—Original Article—

Histone H4 Modification During Mouse Spermatogenesis

Yoshiki SHIRAKATA[1], Yuuki HIRADATE[1], Hiroki INOUE[1], Eimei SATO[2] and Kentaro TANEMURA[1]

[1]*Laboratory of Animal Reproduction and Development, Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan*
[2]*National Livestock Breeding Center, Fukushima 961-8511, Japan*

Abstract. The core histone is composed of four proteins (H2A, H2B, H3 and H4). Investigation of the modification patterns of histones is critical to understanding their roles in biological processes. Although histone modification is observed in multiple cells and tissues, little is known about its function in spermatogenesis. We focused on the modification patterns of histone H4 during murine spermatogenesis. We demonstrated that the individual N-terminal sites of H4 show different modification patterns during the differentiation of male germ cells. The methylation pattern varied depending on the residues that were mono-, di-, or tri-methylated. All the H4 modifications were high during the meiotic prophase, suggesting that histone H4 modification plays an important role during this stage of spermatogenesis. Elongating spermatids showed increased acetylation of histone H4, which may be associated with a histone-to-protamine substitution. Our results provide further insight into the specific relationship between histone H4 modification and gene expression during spermatogenesis, which could help to elucidate the epigenetic disorders underlying male infertility.
Key words: Acetylation, Histone H4, Immunohistochemistry, Mouse, Spermatogenesis

Epigenetics is the study of mitotically or meiotically heritable changes in gene expression or cellular phenotype that are caused by mechanisms other than changes in the underlying DNA sequence [1]. As an epigenetic mechanism, histone modification is as important as DNA methylation and plays essential roles in gene inactivation and activation. The core histone is composed of four proteins (H2A, H2B, H3 and H4), and their N-terminal ends can be chemically modified by methylation, acetylation, and phosphorylation [2]. These modifications change the chromatin structure, thereby influencing gene expression [3–5]. Histone methylation is associated with transcriptional activation and inactivation, depending on the histone N-terminal residues involved [6]. Histone acetylation is associated with the activation of gene expression [7, 8], while histone phosphorylation is related to transcriptional activation. Histone modification is a reversible process catalyzed by enzymes such as methyltransferases, demethylases, acetyltransferases and deacetyltransferases [9, 10].

During the process of female germ cell differentiation, histone modification patterns undergo dramatic changes [11–16] that play an important role in oocyte maturation and oogenesis. In comparison, the effect of histone modification on the differentiation of male germ cells remains understudied.

Spermatogenesis is a very unique cell differentiation process, in that it consists of gene recombination, meiosis and the exchange from histones to protamines. Histone modification patterns during spermatogenesis have been reported to perform specific roles [17, 18]. Although the role of histone H3 modifications during spermatogenesis has been examined [19], the functions of the other histone proteins are unknown. Therefore, our research focused on the histone H4 protein. Histone H4 can be acetylated at lysine 5, 8, 12 and 16 and methylated at arginine 3 and lysine 20 on its N-terminal tail. Histone H4 modifications are involved in the regulation of chromatin structure, protein-protein interactions and transcriptional activity through the nuclear hormone receptor [20–23]. In this study, we used immunohistochemical techniques to analyze the modification of histone H4 during spermatogenesis.

Materials and Methods

Animals

We purchased 12-week-old male C57/BL6 mice from SLC (Shizuoka, Japan). The mice were anesthetized with 2,2,2-tribromoethanol. Their testes were surgically removed, fixed with the methacarn fixative (methanol:chloroform:acetic acid = 6:3:1), treated with 100% ethanol and xylene, and then embedded in paraffin. The care and use of all the mice conformed to the Regulations for Animal Experiments and Related Activities at Tohoku University.

Antibodies

Mouse monoclonal antibodies against histone H4 mono-methylation (H4me, sc-134221; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and histone H4 tri-methylation (H4me3, sc-134216; Santa Cruz Biotechnology) and rabbit polyclonal antibodies against histone H4 lysine 5 acetylation (H4K5ac, sc-34264; Santa Cruz Biotechnology), histone H4 lysine 8 acetylation (H4K8ac, sc-8661-R; Santa Cruz
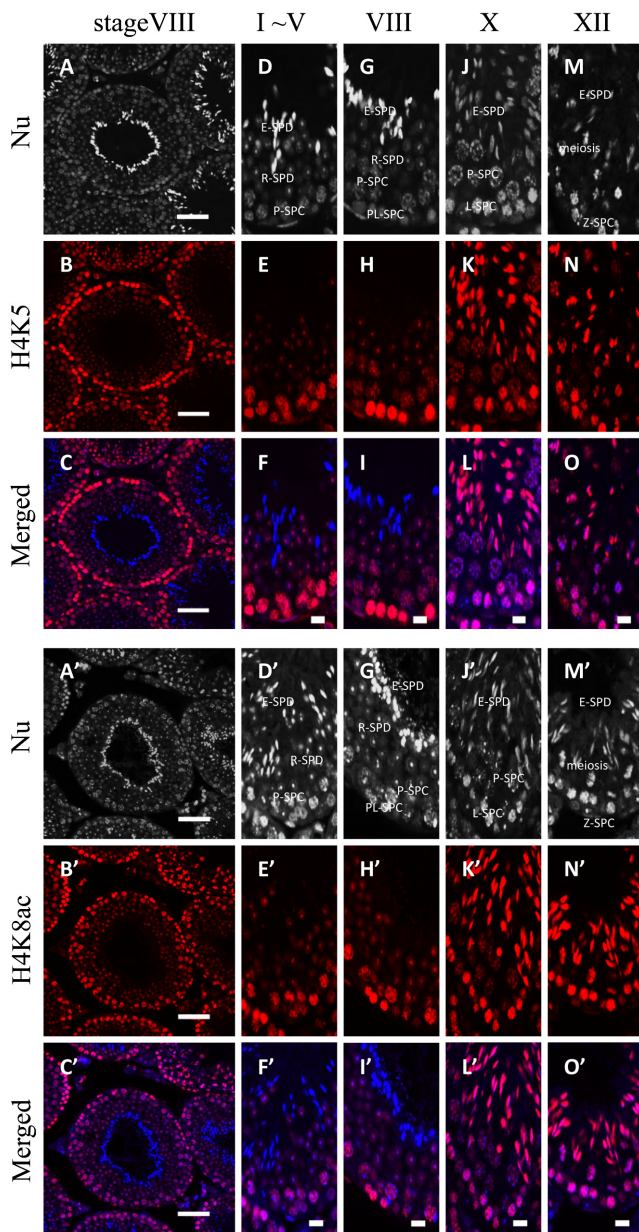
Fig. 1. Immunohistochemical analysis of H4K5ac and H4K8ac. The signals represent nuclear (A, A', D, D', G, G', J, J', M, M') histone H4 lysine 5 acetylation (H4K5ac) (B, E, H, K, N) and histone H4 lysine 8 acetylation (H4K8ac) (B', E', H', K', N'). Stage VIII seminiferous tubules are shown (A– C, A'–C') (G–I, G'–I'). Stage I–V (D–F, D'–F'), stage X (J–L, J'–L') and stage XII (M–O, M'–O') seminiferous tubules are shown. The scale bars represent 50 µm (A, A', B, B', C, C') and 10 µm (F, F', I, I', L, L', O, O'). P-SPC, pachytene spermatocyte; PL-SPC, preleptotene spermatocyte; L-SPC, leptotene spermatocyte; Z-SPC, zygotene spermatocyte; R-SPD, round spermatid; and E-SPD, elongated spermatid.
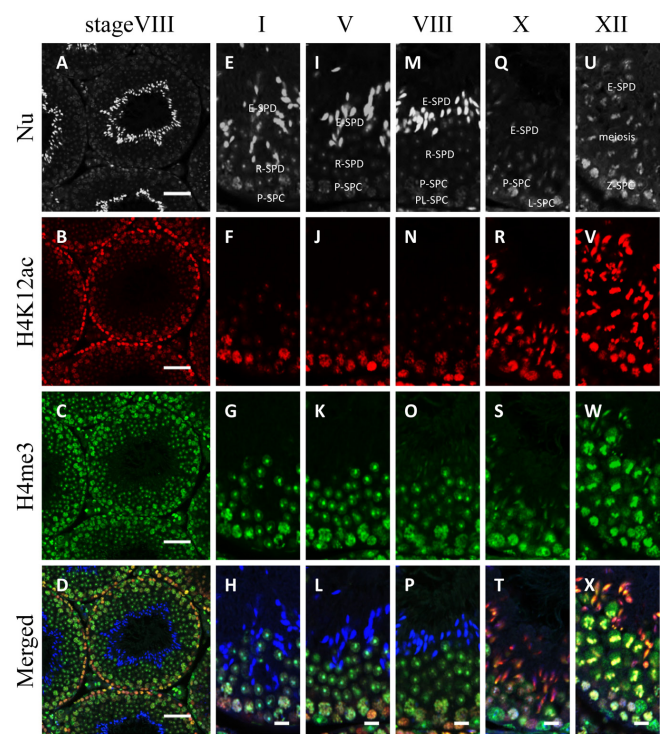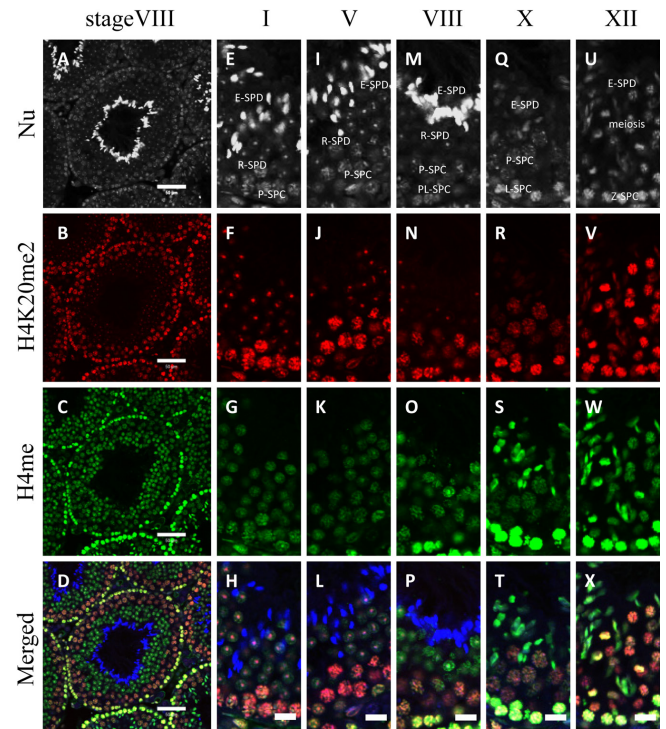
Fig. 2.

Fig. 3.

Biotechnology), histone H4 lysine 12 acetylation (H4K12ac, sc-8661-R; Santa Cruz Biotechnology) and histone H4 lysine 20 di-methylation (H4K20me2, #9759S; Cell Signaling Technology, Danvers, MA, USA) were used as primary antibodies. Alexa Fluor 488-labeled anti-mouse secondary antibodies (Invitrogen, Life Technologies, Carlsbad, CA, USA) were used against H4me and H4me3. Alexa Fluor 594-labeled donkey anti-rabbit secondary antibodies (Invitrogen) were used against H4K5ac, H4K8ac, H4K12ac and H4K20me2.

Immunohistochemistry

Paraffin-embedded sections (10 µm) were mounted on glass slides. The sections were deparaffinized with xylene, dehydrated with ethanol and subsequently incubated with HistoVT One (Nacalai Tesque, Kyoto, Japan) at 90 C for 30 min to mediate antigen retrieval. The sections were then washed with distilled water, incubated with Blocking One (Nacalai Tesque) at 4 C for 1 h and subsequently incubated with primary antibodies at 4 C overnight [the primary antibodies were added to Blocking One (Nacalai Tesque) and phosphate-buffered saline-mixed liquor (diluted 1:200)]. After incubation, the sections were incubated with Alexa Fluor 488-labeled anti-mouse and Alexa Fluor 594-labeled anti-rabbit secondary antibodies (diluted 1:1000) at 4 C for 3 h. The nuclei were counterstained with Hoechst 33342 (diluted 1:5000; Molecular Probes, Eugene, OR, USA). The stained images were obtained using an LSM-700 confocal laser microscope (Carl Zeiss; Oberkochen, Germany), and the fluorescent brightness was analyzed with the ZEN2010 software in conjunction with the LSM-700 microscope. The stage of each seminiferous tubule was determined following the criteria described previously [24].

Comparison of brightness of fluorescence

We used the ZEN-2010 software in conjunction with the LSM-700 microscope and analyzed the mean of fluorescence intensity in the sperm cells. Fluorescence brightness was classified into 255 levels. We categorized the intensity levels over 200 as "strong," those between 100 and 200 as "moderate," those below 100 as "weak" and those that were extra low as "negative."

Results

Histone H4 lysine 5, 8 and 12 acetylation (H4K5ac, H4K8ac and H4K12ac)

H4K5ac, H4K8ac and H4K12ac showed dynamic changes during the differentiation of male germ cells (Figs. 1 and 2). In spermatogonia, the levels of H4K5ac, H4K8ac and H4K12ac were moderately intense. In preleptotene and leptotene spermatocytes,

H4K5ac, H4K8ac and H4K12ac were highly acetylated (Fig. 1H, K, H', K'; Fig. 2N, R). The immunostaining intensity was similar in zygotene spermatocytes (Fig. 1N, N'; Fig. 2V). The expression of H4K5ac, H4K8ac and H4K12ac gradually decreased in pachytene spermatocytes (Fig. 1E–K, E'–K'; Fig. 2F–R). However, H4K5ac, H4K8ac and H4K12ac were highly acetylated during meiosis (Fig. 1N, N'; Fig. 2V). In spermatids, H4K5ac, H4K8ac and H4K12ac were detected in only a portion in step 1–8 spermatids and were highly acetylated in steps 9–12 spermatids (Fig. 1K, N, K', N'; Fig. 2R, V). No acetylation of the histone H4 N-terminal tails was observed in spermatids after step 13.

Histone H4 tri-methylation (H4me3)

H4me3 showed specific exchange during spermatogenesis (Table 1). It showed a weak staining intensity in preleptotene and leptotene spermatocytes (Fig. 2O, S), high intensity staining in zygotene spermatocytes and moderate intensity staining in pachytene spermatocytes (Fig. 2G, K, O, S). High staining intensity was observed during meiosis (Fig. 2W). In the case of round spermatids, a highly modified state was observed in only a portion of the spermatids (Fig. 2G, K, O), and the staining intensity of H4me3 gradually increased in step 9 to 12 spermatids (Fig. 2S, W). However, no stained spermatids were observed after step 13.

Histone H4 lysine 20 di-methylation (H4K20me2)

H4K20me2 varied dynamically during spermatogenesis [25]. Its staining was moderate in spermatogonia, high at the preleptotene spermatocyte stage and similar in leptotene and zygotene spermatocytes (Fig. 3N, R, V). A highly modified state was observed in early pachytene spermatocytes, and this decreased during pachytene stage (Fig. 3F, J, N, R). The expression level of H4K20me2 increased again from diplotene stage to meiotic prophase (Fig. 3V). In round spermatids, H4K20me2 was detected in only a portion of the spermatids and showed a weak intensity by step 9 (Fig. 3F, J, N, R, V). H4K20me2 was not observed in spermatids by step 13.

Histone H4 mono-methylation (H4me)

H4me showed weak expression in spermatogonia. The immunostaining intensity of H4me increased in preleptotene spermatocytes, was similar in leptotene and zygotene spermatocytes (Fig. 3O, S, W), and decreased in pachytene spermatocytes (Fig. 3G, K, O, S). The expression of H4me increased during meiosis (Fig. 3W) and decreased in round spermatids. However, the staining intensity gradually increased in spermatids at around step 8 (Fig. 3O). This state was maintained in spermatids until step 12 but was not observed by step 13.

Fig. 2. Immunohistochemical analysis of H4 tri-methylation and H4 lysine 12 acetylation. The signals represent nuclear (A, E, I, M, Q, U) histone H4 lysine 12 acetylation (H4K12ac) (B, F, J, N, R, V) and histone H4 tri-methylation (H4me3) (C, G, K, O, S, W). Stage VIII seminiferous tubules are shown (A, B, C, D). Stage I (E, F, G, H), stage V (I, J, K, L), stage VIII (M, N, O, P), stage X (Q, R, S, T) and stage XII (U, V, W, X) seminiferous tubules are shown. The scale bars represent 50 µm (A, B, C, D) and 10 µm (H, L, P, T, X). P-SPC, pachytene spermatocyte; PL-SPC, preleptotene spermatocyte; L-SPC, leptotene spermatocyte; Z-SPC, zygotene spermatocyte; R-SPD, round spermatid; and E-SPD, elongated spermatid.

Fig. 3. Immunohistochemical analysis of histone H4 lysine 20 di-methylation and mono-methylation. The signals represent nuclear (A, E, I, M, Q, U) histone H4 lysine 20 di-methylation (H4K20me2) (B, F, J, N, R, V) and histone H4 mono-methylation (H4me) (C, G, K, O, S, W). Stage VIII seminiferous tubule is shown (A, B, C, D). A stage I (E, F, G, H), stage V (I, J, K, L), stage VIII (M, N, O, P), stage X (Q, R, S, T) and stage XII (U, V, W, X) seminiferous tubules are shown. The scale bars represent 50 µm (A, B, C, D) and 10 µm (H, L, P, T, X). P-SPC, pachytene spermatocyte; PL-SPC, preleptotene spermatocyte; L-SPC, leptotene spermatocyte; Z-SPC, zygotene spermatocyte; R-SPD, round spermatid; and E-SPD, elongated spermatid.

Table 1. Modification pattern of histone H4 during mouse spermatogenesis

	Staining intensity										
Spermatogonium	Spermatocyte								Spermatid		
	A+B	PL	L	Z	PI	PVIII	PX	Meiosis	Steps 1–8	Steps 9–12	Steps 13–16
H4K5ac	m	s	s	s	m	m	w	s	w	s	n
H4K8ac	m	s	s	s	m	m	w	s	w	s	n
H4K12ac	m	s	s	s	m	m	w	s	w	s	n
H4me3	m	w	w	s	m	m	m	s	w	m	n
H4K20me2	m	m	s	s	s	m	w	s	w	w	n
H4me	w	s	s	s	w	w	w	s	w m	s	n

PL, preleptotene spermatocyte; L, leptotene spermatocyte; Z, zygotene spermatocyte; PI, pachytene spermatocyte stage I; PVIII, pachytene spermatocyte stage VIII; PX, pachytene spermatocyte stage X; s, strong intensity; m, moderate intensity; w, weak intensity; n, negative.

Discussion

In this study, we demonstrated that modification of the histone H4 N-terminal tails undergoes dramatic changes in male germ cells during spermatogenesis. Spermatocyte development consists of the preleptotene, leptotene, zygotene, pachytene and diplotene stages. Each of the spermatocyte stages shows specific patterns of H4 modification. In preleptotene spermatocytes, the expression of H4me, H4K20me2, H4K5ac, H4K8ac and H4K12ac was high, and that of H4me3 was relatively low. The patterns of staining intensity were similar in leptotene and zygotene spermatocytes. DNA and histone proteins are synthesized in the preleptotene stage [26], and histone acetylation is usually associated with transcriptionally active events [7, 8]. Therefore, it is possible that H4 acetylation is involved in these events during this phase. In preleptotene and leptotene spermatocytes, the histone acetylation state was found to be relatively low compared with that in elongating spermatids [27, 28]. However, since we defined strong intensity as an intensity above a certain fluorescence brightness level, our results showed that both spermatocytes and elongating spermatids had strong intensities. The histone H3 acetylation state in spermatocytes is known to be relatively high [19]. Additional investigation of the relationship between histone acetylation and spermatocyte differentiation is necessary.

The expression of H4K5ac, H4K8ac, K4K12ac and H4me decreased in spermatocytes during the pachytene stage, but H4K20me2 and H4me3 were in a highly modified state. However, H4K20me2 decreased in staining intensity until the late pachytene stage, whereas H4me3 remained relatively high during the pachytene stage. The transcriptional activity is thought to be low in pachytene spermatocytes [26, 29, 30], and it may be associated with H4me3.

During meiosis, all the histone H4 N-terminal tails showed relatively high levels of modification. Since the loss of histone methyltransferase in mouse spermatogenesis is inhibited during meiosis [31–33], it is possible that histone modification plays an important role during the meiotic phase. Histone methylation is associated with both transcriptional activation and inactivation, depending on the histone N-terminal residue involved, while acetylation is associated with transcriptional activation [6, 34]. Both methylation and acetylation states exist in meiosis. The conflicting patterns of histone modification observed in many cells such as embryonic stem cells and spermatogonia may be associated with totipotency [19, 35]. Since numerous biological events are involved in male germ cell meiosis, the conflicting histone modification patterns need further examination.

We found moderate modification of only a portion of the spermatids from step 1 to step 8. Only H4me increased in expression from step 1 to step 8. The nuclei were agglutinated gradually in round spermatids, and then gene expression was generally repressed. These results suggest that low levels of H4 acetylation are perhaps associated with the round spermatid state.

The staining intensity of H4K5ac, H4K8ac, H4K12ac and H4me increased in spermatids from steps 9 to 12. However, those of H4K20me2 and H4me3 were maintained in a lower state. In particular, histone H4 acetylation increased dramatically at around step 10 of the spermatid stage. Histone-to-protamine exchange is known to take place during this period [36]. Although little is known about the mechanism of this exchange, histone acetylation is probably associated with this event [28, 37]. Histone acetylation may also contribute to weakening of the binding between DNA and histone and may be involved in histone-to-protamine exchange. Multiple modification patterns depending on mono-, di-, and tri-methylation may also contribute to the histone-to-protamine exchange.

During spermiogenesis, histone proteins are substituted by transition proteins and then subsequently by protamines. However, a small amount of histone proteins is retained in spermatids, and chemical modification of these histones plays an important role in sperm formation [38]. Nevertheless, the retention of histone H4 modifications was not observed in our experiments.

Comparison of the modification patterns of histones H3 and H4 showed that the H3 and H4ac patterns were similar; however, the patterns of H4me3 differed from those of H3K4me3 and H3K27me3. Thus, it is possible that H4me3 and H3 play different roles during murine spermatogenesis [19, 25].

In this study, we used immunohistochemical methods to show that specific patterns of histone H4 modification are present during murine spermatogenesis. These results can provide further insight into the genetic control (e.g., chromatin remodeling, telomere repair, meiosis and histone-to-protamine exchange) of spermatogenesis and shed light on the epigenetic disorders that involve histone H4.

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