Replacement of nuclear protein by histone in pig sperm nuclei during in vitro fertilization

Y. Nakazawa1,2*, A. Shimada3, J. Noguchi1, I. Domeki2, H. Kaneko1 and K. Kikuchi1†

1Genetic Diversity Department and 2Developmental Biology Department, National Institute of Agrobiological Sciences, Kannondai 2-1-2, Tsukuba, Ibaraki 305-8602, Japan; and 3Laboratory of Animal Reproduction, Department of Zootechnical Science, Tokyo University of Agriculture, Atsugi, Kanagawa 243-0034, Japan

Sperm-specific nuclear protamines are dissociated before decondensation of sperm nuclei during fertilization in pigs. In the present study, replacement of nuclear protein by histone in boar spermatozoa during in vitro fertilization was evaluated by immunohistochemistry using anti-histone antibody. First, the specificity of the antibody used in this study was examined. Immunohistochemistry of the testes and epididymides indicated that somatic nuclei, but not elongated spermatids or maturing spermatozoa, were immunoreactive. Furthermore, immunoreaction was diminished after the antibody had been preincubated with unfractionated histone, indicating that the antibody was specific for the somatic nuclear histone. Immunohistochemistry of serial sections of oocytes, which were matured and co-cultured with boar spermatozoa for 2 to 6 h indicated that, at 2 to 3 h after insemination, penetrating sperm nuclei in the condensed state were not immunoreactive. At 4 to 5 h after insemination, some of the condensed sperm nuclei were immunoreactive in part or over the whole area of the nucleus, and all of the decondensing nuclei and male pronuclei were immunoreactive. At 6 h after insemination, the decondensing sperm nuclei and well-developed male pronuclei were immunoreactive. These results imply that, in pigs, remodelling of sperm nuclear protein from protamine to histone is initiated at the time of sperm penetration, before onset of decondensation and male pronuclear formation.

*Present address: Organogenesis and Neurogenesis Group, Center for Developmental Biology, RIKEN, Kobe 650-0047, Japan
†Correspondence
Email: kiku@nias.affrc.go.jp

Introduction

Fertilization is the first step for embryonic development. Before fertilization, sperm nuclear DNA is packed tightly with strongly basic DNA-binding proteins termed protamines or sperm-specific nuclear proteins. For completion of fertilization, penetrating sperm heads undergo cytological and biochemical changes different from those of oocyte chromatin. In summary, the cytological changes are: fusion of the plasma membrane of acrosome-reacted spermatozoa with the oocyte membrane; decondensation and then recondensation into a mass of chromatin, but not back to the original shape (oval spatulate nuclei); and decondensation of the nuclei again, resulting in male pronucleus formation. During these events, biochemical changes occur to the sperm nuclei. One of the most important events is chromatin remodelling from sperm-specific protamine to somatic nuclear histone. Yanagimachi (1994) suggested that the remodelling requires two steps: first, reduction of S–S to S–H in protamines, and second, replacement of protamines by histones. The ability for S–S reduction in ooplasm correlates with the concentration of free thiol glutathione (Perreault et al., 1988; Zirkin et al., 1989). Biochemical analysis of the replacement process of sperm nuclear protein has been carried out using amphibian egg extracts (Lohka and Masui, 1983; Katagiri and Ohsumi, 1994) because these physiological events terminate quickly in the fertilized oocytes of organisms such as sea urchins. However, these events progress slowly in mammalian fertilized oocytes, which might be an advantage for analysis. Shimada et al. (2000) demonstrated that protamine dissociation occurs in fertilized pig oocytes just after sperm penetration but before sperm nuclear decondensation. This observation indicates that replacement by histones is likely to be initiated during the period just after sperm penetration and completed during sperm chromatin decondensation before DNA synthesis in a male pronucleus; this contention differs slightly from conclusions drawn by Nonchev and Tsanev (1990) in mice.

Understanding of the mechanism of sperm nuclear chromatin remodelling during fertilization may be advantageous not only for studies in developmental biology but also for the improvement of the successful rates of using the developing reproductive techniques of intracytoplasmic sperm injection (ICSI) or cloning by nuclear transfer. The
injected sperm heads should complete their chromatin remodelling in the same way as they do in sperm penetration; however, the relationship between this phenomenon and successful development of sperm-injected oocytes has not yet been determined. In transplanted nuclei (Adenot et al., 2000), interactions may occur between somatic histones brought by the nuclei and oocyte-specific linker histones originating from oocytes, as has been reported in sea urchins (Mandl et al., 1997), frogs (Ohsumi and Katagiri, 1991a) and mice (Tanaka et al., 2001).

In the present study, the correlation between the morphological changes of penetrated sperm nuclei and the dynamics of histone transfer onto sperm nuclei was evaluated by immunohistochemical methods during in vitro fertilization of pig oocytes. Chromatin remodelling during the early period of pig fertilization is discussed in the light of results from this and a previous study (Shimada et al., 2000).

Materials and Methods

In vitro maturation and fertilization

Pig ovaries were obtained from prepubertal cross-bred gilts (Landrace, Large White and Duroc breeds) at a local abattoir and transported to the laboratory at 35°C. Cumulus–oocyte complexes (COCs) were collected from follicles 2–5 mm in diameter in TCM-199 with Hanks’ salts (Gibco, Life Technologies Inc., Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (Gibco), 20 mmol Hepes l–1 (Dojindo Laboratories, Kumamoto), 100 iu penicillin G potassium ml–1 (Sigma Chemical Co., St Louis, MO), and 0.1 mg streptomycin sulphate ml–1 (Sigma; Kikuchi et al., 1995). In vitro maturation (IVM) of oocytes was carried out as described by Kikuchi et al. (1999, 2002). In brief, about 50 COCs were cultured in each 500 μl of maturation medium, a modified NCSU-37 solution (Petters and Wells, 1993) containing 10% (v/v) pig follicular fluid, 0.6 mmol cysteine l–1, 0.05 mmol β-mercaptoethanol l–1, 1 mmol dibutyryl cAMP l–1 (dbcAMP; Sigma), 10 iu eCG ml–1 (PMS 1000 iu; Nihon Zenyaku Kogyo, Koriyama), and 10 iu hCG ml–1 (Puberogen 500 unit; Sankyo, Tokyo), in four-well dishes (Nunclon Multidishes, Nalge Nunc International, Roskilde) for 20 h. COCs were subsequently cultured in the maturation medium without dbcAMP and hormones for 24 h. The maturation culture was carried out under 5% CO2 in air at 39°C. After maturation culture, oocytes with expanded cumulus cells were treated briefly with 150 iu hyaluronidase ml–1 and denuded of cumulus cells by gentle pipetting. The denuded oocytes with the first polar body were harvested under a stereomicroscope and served as matured oocytes for in vitro fertilization (IVF). IVF was carried out as under a stereomicroscope and served as matured oocytes denuded oocytes with the first polar body were harvested.

Results from this and a previous study (Shimada et al., 2000). The final sperm concentration was 1 × 105 cells ml–1.

Examination of fertilization status

After co-incubation of gametes for 2, 3, 4, 5, 6, 8 or 10 h, some oocytes were freed from the attached spermatozoa, whole-mounted on to a glass slide, fixed in acetic alcohol (1:3), stained with 1% (w/v) aceto-orcein solution, and examined under a phase-contrast microscope.

Evaluation of specificity of anti-histone antibody

Before examination in samples from fertilized oocytes, the specificity of anti-histone antibody (monoclonal anti-histone H1; Leinco Technologies, Inc., St Louis, MO) used for immunohistochemistry was evaluated. Immunohistochemical approaches were conducted essentially as described by Noguchi et al. (1997). Tissue samples of testsis and epididymis from adult Landrace boar were fixed with Methacarn solution (methanol–chloroform–acetic acid, 6:3:1 by volume), and dehydrated with a series of alcohols, and embedded in paraffin wax. Sections were cut at 4 μm, deparaffinized, treated with 0.5% (w/v) casein Tris-buffered saline (TBS), the sections were incubated overnight at 4°C with a 1: 5 in casein TBS. The Elite ABC kit (Vector Laboratories Inc., Burlingame, CA) with diaminobenzidine was used to visualize the reaction.

The specificity of the antibody was also examined by testing whether immunopositive reactions were diminished by the use of immunoabsorbed antiserum, which was prepared as follows. The antibody at a dilution of 1: 40 in TBS was mixed with unfractionated whole histone (Type II-A from calf thymus; Sigma) to a final concentration of 2560 μg ml–1. After incubation at 37°C for 1 h and then at 4°C overnight, the mixture was centrifuged at 10 000 g for 30 min for generation of antigen, and then all sections were incubated in 0.5% (w/v) periodic acid to block endogenous peroxidase. After blocking with 0.5% (w/v) casein TBS, the sections were incubated overnight at 4°C with the anti-histone antibody to a dilution of 1: 5 in casein TBS. The Elite ABC kit (Vector Laboratories Inc., Burlingame, CA) with diaminobenzidine was used to visualize the reaction.
of oocytes was the same as described above, except for autoclaving at 121°C for 5–15 min in 0.1 mol citric acid l⁻¹ (pH 6.0) and blocking with 1.5% normal goat serum (Vector).

Immunohistochemical evaluation of IVF oocytes

The IVF oocytes at 2, 3, 4, 5, 6, 8 or 10 h after insemination were fixed and processed for immunohistochemistry as described above. After immunohistochemical staining, serial sections were counterstained with haematoxylin. Morphological changes of penetrating sperm nuclei and immunoreactivity with anti-histone antibody were evaluated.

Results

Specificity of the anti-histone antibody in pig tissues

Immunohistochemical staining of boar testis showed specific reaction in nuclei of interstitial cells or those of germ cells such as spermatogonia, spermatocytes and round spermatids in seminiferous epithelium but not in nuclei of elongated spermatids (Fig. 1a). When immunostaining was followed by staining with haematoxylin, nuclei of elongated spermatids located in the luminal area were stained with haematoxylin alone, whereas the other cells in the seminiferous epithelium were dark brown, indicating staining by both dianaminobenzidine and haematoxylin (Fig. 1b). No positive reaction was detected in the matured epididymal spermatozoa (data not shown). An immunoabsorbance test with the antibody preincubated with unfractionated histone was conducted to confirm the specificity of the staining. Nuclei of germ cells such as spermatogonia, spermatocytes and round spermatids were positive without immuno-absorbance (Fig. 1a); however, most of the positive reactions diminished after preincubation with histone, as the histone concentration increased (Fig. 1c). The ability of the antibody to detect histone in maturing, matured and activated oocytes was also examined. Metaphase I and II and anaphase II chromosomes (Fig. 1d–f) and nuclei of first and second polar bodies (Fig. 1e,f) were detected by immunohistochemistry with this antibody.

Sequential nuclear changes of inseminated oocytes and penetrating spermatozoa

Sequential changes of penetrating sperm nuclei were observed in whole-mount preparations of inseminated oocytes.
oocytes. The rates of sperm penetration, polyspermy and the number of penetrated spermatozoa per oocyte are shown (Fig. 2). Sperm penetration was observed in > 10% of the oocytes at 2 h after insemination. At 4–10 h, about 80% of inseminated oocytes were penetrated, and most of them showed polyspermic fertilization, indicating increased numbers of penetrating spermatozoa. Inseminated oocytes were activated to be at anaphase II at 2 h, proceeded to telophase II at about 4 h, and began to form a female pronucleus from 4 h after insemination (Fig. 3a). Whole-mount preparations of inseminated oocytes also showed the detail of morphological changes of sperm nuclei (Fig. 3b). Sperm entry was first observed at 2 h after insemination, when sperm nuclei were in a condensed state. Decondensation of the nuclei started from 3 h and continued to 10 h after insemination. A male pronucleus was first observed to form at 4 h after insemination, but was small. Well-developed pronuclei with a nucleolus appeared from 6 h after insemination. This development almost matches female pronuclear development.

Histone status in penetrated sperm nuclei

Examination of sectioned oocytes under light microscopy enabled visualization of (i) condensed sperm nuclei of the original shape, (ii) decondensing (including recondensing) sperm nuclei, and (iii) male and female pronuclei. Immunoreactivity in the sperm nuclei after penetration is shown (Fig. 4). Three types of condensed sperm nuclei were identified by the pattern of staining (Fig. 4a–c): (i) histone not associated with the sperm nucleus; no diaminobenzidine signal observed and sperm nuclei stained with haematoxylin only; (ii) histone associated with part of the nucleus; part of the area of sperm nuclei stained with diaminobenzidine and the remaining area stained with haematoxylin; and (iii) histone associated with the entire nucleus: diaminobenzidine signal observed and sperm nuclei stained with diaminobenzidine only. Means ± SEM are presented. At least three replicated trials were performed and a total of 90–153 oocytes were examined for each category.
benzidine signal observed all over the sperm nuclei and haematoxylin staining not visible. All the decondensing sperm nuclei (Fig. 4d) and both male and female pronuclei (Fig. 4e) were stained with diaminobenzidine and the signal from haematoxylin only was not detected. Because distinguishing the male from the female pronucleus was impossible in the sections, one was subtracted as a female pronucleus in the case of multiple pronuclear formation. In the case of polyspermy, all the decondensing sperm nuclei and pronuclei were equally labelled with diaminobenzidine.

The sequential changes of penetrated spermatozoa according to their morphological status and their immunoreactions are summarized (Fig. 5). Before 4 h after insemination, all of the penetrated sperm nuclei were condensed and showed staining only with haematoxylin. At 4–5 h, some condensed sperm nuclei were immunoreactive in part of (haematoxylin and diaminobenzidine) or over the whole area (diaminobenzidine) of the nuclei. Up to 6 h, all of the decondensing sperm nuclei and male pronuclei were immunoreactive (diaminobenzidine). Pronucleus, diaminobenzidine; : decondensing, diaminobenzidine; : condensed, diaminobenzidine; : condensed, haematoxylin and diaminobenzidine; and : condensed, haematoxylin. Numbers on the bars represent the total number of sperm nuclei used for analysis.
part of the nucleus’ and ‘histone associated with the entire nucleus’) were observed. Decondensing sperm nuclei and pronuclei, which were judged to have developed from sperm nuclei, were also immunoreactive and considered to show ‘histone associated with the entire nucleus’. At 6 h after insemination, most of the penetrated sperm nuclei were decondensing and developing into male pronuclei; these were considered to show ‘histone associated with the entire nucleus’. A small percentage of condensed sperm nuclei without immunoreaction was also observed.

**Discussion**

During fertilization, protamines dissociate from penetrating boar sperm nuclei that are still condensed at 2–3 h after insemination (Shimada et al., 2000), at which time the spermatids begin to penetrate the oocytes. The results of the present study clearly indicate that some of the condensed sperm nuclei at 4–5 h after insemination are immunoreactive and that all of the decondensing nuclei and pronuclei are immunoreactive after 4 h. On the basis of these results, it is suggested that protamines dissociate from the boar nuclei just after penetration and, just after the protamine dissociation, histones began to associate in the nuclei before decondensation or formation of the pronucleus (Fig. 6). In mice, although the idea that protamine loss occurs as histones appear in the well-developed male pronucleus is accepted (Nonchev and Tsanev, 1990), it can be concluded that sperm basic proteins are retained in the decondensed nuclei (Rodman et al., 1981). As the timing of the remodelling in mice (whether in condensed or decondensed sperm nuclei) is not yet known, the present study in pigs is the first to have used immunohistochemistry to clarify the remodelling of penetrated sperm nuclei. In other mammalian species, further attention should be paid to the mechanism of remodelling of the nuclei after the sperm penetration.

After sperm penetration, protamine dissociation occurs and histone association and then somatic nucleosome reconstruction are observed (Yanagimachi, 1994). In frogs, remodelling of nuclear protein from protamine to histone occurs just after penetration (Ohsumi and Katagiri, 1991b). In mammals, especially in domestic animals such as pigs, the remodelling processes are not yet well understood. However, the observations in the previous (Shimada et al., 2000) and present studies indicate that the dissociation of protamine and association of histone in penetrating sperm nuclei occur just after sperm penetration and before decondensation of nuclei, indicating that the nucleosome structure in sperm chromatin is reconstructed at the initial stage of fertilization and is similar to that of oocyte chromatin. The equality in the molecular structure between male and female chromatin appears to be important for the synchronization of decondensation and subsequent pronucleus formation between male and female chromatin, and may affect pronuclear functions such as DNA replication or transcriptional activity. In fact, synchronization has been detected between formation of male and female pronuclei in the present study, and in DNA replication within the pronuclei in fertilized mammalian oocytes (hamster: Naish et al., 1987; pig: Laurincik et al., 1995). Further functional analysis, for example examining transcriptional activity, of the pronuclei is expected to produce similar results in pigs as it has in mice (Aoki and Schultz, 1999).

**Fig. 6.** Schematic diagram of remodelling of boar sperm nuclear protein from protamine to histone in relation to changes in sperm nuclear morphology. Protamine dissociation occurs at 2–3 h and histone association occurs until 4 h after insemination, before decondensation or male pronucleus (MPN) formation.
In general, histones are well-conserved; however, many subtypes of linker histone (H1) are known (van Holde, 1988). Oocyte- or embryo-specific H1 subtypes, which can be distinguished by immunological approaches, have been reported, in particular, in sea urchins and frogs (Pehrson and Cohen, 1984; Smith et al., 1988). Somatic H1 subtypes have not been detected in early embryos of mammals or non-mammalian animals. In mice, somatic H1 subtypes are not detected in germinal vesicles, the nuclei of polar bodies or the nuclei of one- or two-cell-stage embryos, but are detected in the nuclei of embryos beyond the four-cell stage (Clarke et al., 1992). In cattle, somatic H1 assembly is similar to that found in mice (Smith et al., 1995). Tanaka et al. (2001) reported evidence of a mouse oocyte- or embryo-specific linker histone subtype, named H1oo. H1oo has been detected in germinal vesicles, metaphase II chromosomes, the nuclei of polar bodies, pronuclei, and nuclei at the two-cell stage, and shows similarity to the oocyte-specific linker histone (B4/H1M) of frogs (Dworkin-Rasl et al., 1994) and the cleavage stage-specific linker histone (cs-H1) of sea urchins (Poccia et al., 1981). The antibody used in the present study detected somatic histone in pig testes and epididymides and its specificity was confirmed by an immunoblotting test. Penetrated sperm nuclei, oocyte chromatin in both metaphase to anaphase chromosomes, the nuclei of polar bodies and the female pronucleus were all shown to be immunoreactive with the antibody. This finding indicates that the anti-histone antibody used in the present study might detect both oocyte-specific H1 and somatic H1 subtypes in pigs; however, there is no evidence for a pig oocyte-specific H1 subtype. Further investigation is necessary to determine the H1 subtype involved in the remodelling during pig fertilization.

In mammals, little is known about the oocyte factors that are responsible for protamine–histone exchange and nuclear decondensation during fertilization. Cytoplasmic proteins called nuclear chaperones mediate protamine–histone exchange and decondensation of sperm nuclei in oocytes (for a review, see Philpott et al., 2000). One of the well-studied nuclear chaperones is nucleoplasmin. In amphibians, nucleoplasmin removes protamine or sperm-specific basic proteins, while it deposits an H2A–H2B dimer (Ohsumi and Katagiri, 1991b; Philpott et al., 1991; Philpott and Leno, 1992). Itoh et al. (1993) demonstrated that remodelling of human sperm chromatin is observed in amphibian egg extracts and is mediated by nucleoplasmin from egg extracts. Furthermore, there is a nuclear factor in interphase egg nucleus that affects sperm decondensation and is released into the ooplasm as oocytes mature (Maeda et al., 1998). These results indicate the presence of nucleoplasmin-like substances in mammalian oocytes. Members of the nucleoplasmin family of nuclear chaperones have been isolated in mice (MacArthur and Shackleford, 1997) and humans (Shackleford et al., 2001). However, there is no firm evidence as yet for nucleoplasmin-like chaperone molecules functioning in protamine–histone exchange of mammalian sperm. In addition, the mechanism for transfer of histone H1 to sperm nuclei has not yet been identified even in amphibians. Further attempts to clarify the mechanism are needed.

In conclusion, the results of the present study indicate that somatic histone or its variant associates with boar sperm nuclei just after their penetration and after dissociation of protamine from the nuclei.

The authors would like to thank T. Aoki, E. Yamauchi, M. Irie and M. Sakurai for technical assistance.

References

Adenot PG, Campion E, Legouy E, Allis CD, Dimitrov S, Renard JP and Thompson EM (2000) Somatic linker histone H1 is present throughout mouse embryogenesis and is not replaced by variant H1+ Journal of Cell Science 113 2897–2907


Clarke HJ, Oblin C and Bustin M (1992) Developmental regulation of chromatin composition during mouse embryogenesis: somatic histone H1 is first detectable at the 4-cell stage Development 115 791–799

Dworkin-Rasl E, Kandolf H and Smith RC (1994) The maternal histone H1 variant, H1M (B4 protein), is the predominant histone H1 in Xenopus progametogenic embryos Developmental Biology 161 425–439


Mandl B, Brandt WF, Superti-Furga G, Graninger PG, Birmstiel ML and Busslinger M (1997) The five cleavage-stage (CS) histones of the sea urchin are encoded by a maternally expressed family of replacement histone genes: functional equivalence of the CS H1 and frog H1M (B4) proteins Molecular and Cellular Biology 17 1189–1200


Ohsumi K and Katagiri C (1991a) Occurrence of H1 subtypes specific to pronuclei and cleavage-stage cell nuclei of anuran amphibians Developmental Biology 147 110–120


Perreault SD, Barbee RR and Slott VL (1988) Importance of glutathione in the acquisition and maintenance of sperm nuclear decondensing activity in maturing hamster oocytes Developmental Biology 125 181–186


Philpott A, Leno GH and Laskey RA (1991) Sperm decondensation in Xenopus egg cytoplasm is mediated by nucleoplasmin Cell 65 569–575


Shackleford GM, Ganguly A and MacArthur CA (2001) Cloning, expression and nuclear localization of human NPM3, a member of the nucleophosmin/nucleoplasmin family of nuclear chaperones BioMed Central Genomics 2 8


Received 26 March 2002.
First decision 17 May 2002.
Revised manuscript received 5 June 2002.
Accepted 12 July 2002.