprod.org). Similarly, BEP treatment did not affect the corresponding unacetylated H4 protein (Supplemental Fig. S1). In round spermatids, H4K5ac and unacetylated H4 protein expression levels were unaffected in BEP-treated animals compared to saline-treated controls. However, there was a clear trend for BEP to increase the level of H4K8ac in round spermatids (fourfold; $P = 0.06$) (Fig. 1A). The corresponding unacetylated H4 protein was unaffected after BEP treatment (Supplemental Fig. S1). In addition, a trend towards a decrease ($P = 0.058$) was observed in the expression of H4K12ac in round spermatids, whereas unacetylated H4 remained unchanged (Fig. 1A and Supplemental Fig. S1).

We then assessed the effects of BEP treatment on the protein expression levels of two repressive marks considered to aid in chromatin compaction, namely H3K9m and H2AK119u (Fig. 1B). In pachytene spermatocytes, a significant increase in the expression of H3K9m was observed after BEP treatment, whereas nonmethylated H3 protein expression level was not affected (Supplemental Fig. S1). BEP treatment did not affect the expression of H3K9m in round spermatids. Moreover, no significant difference was observed in the protein expression of histone H2AK119u in pachytene spermatocytes after BEP treatment (Fig. 1B and Supplemental Table S1). Interestingly, whereas histone H2A was present in round spermatids, the monoubiquitinated form was not observed in this germ cell type (Fig. 1B). Overall, BEP treatment significantly increased the level of methyl-K9 on H3 and decreased tH2B in pachytene spermatocytes.

To investigate the link between BEP treatment and histone replacement, tH2B and HIT2 protein expression levels were quantified in pachytene spermatocytes and round spermatids (Fig. 1C). Expectedly, we did not observe HIT2 in pachytene spermatocytes and its expression in round spermatids was not significantly altered after BEP treatment. Strikingly, BEP treatment significantly reduced the expression of tH2B in pachytene spermatocytes but not in round spermatids. Overall, BEP treatment significantly decreased the level of methyl-K9 on H3 and tH2B in pachytene spermatocytes; in round spermatids, there was a clear trend for BEP treatment to increase the level of acetyl-K8 on H4 and decrease acetylated K12 on H4 ($P = 0.06$).

Histone Marks Involved in Chromatin Remodeling Display Stage and Cell Specificity in Rat Spermatogenesis

We used immunohistochemical analysis to determine the stage- and cell-specific distribution of activating and repressive histone marks involved in chromatin remodeling in spermatogenesis. Our analysis revealed that levels of both activating marks, H4K5, K8, and K12 acetylation, and repressive marks, H3K9m and H2AK119u, were highly dynamic during germ cell development (Fig. 2 and Table 2). These epigenetic modifications were all observed at the onset of spermatogenesis, with strong signals present in type A spermatogonia to stage VIII pachytene spermatocytes, inclusively. Differences in expression pattern and stage and cell specificity were apparent in germ cell types that followed pachytene spermatocytes. H4K8 and K5 acetylation displayed similar stage and cell specificity and expression patterns; positive signals were found in stages IX–XII pachytene spermatocytes, germ cells undergoing meiosis, and steps 1–8 round spermatids, at a progressively weaker signal. A strong signal reappeared in step 9 round spermatids that was maintained in elongating spermatids steps 10–13 but disappeared entirely in the elongated spermatids and spermatozoa. H4K12ac displayed a similar pattern of expression with the exception of steps 7 and 8 round spermatids, where no intensity was observed (Fig. 2B and Table 2).

Interestingly, the repressive marks, H3K9m and H2AK119u, displayed very different stage and cell specificities, and dramatic expression pattern differences (Fig. 2, D and E, and Table 2). For H3K9m, we observed a progressive change from a weak to a very weak positive signal in type A spermatogonia to stage VIII pachytene spermatocytes; the signal was not apparent in stage IX–XII pachytene spermatocytes but reappeared at a weak intensity in germ cells undergoing meiosis. A very weak signal was maintained in step 1–9 round spermatids. The signal appeared weak in step 10–13 elongating spermatids; it was nondetectable in steps 14–18 but reappeared as a weak signal in step 19 elongated spermatids (Fig. 2D). H2AK119u staining was observed exclusively in premeiotic germ cells, contrasting to what had been previously illustrated in mouse spermatogenesis (Fig. 2E) [28]. Overall, our immunohistochemical analyses illustrate the species-specific dynamicity of epigenetic markers involved in chromatin remodeling.

BEP Treatment Has a Stage-Specific Effect on the Expression of Histone Marks

Our Western blot analyses indicated that BEP treatment affected the protein expression of H4K8ac and H4K12ac in round spermatids; we confirmed these results by confocal microscopy and mapped these changes to different spermatogonia.

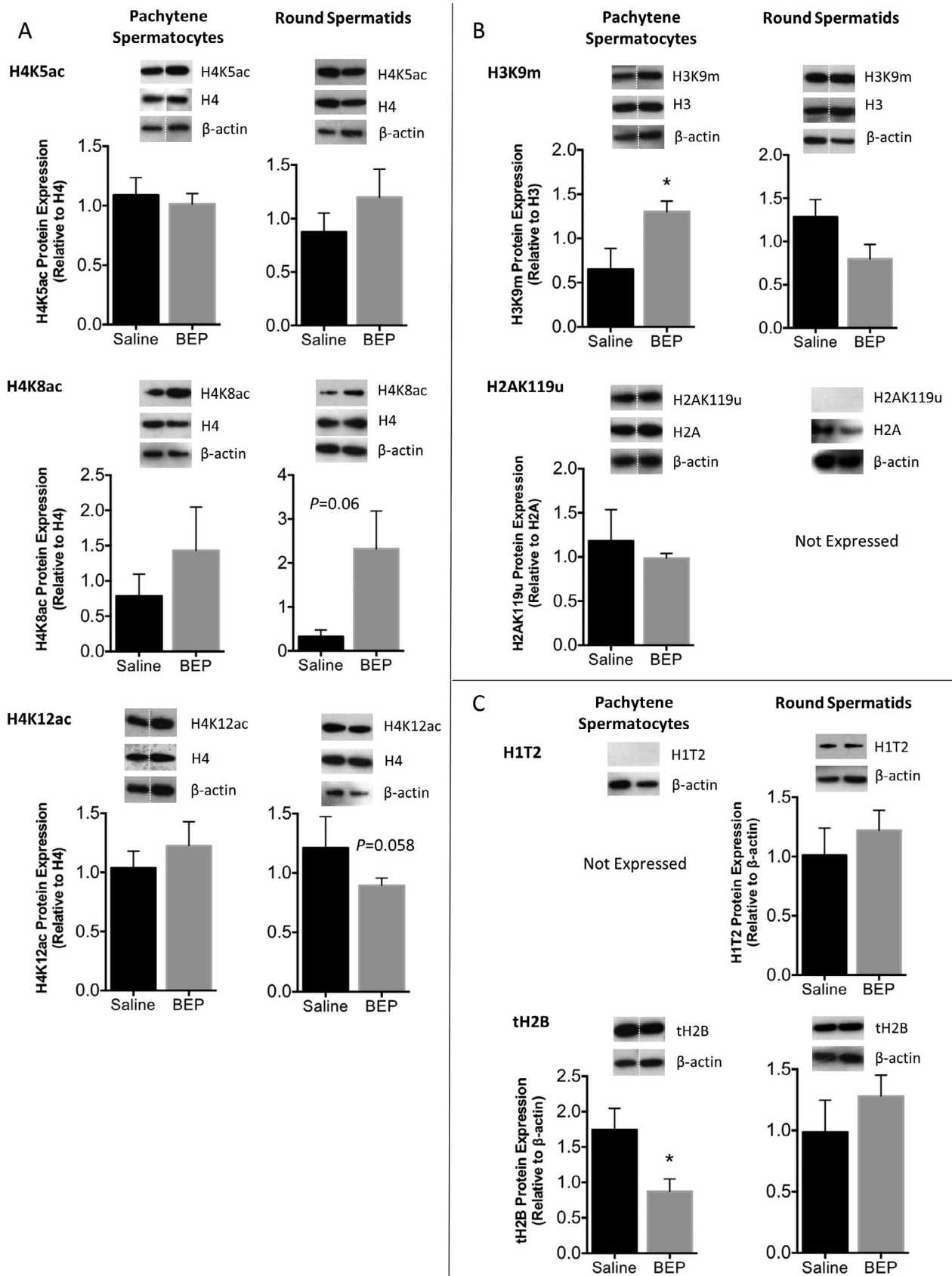


FIG. 1. The effects of a 3-wk treatment with BEP on histones and epigenetic regulators of chromatin remodeling in pachytene spermatocytes and round spermatids. **A**) Activating epigenetic modifications, H4K8ac, H4K5ac, and H4K12ac. **B**) Repressive epigenetic modifications, H3K9m and H2AK119u. **C**) Testis-specific histone variants, H1T2 and tH2B. Bars represent the means \pm SEM. $n = 4-5$. * $P < 0.05$.

EFFECTS OF BEP ON CHROMATIN REMODELING

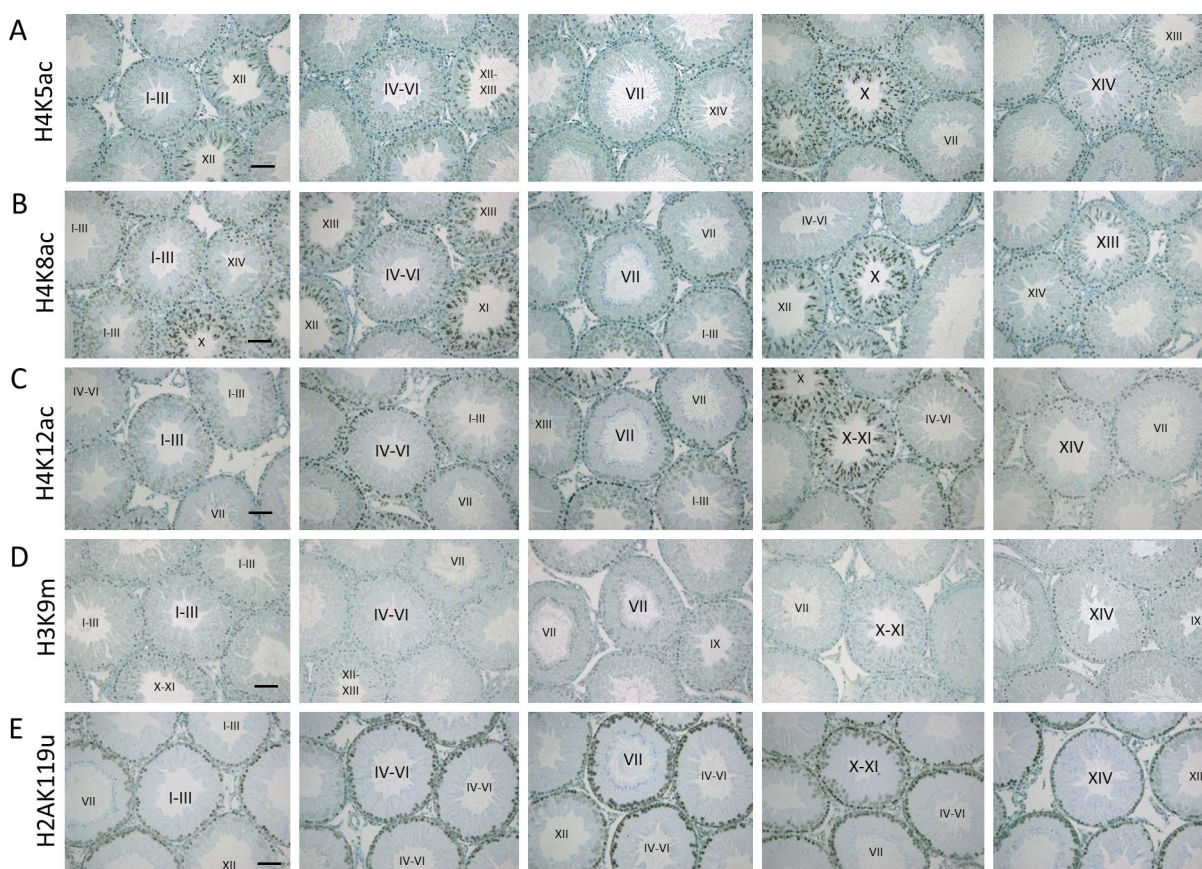


FIG. 2. Immunohistochemical analyses of epigenetic marks involved in chromatin remodeling in rat spermatogenesis. Germ cell and stage specificity and dynamic modulation of epigenetic marks were determined using antibodies specific for H4K5ac (A), H4K8ac (B), H4K12ac (C), H3K9m (D), and H2AK119u (E). Dark brown staining indicates positive reactivity. Roman numerals indicate the stage of spermatogenesis. Bar = 50 μ m.

genic stages. In addition, we determined how BEP affected the protein expression in elongating spermatids for those histone marks that appeared to display a basal expression, as observed in our immunohistochemical analysis. Unexpectedly, BEP treatment significantly decreased the immunofluorescence intensity of H4K8ac in stage I–III round spermatids, and significantly increased its intensity in stage IV–VI round spermatids (Fig. 3A). BEP treatment had no effect on H4K8ac expression in stage VII–VIII round spermatids. However, the fluorescence intensity of H4K8ac was significantly decreased after BEP treatment in stage X–XI elongating spermatids. BEP treatment decreased the fluorescence intensity of H4K12ac in stage I–III round spermatids (Fig. 3B). We were unable to quantify this histone modification in round spermatids of later

stages because it displayed a fluorescence that was below the threshold for quantification (Supplemental Fig. S2). BEP treatment did not affect the fluorescence intensity of H4K12ac, H4K5ac, or H3K9m in elongating spermatids (Fig. 3B and Supplemental Fig. S3, A and B). Overall, these findings confirm our Western blot analysis and demonstrate that the changes induced by BEP treatment on histone marks involved in chromatin remodeling are cell type and stage specific.

DISCUSSION

The sequential replacement of somatic histones with testis-specific variants, TPs, and then with PRMs must be highly regulated in order to produce spermatozoa with properly compacted chromatin. Previously, we showed that

TABLE 2. Spermatogenic cell distribution patterns of H4K5ac, H4K8ac, H4K12ac, H3K9m, and H2AK119u in rat testis.*

Spermatogonia	Staining intensity									
	Spermatocytes					Round spermatids			Elongating spermatids	Elongated spermatids
	A, B	R, L, Z	PI–VIII	PIX–XII	Meiosis	Steps 1–6	Steps 7–8	Step 9	Steps 10–13	Steps 14–19
H4K5ac	S	S	S	M	S	W	S	S	S	N
H4K8ac	S	S	S	M	S	W	S	S	S	N
H4K12ac	S	S	S	W	S	W	N	S	S	N
H3K9m	W	W	W	N	W	V	V	V	W	N (W in s.19)
H2AK119u	S	S	M	W	N	N	N	N	N	N

* A, type A spermatogonia; B, type B spermatogonia; R, preleptotene spermatocytes; L, leptotene spermatocytes; Z, zygotene spermatocytes; PI–VIII, pachytene spermatocytes stages I–VIII; PIX–XII, pachytene spermatocytes stages IX–XII; S, strong intensity; M, medium intensity; W, weak intensity; V, very weak; N, no intensity; s., step.

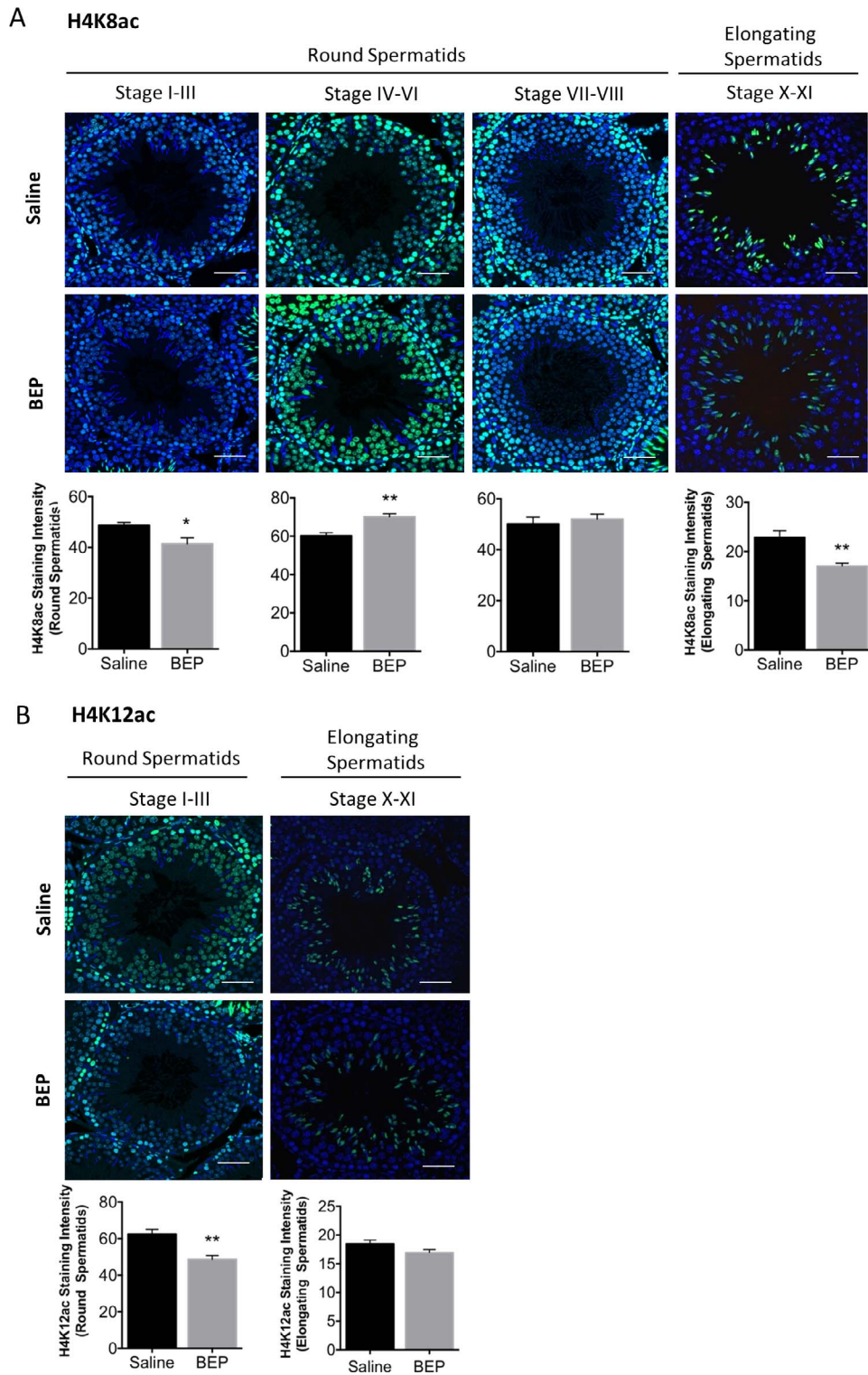


FIG. 3. Confocal microscopy analyses of H4K8ac (A) and H4K12ac (B) in round and elongating spermatids, as indicated. Illustrated are merged images of the indicated antibody staining (green) and nuclear DAPI staining (blue). Immunofluorescent intensity of proteins in representative cell types was quantified using Imaris software. Bars represent means \pm SEM. $n = 4$. * $P < 0.05$, ** $P < 0.01$. Bar = 50 μ m.

histone eviction was compromised after BEP treatment, as there were changes in regulators of histone removal, an increase in the amounts of histones retained, and a decrease in the level of PRM1 in mature spermatozoa [8]. We hypothesized that the germ cell epigenome was the target of this drug effect. Indeed, our results showed that BEP treatment altered histone marks associated with chromatin

remodeling well before histone-PRM exchange, specifically in premeiotic germ cells, at a time when critical postmeiotic genes are being transcribed [29]. The BEP-induced increase in H3K9m, a repressive histone mark, in pachytene spermatocytes suggests that chromatin was not appropriately uncompacted to aid in histone removal, leading to transcriptional repression in these germ cells. In fact, the

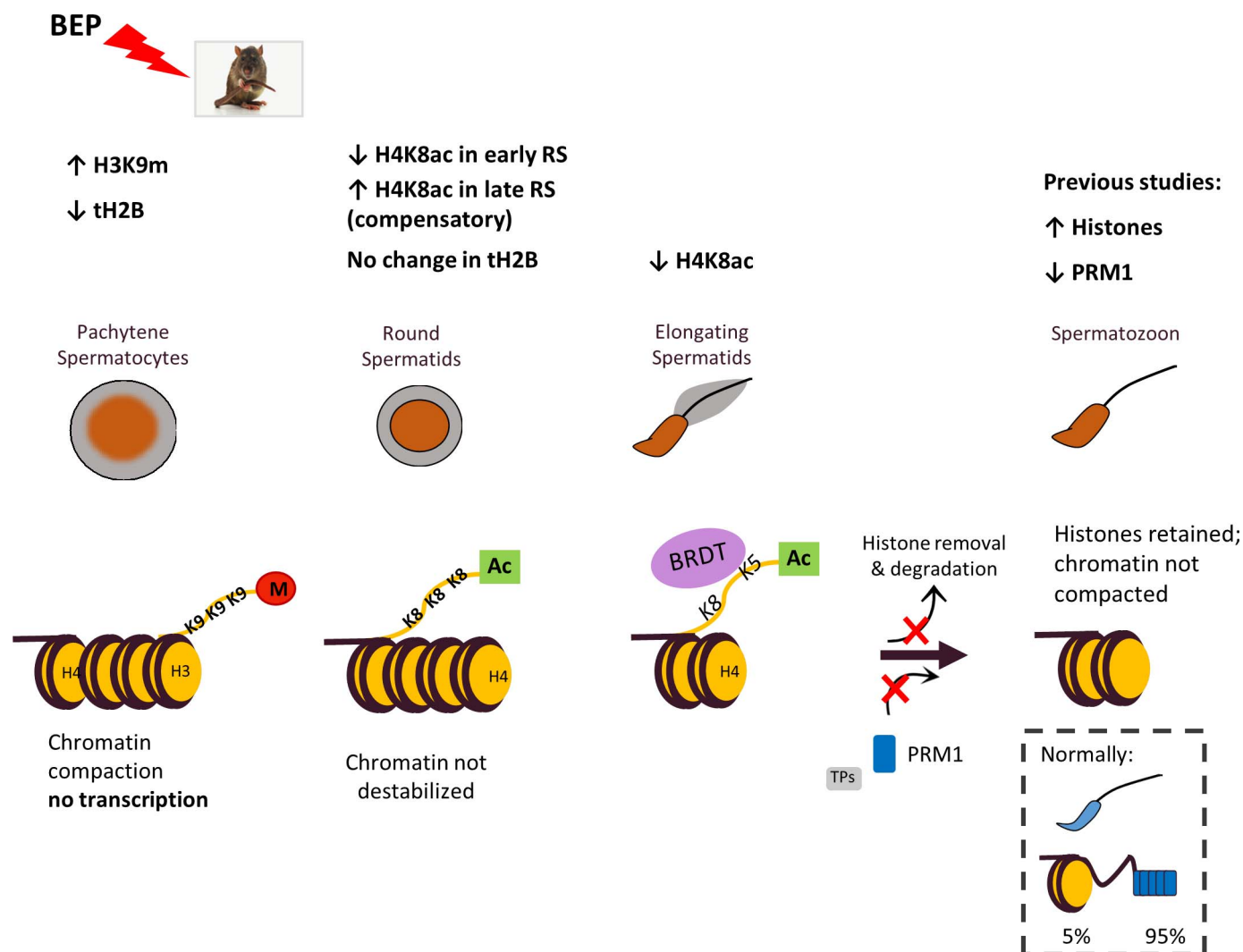


FIG. 4. Treatment of adult male Brown Norway rats with BEP chemotherapy for 3 wk increases H3K9m and decreases tH2B in pachytene spermatocytes, leading to chromatin compaction in these premeiotic germ cells. In round spermatids, BEP treatment reduces H4K8ac to prevent nucleosome destabilization, suggesting that somatic histones are not replaced with testis-specific variants. In elongating spermatids, BEP treatment reduces H4K8ac, suggesting that the affinity of BRDT on histone H4 is reduced. This may prevent the removal and degradation of histone H4 and subsequent TP and PRM1 binding. As a consequence, the nuclei of spermatozoa remain uncondensed.

transcriptional activation of the genes encoding TP and PRM1 is tightly regulated by KDM3A, an H3K9-specific demethylase; a loss of *Kdma3a* is associated with reduced transcription of TP and PRM1 genes [18].

The exact function of testis-specific histone H2B (tH2B) in pachytene spermatocytes is unknown, as its gene shares regulatory elements with neighboring *tH2a* genes [30, 31]. However, it has been proposed that tH2B is a part of the intermediary structures that regulate nucleosomal unwinding for chromatin remodeling in spermiogenesis [32]. This is in agreement with previous studies that have reconstructed chromatin containing human tH2B in vitro and have revealed, by biochemical analysis, that they are quite unstable [33]. Therefore, our results showing a decrease in the protein expression of tH2B in pachytene spermatocytes further support the hypothesis that BEP treatment prevents histone removal in these premeiotic germ cells.

The monoubiquitination of histone H2A on lysine 119 is implicated in transcriptional regulation [20]. In fact, H2AK119u is associated with heterochromatic structures in pachytene spermatocytes, serving as evidence for its role in

transcriptional silencing [21]. Unexpectedly, BEP treatment did not affect the protein expression of H2AK119u in pachytene spermatocytes. We propose that the inability of nucleosomes to be unwrapped in pachytene spermatocytes after BEP treatment may prevent the transcription of genes important for chromatin remodeling in spermiogenesis, including genes for TPs and PRMs [29].

In round spermatids, chromatin remodeling is partly responsible for the replacement of almost all somatic histones with testis-specific variants [34]. The binding of testis-specific variants leads the genome into a transitional state that involves nuclear chromatin destabilization. In contrast to premeiotic germ cells, where this would lead to transcriptional activation, the destabilization in round spermatids is required for and directly precedes TP/PRM loading [34]. In this study, we found that BEP treatment leads to an increasing trend in H4K8ac expression, and we map this trend to a specific group of round spermatids. Unexpectedly, we found that BEP treatment significantly decreases this epigenetic mark in round spermatids at the early stages of development, but increases its protein expression significantly in late round spermatids. Interestingly,

BEP treatment also decreases the expression of H4K12ac in early round spermatids, suggesting that BEP treatment leads to chromatin compaction in these early postmeiotic germ cells. Therefore, we hypothesize that the tendency for the increase of H4K8ac in late round spermatids is a compensatory mechanism to enhance nucleosome destabilization, as molecular events in these germ cells directly lead to histone-PRM exchange, a process that requires nucleosomal unwinding [15].

Testis-specific histones and much of the somatic histone H4 are replaced with PRMs during spermiogenesis. It has been proposed that the transcription-independent hyperacetylation of histone H4 on two specific lysine residues, K5 and K8, precedes this major chromatin remodeling event [35, 36]. The hyperacetylation of H4 sets the epigenetic platform for the binding of BRDT protein that aids in the removal of histone H4. Our results reveal that BEP treatment significantly reduces the protein expression of H4K8ac in stage X–XI elongating spermatids, the window before histone-PRM exchange [14]. These results strongly support our previous findings showing that even after a 9-wk recovery period (during which no BEP was administered), mature spermatozoa had a significant decrease in protamination and persistent retention of histones [8]. In addition, altered chromatin of the fertilizing sperm resulted in a decrease in the number of live progeny, and altered expression of reproduction-related genes in the developing testis of surviving offspring [9]. These results highlight the importance of the sperm epigenome on early embryo development and the potential transgenerational effect of BEP-induced histone retention in spermatozoa.

To the best of our knowledge, this is the first study to report a change in the epigenetic markers of chromatin remodeling in rat germ cells following a drug treatment. In addition, we have mapped the basal expression of these epigenetic marks during rat spermatogenesis. Our findings demonstrate that the effect of BEP treatment on sperm chromatin remodeling is at the level of the histone marks. As outlined in Figure 4, our results suggest that BEP treatment perturbs epigenetic histone marks in pachytene spermatocytes to prevent chromatin destabilization, thus preventing the transcription of postmeiotic genes such as *Tp1* and *Prm1*. This effect is carried through to the round spermatids to block transitional states leading up to histone-PRM exchange. Moreover, we propose that BEP treatment prevents final histone removal in elongating spermatids that await TP1 and PRM1 incorporation, resulting in less-compacted sperm chromatin.

ACKNOWLEDGMENT

We should like to thank Dr. Wolfgang Reintsch (McGill University) for assistance with confocal microscopy, and Yaned Gaitan (McGill University) for assistance with immunohistochemistry.

REFERENCES

- Shanmugalingam T, Soultati A, Chowdhury S, Rudman S, Van Hemelrijck M. Global incidence and outcome of testicular cancer. *Clin Epidemiol* 2013; 5:417–427.
- Elzinga-Tinke JE, Dohle GR, Looijenga LHJ. Etiology and early pathogenesis of malignant testicular germ cell tumors: towards possibilities for preinvasive diagnosis. *Asian J Androl* 2015; 17:381–393.
- Robinson D, Moller H, Horwich A. Mortality and incidence of second cancers following treatment for testicular cancer. *Br J Cancer* 2007; 96: 529–533.
- Einhorn LH, Foster RS. Bleomycin, etoposide, and cisplatin for three cycles compared with etoposide and cisplatin for four cycles in good-risk germ cell tumors: is there a preferred regimen? *J Clin Oncol* 2006; 24: 2597–2598. Author reply 2598–2599.
- Huddart RA, Birtle AJ. Recent advances in the treatment of testicular cancer. *Expert Rev Anticancer Ther* 2005; 5:123–138.
- Kopp HG, Kuczyk M, Classen J, Stenzl A, Kanz L, Mayer F, Bamberg M, Hartmann JT. Advances in the treatment of testicular cancer. *Drugs* 2006; 66:641–659.
- O’Flaherty CM, Chan PT, Hales BF, Robaire B. Sperm chromatin structure components are differentially repaired in cancer survivors. *J Androl* 2012; 33:629–636.
- Maselli L, Hales B, Chan P, Robaire B. Exposure to bleomycin, etoposide, and *cis*-platinum alters rat sperm chromatin integrity and sperm head protein profile. *Biol Reprod* 2012; 86:166.
- Maselli L, Hales B, Robaire B. The effects of chemotherapy with bleomycin, etoposide, and *cis*-platinum (BEP) on rat sperm chromatin remodeling, fecundity and testicular gene expression in the progeny. *Biol Reprod* 2013; 89:85.
- Balhorn R, Brewer L, Corzett M. DNA condensation by protamine and arginine-rich peptides: analysis of toroid stability using single DNA molecules. *Mol Reprod Dev* 2000; 56:230–234.
- Martianov I, Brancorsini S, Catena R, Gansmuller A, Kotaja N, Parvinen M, Sassone-Corsi P, Davidson I. Polar nuclear localization of HIT2, a histone H1 variant, required for spermatid elongation and DNA condensation during spermiogenesis. *Proc Natl Acad Sci U S A* 2005; 102:2808–2813.
- Lanneau M, Loir M. An electrophoretic investigation of mammalian spermatid-specific nuclear proteins. *J Reprod Fertil* 1982; 65:163–170.
- Meistrich ML, Brock WA, Grimes SR, Platz RD, Hnilica LS. Nuclear protein transitions during spermatogenesis. *Fed Proc* 1978; 37:2522–2525.
- Balhorn R. The protamine family of sperm nuclear proteins. *Biology* 2007; 8:227.
- Kota SK, Feil R. Epigenetic transitions in germ cell development and meiosis. *Dev Cell* 2010; 19:675–686.
- Govin J, Caron C, Lestrat C, Rousseaux S, Khoshbin S. The role of histones in chromatin remodeling during mammalian spermiogenesis. *Eur J Biochem* 2004; 271:3459–3469.
- Pivot-Pajot C, Caron C, Govin J, Vion A, Rousseaux S, Khochbin S. Acetylation-dependent chromatin reorganization by BRDT, a testis-specific bromodomain-containing protein. *Mol Cell Biol* 2003; 23: 5354–5365.
- Okada Y, Scott G, Ray MK, Mishina Y, Zhang Y. Histone demethylase JHDM2A is critical for *Tnp1* and *Prm1* transcription and spermatogenesis. *Nature* 2007; 450:119–123.
- Khalil AM, Wahlestedt C. Epigenetic mechanisms of gene regulation during mammalian spermatogenesis. *Epigenetics* 2008; 3:21–28.
- Cao J, Yan Q. Histone Ubiquitination and deubiquitination in transcription, DNA damage response, and cancer. *Front Oncol* 2012; 2:26.
- An JY, Kim EA, Jiang Y, Zakrzewska A, Kim DE, Lee MJ, Mook-Jung I, Zhang Y, Kwon YT. UBR2 mediates transcriptional silencing during spermatogenesis via histone ubiquitination. *Proc Natl Acad Sci U S A* 2010; 107:1912–1917.
- Sheng K, Liang X, Huang S, Xu W. The role of histone ubiquitination during spermatogenesis. *Biomed Res Int* 2014; 2014:1–8.
- Song N, Liu J, An S, Nishino T, Hishikawa Y, Koji T. Immunohistochemical analysis of histone H3 modifications in germ cells during mouse spermatogenesis. *Acta Histochem Cytochem* 2011; 44:183–190.
- Hermo L. Structural features and functions of principal cells of the intermediate zone of the epididymis of adult rats. *Anat Rec* 1995; 242: 515–530.
- Bellve AR, Cavicchia JC, Millette CF, O’Brien DA, Bhatnagar YM, Dym M. Spermatogenic cells of the prepubertal mouse. Isolation and morphological characterization. *J Cell Biol* 1977; 74:68–85.
- Aguilar-Mahecha A, Hales BF, Robaire B. Expression of stress response genes in germ cells during spermatogenesis. *Biol Reprod* 2001; 65: 119–127.
- Wakayama T, Nakata H, Kumchantuek T, Gewaily MS, Iseki S. Identification of 5-bromo-2’-deoxyuridine-labeled cells during mouse spermatogenesis by heat-induced antigen retrieval in lectin staining and immunohistochemistry. *J Histochem Cytochem* 2015; 63:190–205.
- Baarends WM, Hoogerbrugge TW, Roest HP, Ooms M, Vreeburg J, Hoeijmakers JHJ, Grootegoed, JA. Histone ubiquitination and chromatin remodeling in mouse spermatogenesis. *Dev Biol* 1999; 207:322–333.
- Schultz N, Hamra FK, Garbers DL. A multitude of genes expressed solely in meiotic or postmeiotic spermatogenic cells offers a myriad of contraceptive targets. *Proc Natl Acad Sci U S A* 2003; 100:12201–12206.
- Huh NE, Hwang IW, Lim K, You KH, Chae CB. Presence of a bidirectional S phase-specific transcription regulatory element in the promoter shared by testis-specific TH2A and TH2B histone genes. *Nucleic Acids Res* 1991; 19:93–98.
- Choi YC, Chae CB. Demethylation of somatic and testis-specific histone

EFFECTS OF BEP ON CHROMATIN REMODELING

- H2A and H2B genes in F9 embryonal carcinoma cells. *Mol Cell Biol* 1993; 13:5538–5548.
32. Montellier E, Boussouar F, Rousseaux S, Zhang K, Buchou T, Fenaille F, Shiota H, Debernardi A, Hery P, Curtet S, Jamshidikia M, Barral S, et al. Chromatin-to-nucleoprotamine transition is controlled by the histone H2B variant TH2B. *Genes Dev* 2013; 27:1680–1692.
 33. Li A, Maffey AH, Abbott WD, Conde e Silva N, Prunell A, Siino J, Churikov D, Zalensky AO, Ausio J. Characterization of nucleosomes consisting of the human testis/sperm-specific histone H2B variant (hTSH2B). *Biochemistry* 2005; 44:2529–2535.
 34. Govin J, Escoffier E, Rousseaux S, Kuhn L, Ferro M, Thevenon J, Catena R, Davidson I, Garin J, Khochbin S, et al. Pericentric heterochromatin reprogramming by new histone variants during mouse spermiogenesis. *J Cell Biol* 2007; 176:283–294.
 35. Hazzouri M, Pivot-Pajot C, Faure AK, Usson Y, Pelletier R, Sele B, Khochbin S, Rousseaux S. Regulated hyperacetylation of core histones during mouse spermatogenesis: involvement of histone deacetylases. *Eur J Cell Biol* 2000; 79:950–960.
 36. Moriniere J, Rousseaux S, Steuerwald U, Soler-Lopez M, Curtet S, Vitte AL, Govin J, Gaucher J, Sadoul K, Hart DJ, Krijgsveld J, Khochbin S, Müller CW, Petosa C. Cooperative binding of two acetylation marks on a histone tail by a single bromodomain. *Nature* 2009; 46:664–668.