H3K9ac involved in the decondensation of spermatozoal nuclei during spermatogenesis in Chinese mitten crab *Eriocheir sinensis*

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Abstract As a well-known crustacean model species, the Chinese mitten crab *Eriocheir sinensis* presents spermatozoa with decondensed DNA. Our aim was to analyze structural distribution of the histone H3 and its acetylated lysine 9 (H3K9ac) during spermatogenesis for the mechanistic understanding of the nuclear decondensation of the spermatozoa in *E. sinensis*. Using specific antibodies, we followed the structural distribution and acetylated lysine 9 of the histone H3 during spermatogenesis, especially spermiogenesis, of *E. sinensis*. Various spermary samples at different developmental stages were used for histological immunofluorescence and ultrastructural immunocytochemistry. Our results demonstrate a wide distribution of the histone H3 and H3K9ac during spermatogenesis, including spermatogonia, spermatocytes, spermatids, and immature and mature spermatozoa except for absence of H3K9ac in the secondary spermatocytes. Especially during the initial stage of nuclear decondensation, histone H3 lysine 9

was acetylated and then an amount of H3K9ac was removed from within to outside of the nuclei of late spermatids. The portion of remaining H3K9ac was gradually transferred from the nuclei during the stages of spermatozoa maturation. Our findings suggest both the acetylation of histone H3 lysine 9 and the remain of H3K9ac to contribute to the nuclear decondensation in spermatozoa of *E. sinensis*.

Keywords Spermatogenesis · Decondensed nuclei · Histone H3 · Acetylation · *Eriocheir sinensis*

Introduction

Spermatogenesis is the developing process of spermary during which a spermatogonium undergoes a serial morphological and structural change and becomes four mature spermatozoa. The late stage of spermatogenesis during which a spermatid becomes a spermatozoon is called spermiogenesis. Not only the morphology and the structure of spermatozoon but also the state of its nuclear genetic material compactness is significantly different from those of spermatid in most animals. The nuclei of animal spermatozoa are classified as one of three types according to nuclear genetic material compactness. These types are: condensed, semicondensed and decondensed. The nuclei of *Cerebratulus californiensis* and *Carassius auratus* spermatozoa are semicondensed, due to a lack of

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replacement of the four core histones of nucleosomes in spermatozoa nuclei by other basic proteins during spermiogenesis (Muñoz-Guerra et al. 1982; Wang and Ausió 2001). However, the majority of histones in mammalian and human spermatozoal nuclei are replaced by transition proteins and subsequently by protamines during spermiogenesis. As a result, the nuclei in those spermatozoa are condensed. Contrary to the examples mentioned above, nuclei are decondensed in many decapod crustacean spermatozoa (Susan 1969; Vaughn et al. 1969; Vaughn and Thomson 1972; Du et al. 1987b; Shangguan and Li 1994; Simeó et al. 2010; Stewart et al. 2010). The state of decondensation of nuclear genetic material correlates with histone acetylation of the nucleosomes during spermatogenesis, which role is found in most species (Oliva et al. 1990; Nishida et al. 2006; Kurtz et al. 2009a, b; Michael et al. 2010; Bergmann et al. 2012; Dai et al. 2015).

Histones usually are modified including acetylation (Weake and Workman 2008; Berger 2010; Pérez-Cadahía et al. 2010; Du 2012; Healy et al. 2012; Qiao et al. 2015). Acetylation of histones is universal in physiological and pathological somatic cells, where it plays a key role in the regulation of cell function, with special emphasis on increasing the expression of genes associated with the acetyl-histones (Marmorstein and Zhou 2014; Zhang et al. 2015; Qiao et al. 2015). This is also present in male germ cells of many animals and humans where the histone acetylation regulates cell function (Oliva et al. 1990; Rousseaux et al. 2009; Kurtz et al. 2009a, b; Michael et al. 2010; Xia et al. 2012; Bergmann et al. 2012; Qian et al. 2013; Asselman et al. 2015; Gómez et al. 2016; He et al. 2015; Strepetkaitė et al. 2015; Dai et al. 2015). During spermatogenesis in many animals, histones undergo acetylation or hyperacetylation three times, including in the spermatogonia, the spermatocytes and the spermatids (Christensen et al. 1984; Sonnack et al. 2002; Govin et al. 2006; Song et al. 2011; Goudarzi et al. 2014). Especially in the spermatids, histones are widely (hyper)acetylated. This plays a significant role in facilitating the replacement of histones by other basic proteins, which is associated with changes in chromatin compactation (Christensen et al. 1984; Sonnack et al. 2002; Awe and Renkawitz-Pohl 2010; Song et al. 2011). The acetylation or hyperacetylation of histones weakens the affinity between these histones and DNA and subsequently, other basic nuclear proteins such as

transition proteins in the late spermatids replace them (Christensen et al. 1984; Govin et al. 2006; Rousseaux et al. 2009; Lu et al. 2010; Miller et al. 2010; Xia et al. 2012; Qian et al. 2013). Protamines replacing the transition proteins to combine DNA further compacts the chromatin of spermatozoa, which is one of the major events during mammalian spermiogenesis and results in condensed nuclei of mature mammalian spermatozoa (Christensen et al. 1984; Awe and Renkawitz-Pohl 2010; Miller et al. 2010).

Spermatozoa of many decapod crustaceans, however, are decondensed (Susan 1969; Vaughn et al. 1969; Vaughn and Thomson 1972; Du et al. 1987b; Shangguan and Li 1994; Stewart et al. 2010; Simeó et al. 2010). For the last decades, researchers assumed that there is simply no histone present in mature spermatozoa of these decapod crustaceans (Susan 1969; Vaughn et al. 1969; Vaughn and Thomson 1972; Du et al. 1987b; Shangguan and Li 1994; Simeó et al. 2010; Stewart et al. 2010). Interestingly, some histones have recently been identified in nuclei of the mature spermatozoa in several crustaceans of decapod: the brachyuran crab, *Maja brachydactyla*, *Cancer pagurus*, *C. magister*, the blue swimming crab, *Portunus pelagicus*, and the Chinese mitten crab, *Eriocheir sinensis* (Decapod: Crustacea) (Kurtz et al. 2008, 2009a, b; Michael et al. 2010; Wu et al. 2015). Moreover, some acetyl-histones in nuclei of mature spermatozoa in the brachyuran crab, *M. brachydactyla* and *Cancer* were identified via western blot (Kurtz et al. 2008, 2009a, b). However, it remains unclear whether acetyl-histones appear in *E. sinensis* and other crustaceans as well and if it does, it remains to be seen how the acetyl-histones are distributed or modified during spermatogenesis. Tracking the histones and acetyl-histones during spermatogenesis can be helpful to solve these problems and improve our understanding of the mechanisms of nuclear decondensation in the Crustacea spermatozoa.

Eriocheir sinensis is a commercially aquatic crustacean widely bred in China and has a classic decondensed nucleus in their spermatozoon. During the recent three decades, this species has been used as a model system for studying the crustacean spermatogenesis (Du et al. 1987a; Yu et al. 2009; Mao et al. 2012; Hou and Yang 2013; Wang et al. 2014; He et al. 2015; Wang et al. 2015; Wu et al. 2015). Spermatogenesis in this crab also includes the stages of spermatogonia, spermatocytes and spermiogenesis as

in other animals (Du et al. 1987a; Wu et al. 2015). The male germ cells from spermatogonia to spermatocytes to spermatids to spermatozoa are orderly arranged from the germinal zone at one side of seminiferous tubule wall to the opposite side, which present a series of reproductive waves. The reproductive waves refer to a certain type of cells that appear in a given zone of the seminiferous tubule wall. There are different male germ cells in the spermaries at the diverse developmental stages. Spermatogonia are the only or major male germ cells on the seminiferous tubule wall at the spermatogonia stage while spermatocytes are major ones at the spermatocyte stage. The late stage of spermatogenesis is called spermiogenesis. The spermiogenesis is divided into three stages: the early stage, the mid stage and the late stage. During spermiogenesis, the round nuclei of spermatids undergo a metamorphosis and shape into a cup-shaped form to surround a new acrosome, omitting most of the cytoplasm. And ultimately, the spermatids become mature Spermatozoa (Du et al. 1987a). Acetylated lysine 9 of histone H3 (H3K9ac) is essentially related to transcriptional activation in human cell, human embryonic stem cell differentiation and mammalian complex chromatin remodeling during spermiogenesis (Yan et al. 2003; Nishida et al. 2006; Bergmann et al. 2012; Qiao et al. 2015). Therefore, in this study, we explored the structural distributions of the histone H3 and H3K9ac during spermatogenesis of *E. sinensis*. Using specific antibodies, histological immunofluorescence and ultrastructural immunocytochemistry, we sought to gain understanding of the mechanism of nuclear decondensation of the *E. sinensis* spermatozoa.

Materials and methods

Animals

Immature males of *E. sinensis* at different developmental stages as well as adult males were collected from the Baiyangdian Lake in Anxin County, Hebei Province, and purchased from a local fishery market in Baise City, Guangxi Zhuang Autonomous Region, China. The crabs were maintained in aerated tanks (100 cm × 40 cm × 50 cm) with a water depth of about 10 cm for one week. To ensure a sufficient amount of dissolved oxygen in the water, inflatable pumps worked continuously. The crabs were

regularly fed with mixed food (fish, potatoes, grass, etc.) under a natural light cycle. To ensure high water quality, we changed the water in the tanks daily to remove food residues and excreta.

Antibodies

Rat anti-histone H3 monoclonal antibody (DCABH-50) and anti-H3K9ac produced in rabbit, affinity isolated antibody (H9286) were purchased from Creative Diagnostics (Shirley, NY, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. FITC-conjugated affinipure goat anti-rat IgG antibodies were purchased from Proteintech Group, Inc. (Chicago, IL, USA). Red-conjugated goat anti-rabbit IgG antibodies, goat anti-rat IgG antibodies and anti-rabbit IgG antibodies conjugated to 25 nm gold were purchased from Solarbio (Beijing, China).

Tissue preparation

The males were divided into four groups according to the size: the first group 1.5–3.5 g, the second one 6.0–8.0 g, the third one 10.5–12.5 g, and the fourth one beyond 25 g. The spermaries were separated from each group, respectively, immediately placed in 4 °C precold PBS and cut into no more than $0.5 \times 0.5 \times 0.5$ mm³ sections. The specimens were immediately submerged in a fixing buffer (50 mmol/L PBS, pH 7.3, containing 4% (v/v) paraformaldehyde and 0.25% (v/v) glutaraldehyde) in 1.5 ml centrifugal tubes for 2 h at 4 °C and subsequently underwent a series of processes of dehydration. For this, we used increasing ethanol concentrations and 100% acetone, and penetration with 3:1, 1:1, and 1:3 (acetone: Epon812) (epoxy resin). The resulting samples were transferred to and embedded in Epon812 in gelatin capsules.

Immunofluorescence

The resin-embedded 1.6 µm semi-thin sections of the samples were cut on a Reichert ultramicrotome (Optische Werke AG, Vienna, Austria). All sections were incubated with our primary antibodies diluted 1:100 and the secondary antibody diluted 1:500 in 50 mmol/L TBST at room temperature for 2 h, respectively. Data extraction was carried out under a BX51+DP71 fluorescence microscope fitted with

Fig. 1 Localization of histone H3 and H3K9ac (acetyl-H3 lysine 9) in the spermatogonia in *E. sinensis*. **a–d** (Immuno)fluorescent localization of histone H3 (green), H3K9ac (red) and DNA (blue). **e, f** Subcellular distribution (white arrows) of histone H3 and H3K9ac: both histones H3 and H3K9ac are present in the nucleus (N) but not in the cytoplasm (CP). (Color figure online)

appropriate filters (Olympus, Tokyo, Japan). All images were captured with an IPE (Image-Pro Exoress 6.0) (Olympus, Tokyo, Japan). The nuclei were stained with DAPI contained in the mounting medium (Vectashield, Vector Laboratories, Burlingame, CA, USA) (Wu et al. 2015). Negative control sections were processed similarly but were incubated with 10% (m/v) goat serum albumin (Sigma, St. Louis, MO, USA) in TBST in place of the primary antibodies.

Colloidal gold labeling and electron microscopy

All resin-embedded samples were cut to ultrathin sections of 80 nm on a Reichert ultramicrotome and subsequently collected on gold grids coated with a coat buffer consisting of chloroform containing 0.25% (m/v) Formval (Feinbio Chemical, Heidelberg, Germany). The gold grids with the ultrathin sections were

incubated for 1.5 h at room temperature with primary antibodies diluted 1:10 and secondary antibodies diluted 1:100 in 50 M TBST, containing 0.5% (m/v) goat serum albumin, respectively. The primary antibodies were replaced with 0.5% (m/v) goat serum albumin in control sections of the TBST. Ultrathin sections were examined under a JEM-100SX electron microscope at 75 kV (NEC, Tokyo, Japan).

Quantitative evaluation of gold particle distribution

Primary spermatocytes, early and late spermatids, and mature spermatozoa were chosen to measure the gold labeling density in the nuclei using Image-Pro Plus 5.1 software (Media Cybernetics, Silver Spring, MD, USA) (Wu et al. 2015). Statistics were performed by

Fig. 2 Localization of histone H3 and H3K9ac (acetyl-H3 lysine 9) in the spermatocytes in *E. sinensis*. **a–f** Primary spermatocytes; **a–d** (Immuno)fluorescent localization of histone H3 (green), H3K9ac (red) and DNA (blue). **e** and **f** Subcellular distribution (white arrows) of histone H3 and H3K9ac: both histones H3 and H3K9ac are present in the nucleus (N) but not in the cytoplasm (CP). **g–l** Secondary spermatocytes (purple arrows; yellow arrows indicate spermatids); **g–j** (Immuno)fluorescent localization of histone H3 (green), H3K9ac (red) and DNA (blue). **k, l** Subcellular distribution (white arrows) of histone H3 and H3K9ac: only histone H3 but no H3K9ac is present in the nucleus (N) and there were neither histone H3 nor H3K9ac in the cytoplasm (CP). (Color figure online)

Fig. 3 Localization of histone H3 and H3K9ac in the spermatids in *E. sinensis*. **a–h** (Immunofluorescent localization of histone H3 (green), H3K9ac (red) and DNA (blue). **i–n** Subcellular distribution (black arrows) of histone H3 and H3K9ac. We observed both histones H3 and H3K9ac in the nuclei (N) of the early and mid spermatids. Some of H3K9ac has moved out of the nuclei into the proacrosomal vesicles (PV) and the rest remained in the nuclei of late spermatids. Our results indicated that histone H3 was acetylated at the early spermatid stage and then some was transported from the nuclei into the PV at the late spermatid stage. **a–d, i** and **j** Early spermatids. **e–h, k, l** Mid spermatids (white (e–h)/black (k, l), thin arrows). **e–h, m, n** Late spermatids (white (e–h)/black (m, n), bold arrows) in which the cytoplasm (CP) had been discarded and the round nuclei had transformed into cup-shaped nuclei. (Color figure online)

SPSS19.0. The significances of difference were compared among the four typical cell types using analysis of variance and $p < 0.05$ was assumed significant.

Results

Localization of histone H3 during spermatogenesis

The localization via immunofluorescence and immunoelectron microscopy with anti-histone H3 antibodies revealed histone H3 distribution within the nuclei of spermatogonia (Fig. 1b, e), spermatocytes (Fig. 2b,

e, h, k), spermatids (Fig. 3b, f, i, k, m), immature spermatozoa (Fig. 4b, e) and mature spermatozoa (Fig. 5b, e) during spermatogenesis. We saw no histone H3 outside of the nuclei. No immunoreactivity was observed in the negative controls (control data not shown, similarly hereinafter).

H3K9ac distribution in spermatogonia and primary spermatocytes but no in secondary spermatocytes

Both the localization via immunofluorescence and the localization via immunoelectron microscopy with

Fig. 4 Localization of histone H3 and H3K9ac (acetyl-H3 lysine 9) in immature spermatozoa in *E. sinensis*. **a–d** (Immuno)fluorescent localization of histone H3 (green), H3K9ac (red) and DNA (blue). **e, f** Subcellular distribution (short, white arrows) of histone H3 and H3K9ac. Both histone H3 and H3K9ac remained in the nuclei (N) and a little of H3K9ac was also present in the acrosome (A) in the spermatozoa in which the cup-shaped nuclei further became thin and the apical cap (AC) and acrosome tubule (AT) appeared. Our results indicated that a little of H3K9ac continued to be transported out of the nuclei during the spermatozoa stage. (Color figure online)

anti-H3K9ac antibody resulted in immunoreactivity of the nuclei both in the spermatogonia (Fig. 1c, f) and in the primary spermatocytes (Fig. 2c, f), but not outside of the nuclei (Figs. 1c, f, 2c, f). By contrast, there was no immunoreactivity in the secondary spermatocytes (Fig. 2i, l). No immunoreactivity was observed in the negative controls.

Acetylation and the remain of histone H3 during the spermiogenesis

At the metamorphic stage of spermiogenesis, the spermatids gradually changed their shape from spheroid to irregular, accompanied by omission of cytoplasm, emergence of small vesicles in their cytoplasm, and restructuration of cells. After that, vesicles in the cytoplasm fused to form a large proacrosomal vesicle close to the nuclei. Similar to

spermatogonia and primary spermatocytes, localization via immunoelectron microscopy with anti-H3K9ac antibodies showed that the H3K9ac likewise were only distributed in the nuclei of both the early spermatids (Fig. 3j) and the mid ones (Fig. 3l). However, the H3K9ac appeared not only inside but also outside the nuclei of the late spermatids (Fig. 3n) after the nuclei changed shape dramatically from spherical to cup-shaped (Fig. 3n). As for the spermatozoa, we found H3K9ac both within and outside the nuclei of both the immature spermatozoa (Fig. 4f) and the mature ones (Fig. 5f) as well. We observed no labeling on the negative control grids incubated in the absence of the primary antibodies. Localization via immunofluorescence displayed a similar result (Figs. 3c, g, 4c, 5c).

Those results indicated that the lysine 9 of histone H3 was acetylated in the early spermatid during the

Fig. 5 Localization of histone H3 and H3K9ac (acetyl-H3 lysine 9) in mature spermatozoa in *E. sinensis*. **a–d** (Immuno)fluorescent localization of histone H3 (green), H3K9ac (red) and DNA (blue). **e, f** Subcellular distribution (short, white arrows) of histone H3 and H3K9ac. Both histone H3 and H3K9ac remained in the nuclei (N) and a little of H3K9ac was also present in the acrosome (A) in the spermatozoa in which the radial arms (RA) appeared. AC, apical cap; AT, acrosome tubule. (Color figure online)

spermiogenesis, followed by transfer of the H3K9ac from within the nuclei to the cytoplasm during the late spermatid stage. In addition, that H3K9ac appeared outside of the nuclei during the spermatozoa stage indicated that the transfer of H3K9ac also occurred from inside to outside of the nuclei during the stage of spermatozoal mature. However, the most important fact is, that the H3K9ac was always present within the nuclei of both spermatids and spermatozoa, which suggested that the H3K9ac played an important role in nuclei during spermiogenesis.

Quantitative changes of histones H3 and H3K9ac

The quantitative results for histones H3 and H3K9ac at different periods are shown in Fig. 6. The labeling density of histone H3 in the nuclear areas did not change significantly among the primary

spermatocytes, the early spermatids, the late spermatids, and the mature spermatozoa. The labeling density of H3K9ac in the nuclear areas both in the primary spermatocytes and in the early spermatids was significantly higher than that in the late spermatids or in the mature spermatozoa ($p < 0.05$). The H3K9ac labeling was not significantly different between spermatocytes and early spermatids or between late spermatids and mature spermatozoa ($p > 0.05$).

Discussion

Our study showed that both histone H3 and H3K9ac were widely distributed in almost all male germ cells during spermatogenesis. We could locate histone H3 only in the nuclei. In addition, H3K9ac was localized in the nuclei of the various male germ cells except for

Fig. 6 Patterns of nuclear labeling for histones H3 and H3K9ac in different cell types during spermatogenesis in *E. sinensis*. Vertical bars represent the mean $\pm$ SD of the numbers of gold particles. The nuclear labeling density of histone H3 did not show significant differences among the primary spermatocytes, the early spermatids, the late spermatids or the mature spermatozoa. However, H3K9ac labeling in the late spermatids or in the mature spermatozoa decreased significantly compared with that in the primary spermatocytes and in the early spermatids. Different letters above the error bars indicate significant differences ($p < 0.05$) of the given protein labeling in different cell types

the secondary spermatocytes. Interestingly, H3K9ac also appeared outside of the nuclei of both the late spermatids and the spermatozoa. Especially, a large amount of H3K9ac was present in the nuclei of early and mid spermatids, followed by the emergence out of the nuclei of late spermatids. Our results demonstrated that histone H3 lysine 9 was acetylated and then transferred out of the nuclei of male germ cells during the spermatogenesis in *E. sinensis*. The transfer is especially visible at the late spermatid stage. The transfer of H3K9ac also took place during the transformation stage from the immature spermatozoa to the mature ones because H3K9ac appeared outside of the nuclei of both the immature and the mature spermatozoa. It is in the spermatids where the nuclear genetic material of spermatozoa alternates from the semi-condensed state to the decondensed state. Therefore, the present results showed the decondensation of nuclear genetic material during crab spermatogenesis to be accompanied by the processes of both the acetylation of histone H3 and the removal of some H3K9ac from within the nuclei to the vicinity of the nuclei. Wu et al. (2015) found that histone H3 was present in the nuclei of spermatogonia, spermatocytes,

spermatids and mature spermatozoa and the mRNA expression levels of histones H3 were higher at the mitotic and meiotic stages than in later spermiogenesis although the contents of histones H3 were not changed significantly among the three types of cells (spermatocyte, spermatozoon and somatic cell). The difference among the mRNA expression levels of histones H3 might be related to the quantitatively different distribution and modification of histone H3 in various male germ cells during these different stages of *E. sinensis* spermatogenesis. In our study, we explored the distribution of histone H3 and H3K9ac in the spermatogonia, spermatocytes, spermatids and spermatozoa and quantitatively analyzed the distribution of histone H3 and H3K9ac in the primary spermatocytes, the early spermatids, the late spermatids, and the mature spermatozoa. The results showed that there was a significant difference ($p < 0.05$) of the contents of H3K9ac between in the primary spermatocytes or in the early spermatids and in the late spermatids or in the mature spermatozoa, however, there was no statistical difference of the contents of histones H3 between the four types of cells mentioned above. Histones including the H3K9ac directly participate in the construction of chromosome (Nishida et al. 2006; Bergmann et al. 2012; Qiao et al. 2015). However, based on our results mentioned above, H3K9ac was associated with the decondensation of nuclear genetic material. Therefore, our study suggested that H3K9ac played an important role during the spermatogenesis in *E. sinensis*.

The research on mammals shows that the H3K9ac is a way to regulate gene expression in spermatogonia and spermatocytes as well as somatic cells (Nishida et al. 2006; Bergmann et al. 2012; Qiao et al. 2015). We also found that the distribution of H3K9ac in both the spermatogonia and the primary spermatocytes in *E. sinensis* was similar to that in mammals. This suggested that to synthesize sufficient amounts of proteins for mitotic and meiotic spermatogonia and spermatocytes, H3K9ac was involved in the regulation of gene expression in *E. sinensis* spermatogonia and spermatocytes during the spermatogenesis. Especially, on one hand, the acetylation in the decondensed nuclei of *E. sinensis* spermatids happened concurrent with the acetylation in the condensed nuclei of mammal and human spermatids. On the other hand, both the decondensation of the nuclei of *E. sinensis* spermatids and the condensation of the nuclei of

Fig. 7 A model of histone H3 localization and function during *E. sinensis* spermiogenesis. The *black point* indicates histone H3 and the *purple point* indicates H3K9ac. **a** In the early stage, histone H3 is associated with DNA in the spermatid nucleus (N) and the nuclear genetic material is compact. Following acetylation of histones that could weaken the affinity between those histones and DNA, H3K9ac decondenses the chromatin in the nuclei. **b** In the mid stage, no or little H3K9ac is transported out of the nucleus and into the newly emerged proacrosomal

vesicle (PV). **c–e** An acrosomal tubule (AT) and an apical cap (AC) appear at the late stage. H3K9ac moves from the nuclei into the PV in the late spermatids. Some goes on to be transferred out of the nuclei in the immature spermatozoon. **f** The radial arms (RA) appear at the mature spermatozoon. Both histone H3 and H3K9ac remain within the nucleus. Remaining H3K9ac in the nucleus still contribute to the decondensation of chromatin in the *E. sinensis* spermatozoon. (Color figure online)

mammal and human spermatids as well take place at the spermatid stage (Christensen et al. 1984; Sonnack et al. 2002; Zarnescu 2007). Previous studies showed that during spermatogenesis, the reorganization of nuclear chromatin in the spermatozoa was accompanied by substitution of the testis-specific histones (Yan et al. 2003; Okada et al. 2005; Kristie et al. 2008). In the spermatids of rats, mice and humans, histones are significantly hyperacetylated and then replaced by transition proteins and subsequently protamines (Christensen et al. 1984; Sonnack et al. 2002; Zarnescu 2007). This suggests that the histone hyperacetylation is closely related to histone substitution. Acetylation of histones results in a weakened affinity of histones with DNA, which provides an opportunity

for protamine interaction with DNA (Oliva et al. 1990; Kurtz et al. 2009a, b; Michael et al. 2010). Therefore, acetylation of histones H3 lysine 9 might also loosen the nucleosomes in the nuclei of spermatid in *E. sinensis*. Thus, both the acetylation of histone H3 lysine 9 and the remain of H3K9ac within the nuclei contribute to the decondensation of the spermatozoal nuclei of *E. sinensis*.

In mammals, following hyperacetylation of the histones, these hyperacetyl-histones are replaced with basic nuclear proteins, such as the transition proteins and members of the family of protamines. This leads to nuclear condensation in the mature spermatozoa of mammals and some other animals (Christensen et al. 1984; Sonnack et al. 2002; Govin et al. 2006; Zarnescu

2007; Awe and Renkawitz-Pohl 2010; Goudarzi et al. 2014). Consequently, (hyper) acetylation of histones marks the beginning of nuclei condensation and is the necessary condition for the replacement of basic nuclear proteins in the nuclei of mammalian and some other animal spermatids, such as human, rainbow trout, paddlefish and fruit fly (Christensen et al. 1984; Sonnack et al. 2002; Govin et al. 2006; Zarnescu 2007; Awe and Renkawitz-Pohl 2010; Goudarzi et al. 2014). By contrast, we have shown that the acetylation of histone H3 lysine 9 marked the initiation of nuclei decondensation in *E. sinensis* spermatids. Due to the conversion from basic amino groups to neutral acetyl groups, the acetylation of histones within spermatid nuclei may result in the genetic material being unable to further condense, and even to some extent, being able to decondense in the *E. sinensis* spermatid nuclei.

In conclusion, the histone H3 and H3K9ac are widely distributed in the spermatogonia, spermatocytes, spermatids, immature and mature spermatozoa during the spermatogenesis of *E. sinensis* except for absence of H3K9ac in secondary spermatocytes. Especially during the spermatid stage, we observed acetylation of the histone H3 lysine 9 and the transfer of H3K9ac. It is worth noting that H3K9ac always remained in the nuclei although some moved out of the nuclei during the stages from immature to mature spermatozoa. Therefore, both the acetylation of histones H3 lysine 9 and the remain of H3K9ac contribute to the decondensation of the nuclei in *E. sinensis* spermatozoa. We suspect these epigenetic factors to play unique roles in the regulation of nuclei decondensation during *E. sinensis* spermatogenesis. According to our results, we drew a model of histone H3 and H3K9ac localization and function during *E. sinensis* spermiogenesis to outline our understanding of the mechanism of nuclear decondensation in *E. sinensis* spermatozoa in which H3K9ac is involved (Fig. 7). H3K9ac usually specifically binds the methylated genomic DNA fragments (Fernández-Sánchez et al. 2013; Li et al. 2015). It remains to be further studied whether and how there is a correlation between DNA methylation and H3K9ac in *E. sinensis*.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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