Zygote **8** (February), pp 51–56. © 2000 Cambridge University Press Printed in the United Kingdom

Further evidence that sperm nuclear proteins are necessary for embryogenesis

W. Steven Ward[1], *Hidehumi Kishikawa*[2], *Hidenori Akutsu*[2], *Hiroko Yanagimachi*[2] *and Ryuzo Yanagimachi*[2]

Division of Urology, Robert Wood Johnson Medical School, New Brunswick, New Jersey, USA and Department of Anatomy and Reproductive Biology, University of Hawaii Medical School, Honolulu, Hawaii, USA

Date submitted: 25.7.99. Date accepted: 25.8.99

Summary

We have recently presented evidence that the structural integrity of the mouse sperm nuclear matrix may be necessary for the proper unpackaging of sperm DNA for participation in embryogenesis. It is likely that the sperm nuclear matrix contributes to the organisation of the sperm DNA and its disturbance can seriously damage the paternal genome or its expression. In this work, we confirm our previous data and further suggest that even very subtle changes in the sperm nuclear structure may have a significant impact on embryo development. As reported previously, dithiothreitol (DTT) in the presence of an ionic detergent, ATAB, destabilised the nuclear matrix as measured by the halo assay, and oocytes injected with these nuclei failed to develop. We also discovered that omitting the protease inhibitor PMSF from the buffers used to extract spermatozoa prevented sperm injected into oocytes from participating in development. The organisation of DNA into loop domains by the nuclear matrix in these nuclei appeared normal, as measured by the halo assay. Oocytes injected with sperm nuclei that had been washed with ATAB in the presence of phenylmethylsulphonyl fluoride (PMSF) but in the absence of DTT resulted in live births. Neither DTT treatment nor the absence of PMSF would be expected to disrupt the integrity of the paternal DNA. The data therefore suggest that even very subtle alterations in the structural proteins of the nucleus are enough to deprive sperm DNA of the ability to contribute to embryonic development.

Key words: Embryogenesis, ICSI, Paternal genome, Sperm DNA, Sperm nucleus

Introduction

We have been interested in determining the components of the sperm head that are essential for full development of the embryo. The prime function of the spermatozoa is to deliver the paternal genome to the egg, and to initiate development by activating the oocyte, leading to the development of the normal embryo (Yanagimachi, 1994). It is known that mouse sperm heads isolated from tails are not capable of entering the oocyte by normal physiological means, but they can nevertheless activate the oocyte and participate fully in normal embryo development if injected microsurgically into the oocyte (Kuretake *et al*., 1996; Kimura *et al*., 1998). This approach has allowed us to focus more specifically on the genetic contribution of the mouse sperm nucleus to embryo development. Previous work has demonstrated that at least in the mouse all components that are required for the full participation of the paternal genome in embryogenesis reside within the sperm nucleus and the perinuclear materials (Kimura *et al*., 1998; Perry *et al*., 1999).

Most researchers believe that the only truly essential component in the sperm head for embryogenesis is the DNA itself, and that all other sperm components

All correspondence to: W. Steven Ward, PhD, Division of Urology, Robert Wood Johnson Medical School, 1 RWJ Pl., MEB-588, New Brunswick, NJ 08903, USA. Tel: +1 (732) 235 8 73. Fax: +1 (732) 235 6042.

[1]Division of Urology, Robert Wood Johnson Medical School, New Brunswick, New Jersey, USA.

[2]Department of Anatomy and Reproductive Biology, University of Hawaii Medical School, Honolulu, Hawaii, USA.

https://doi.org/10.1017/S0967199400000824 Published online by Cambridge University Press

evolved solely as the means of transporting the parental DNA into the oocyte. This idea was supported by the successful development of mice from oocytes that had been injected with the nuclei of round spermatids (Ogura *et al*., 1994; Kimura & Yanagimachi, 1995*b*; Sato *et al*., 1998). The chromatin structure of the round spermatid is very different from that of spermatozoa, being much less compact and bound to histones rather than protamines (Balhorn *et al*., 1984; Ward & Coffey, 1991; Green *et al*., 1994). Furthermore, it has been established that naked DNA injected into oocytes will initiate the formation of nuclear-like structures, and that the exogenous DNA will subsequently undergo some DNA replication (Blow & Laskey, 1986; Laskey *et al*., 1993). This suggests that if it were possible to inject the DNA from a single spermatozoon into an oocyte, without any nicks or breaks, the oocyte would form a pronuclear-like structure and at least initiate DNA replication. But whether such oocytes would complete development to a live mouse is still an open question.

Is the DNA the only component in the sperm nucleus that is necessary for development, or is there some important and necessary information in the chromatin structure? The sperm nucleus contains one level of DNA organisation that is independent of protamines and histones: the organisation into loop domains 20–100 kp in size by the nuclear matrix (Nadel *et al*., 1995; Ward & Zalensky, 1996). In somatic cells, this organisation is associated with DNA replication (Pardoll *et al*., 1980; Vogelstein *et al*., 1980) and transcription of RNA (Gerdes *et al*., 1994; Getzenberg, 1994; Chang *et al*., 1995; Kim *et al*., 1996). It is therefore possible that the loop domain organisation of DNA by the nuclear matrix is important in the regulation of transcription and DNA replication of the pronucleus. This type of organisation can be visualised by extracting the histones (from somatic cells) or protamines (from spermatozoa) from isolated nuclei, and staining with a fluorescent DNA binding dye, such as ethidium bromide (Vogelstein *et al*., 1980; Ward *et al*., 1989). Nuclei prepared in this manner have a halo of fluorescence surrounding the nucleus, representing the DNA extending from the nuclear matrix as loops. We have previously demonstrated that the mammalian sperm nuclear matrix is extremely sensitive to extraction conditions. Ionic detergents such as SDS protect the sperm nuclear matrix from dissipation upon subsequent extraction of the protamines with 2 M NaCl and 10 mM dithiothreitol (DTT) (Ward *et al*., 1989), while extraction with non-ionic detergents such as Triton X-100 or NP-40 results in complete dissipation of the sperm nuclear matrix (Ward & Coffey, 1989, 1991).

Since it is technically impossible at present to isolate chromosome-sized DNA molecules completely intact for injection, we cannot perform the experiment described above to answer the question of whether the DNA alone is sufficient for the paternal genome to participate in embryogenesis. However, we have recently presented evidence that an intact sperm nucleus is necessary for normal embryogenesis to occur. We also demonstrated that treatment of mouse spermatozoa with the ionic detergent ATAB were capable of participating fully in embryogenesis when injected into oocytes (Ward *et al*., 1999). However, when 2 mM DTT was included in the original ATAB extraction buffer, the nuclear matrices became unstable, and none of the oocytes injected with such sperm nuclei developed. These data suggested that a stable sperm nuclear matrix was important for normal embryogenesis. In this work, we confirm our previous results, and extend our observations by testing the hypothesis that more subtle variations in the proteinaceous nuclear structure can also affect the participation of the paternal genome in embryogenesis.

Materials and methods

Treatment of mouse spermatozoa with ATAB

The epididymal spermatozoa from one mouse ($B6D_2$ F1) were collected, and suspended in 2 ml of nuclear isolation media (NIM: 121.6 mM KCl, 7.8 mM Na_2HPO_4 ,1.4 mM KH_2PO_4, 0.1% polyvinyl alcohol, 10 mM EDTA, with or without 1.0 mM PMSF; all supplied by Sigma, St Louis, MO) supplemented with an ionic detergent, 0.5% ATAB (mixed alkyltrimethylammonium bromide; supplied by Sigma), and 2 mM dithiothreitol (DTT; supplied by Sigma), and the pH adjusted to 8.2 by adding drops of 1 M KOH. The sperm suspension was occasionally vortexed, then centrifuged at 700 *g* for 5 min. The sperm pellet was washed in 10 ml of NIM without ATAB or DTT, split into two aliquots and centrifuged again. For each experiment, one sperm pellet was used to test for halo formation (gross stability of the nuclear matrix: see below) and the other sperm pellet was used for intracytoplasmic sperm injection (ICSI).

Halo assay for gross nuclear matrix stability

The pelleted sperm (heads and tails were all separated) obtained as described above were resuspended in 100 µl of 2 M NaCl, 25 mM Tris, pH 7.4, with 2 mM DTT. The heads were incubated in the salt extraction buffer for 10 min on ice, then stained with 100 µg/ml ethidium bromide to stain the DNA, and examined by fluorescence microscopy. Sperm heads with stable nuclear matrices

https://doi.org/10.1017/S0967199400000824 Published online by Cambridge University Press

remain intact under these conditions, and the DNA forms a halo of fluorescence surrounding the nuclei. Sperm heads with unstable nuclear matrices decondense completely, and no visible structure remains.

Preparation of oocytes

B6D2F1 females, 8–10 weeks old, were induced to superovulate by consecutive injections of 5 IU eCG and 5 IU hCG 48 h apart. Oocytes, collected from oviducts 14–15 h after hCG injection, were freed from cumulus cells by treatment with 0.1% bovine testicular hyaluronidase (300 USP units/ng; ICN Pharmaceuticals, Irvine, CA) in Hepes-buffered CZB medium (Hepes-CZB) (Kimura & Yanagimachi, 1995*a*). The oocytes were washed thoroughly and kept in CZB for up to 2 h at 37 °C under 5% CO_2 in air. Oocytes to be injected with spermatozoa were transferred into a droplet (6 µl) of Hepes-CZB under mineral oil (Squibb and Sons, Princeton, NJ) in an operation dish on the microscope stage (Kimura & Yanagimachi, 1995*a*).

Sperm head injection into oocytes

One pellet of sperm heads prepared as above was resuspended in 100 µl of NIM with 6% (w/v) polyvinylpyrrolidone (PVP, M_r 360 000; ICN Pharmaceuticals, Irvine, CA) and a single sperm head was used for ICSI. A single sperm nucleus was aspirated into the injection pipette attached to the micromanipulator and injected into an oocyte according to the method described by Kimura & Yanagimachi (1995*a*) except that all operations were carried out at room temperature (about 25 °C) instead of 16–17 °C. Approximately 10 min after sperm injection the oocytes were transferred into Ca^{2+}-free CZB containing 10 mM $SrCl_2$ and incubated for 1 h to activate them (Bos-Mikich *et al.*, 1997). Unlike fresh spermatozoa or Triton- treated sperm heads (Kimura & Yanagimachi, 1995*a*; Kuretake *et al.*, 1996), ATAB + DTT-washed sperm nuclei could not activate oocytes when injected into oocytes, so the oocytes had to be activated. We used Sr^{2+} as the activating agent. Sr^{2+}-treated oocytes were incubated for about 5 h in CZB medium, and examined with an inverted microscope. Those with two distinct pronuclei and the second polar body were considered normally activated and fertilised.

Embryo transfer to foster mothers

Morulae/blastocyst embryos arising from normally fertilised eggs were transferred to uteri of ICR (albino) female mice which had mated with vasectomised males of the same strain 3 days previously (Hogan *et al.*, 1994). Caesarian sections were performed on day 19.5 *post coitum* and pups with black eyes were raised by foster mothers (albino CD-1).

Electron microscopy

Sperm heads were fixed in 2% glutaraldehyde in cacodylate buffer, embedded in Spurr (Ladd Research Industries, Burlington, VT), and thin-sectioned for transmission electron microscopy.

Results

Gross stability of the sperm nuclear matrix

As described in the Introduction, mouse sperm nuclei treated with ATAB + PMSF form halos when subsequently extracted with 2 M NaCl and 10 mM DTT to extract the protamines (Ward *et al.*, 1999). In these nuclei the nuclear matrix appears to be grossly intact, since the organisation of DNA into loop domains is still apparent. The inclusion of DTT caused the nuclei to decondense completely when subsequently extracted with salt. In the present work we repeated these results, and added a second variable: the protease inhibitor PMSF (Table 1). As in our previous work, we found that the presence of DTT destabilised the nuclear matrix. Interestingly, the presence of PMSF in the original extraction buffer had no effect on halo formation. This suggested either that PMSF had no effect on sperm nuclear structure, or that the effect was too subtle to be detected by the halo assay.

Table 1. Gross stability of sperm nuclear matrices after pretreatment with various extraction buffers

Extraction buffer	Nuclear matrix stability
ATAB + PMSF	Halos (stable)
ATAB	Halos (stable)
ATAB + DTT + PMSF	Decondensed (unstable)
ATAB + DTT	Decondensed (unstable)

ATAB, mixed alkyltrimethylammonium bromide; PMSF, phenylmethylsulphonyl fluoride; DTT, dithiothreitol.

Electron microscopy

We have previously published electron micrographs of mouse spermatozoa treated with ATAB and PMSF, with and without DTT (Ward *et al.*, 1999). In this work, we examined the structure of sperm nuclei treated under both conditions without PMSF (Fig. 1). Nuclei after all four treatments look similar: the nuclei are very condensed, and the perinuclear theca has been almost completely removed.

https://doi.org/10.1017/S0967199400000824 Published online by Cambridge University Press

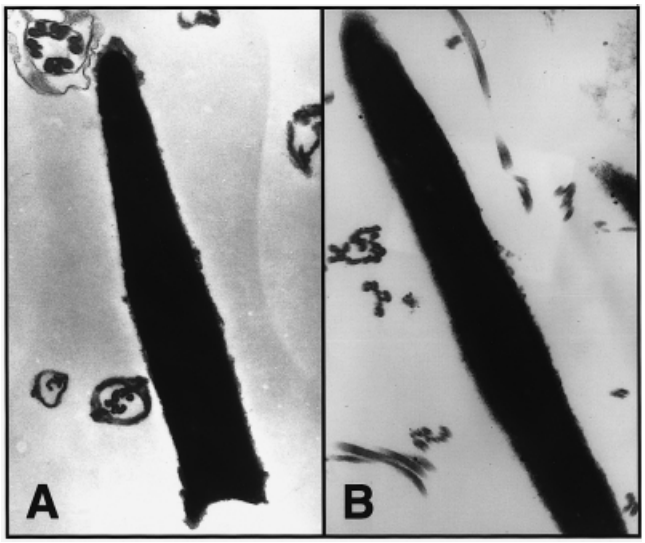


Figure 1 Electron micrographs of mouse sperm nuclei. Mouse sperm nuclei were treated with ATAB (*A*) or ATAB + DTT (*B*), both without PMSF. ATAB, mixed alkyltrimethylammonium bromide; DDT, dithiothreitol; PMSF, phenylmethylsulphonyl fluoride.

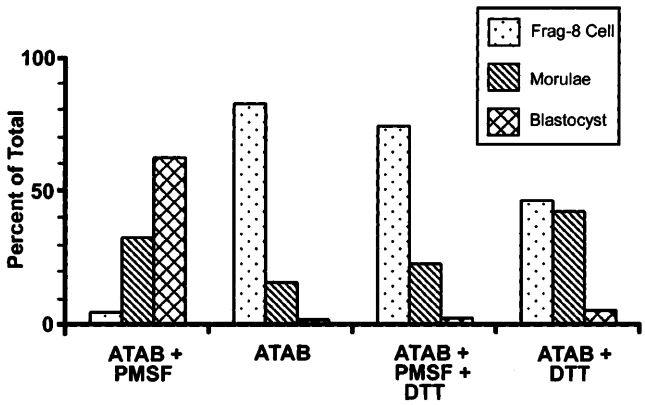


Figure 2 Development of oocytes to the blastocyst stage. Abbreviations as in Fig. 1.

Sperm injection

For every experiment in which oocytes were injected, half the sperm preparation was examined for the stability of the sperm nuclear matrix by the halo assay and the other half was used for injections. In this way, the correlations between sperm nuclear structure and embryonic development could be assessed. The results for the ICSI experiments for development to blastocyst stage are illustrated in Fig. 2. For all four treatments of spermatozoa used, only one allowed the spermatozoa to participate in embryogenesis: washing with ATAB in the presence of PMSF and in the absence of DTT. The differences are striking: 63% of the eggs developed to blastocysts when injected with sperm nuclei treated in this manner, while only 1–7% of the eggs developed when injected with sperm nuclei in all other treatments. Table 2 summarises the results of those embryos transferred to foster mothers that resulted in live offspring. Again, only those oocytes that were injected with spermatozoa treated with ATAB in the presence of PMSF and the absence of DTT had the ability to develop to live young.

Discussion

The results presented here confirm our previous finding (Ward *et al.*, 1999) that sperm nuclei that do not have stable nuclear matrices as measured by the halo assay are not capable of participating in embryogenesis. Oocytes that are injected with these nuclei do not develop normally, even though the DNA is most likely intact. The data support our previous suggestion that the sperm nucleus requires additional factors for embryogenesis in addition to the genetic information encoded within the DNA. We have now demonstrated that a much more subtle modification of the sperm nuclear proteins brought about by the omission of the protease inhibitor PMSF during the nuclear isolation prevents sperm nuclei from participating in embryogenesis. This suggests that even very subtle alterations in the sperm nuclear structure have broad effects on embryogenesis.

These data have caused us to reconsider the widely accepted notion that the only function of the sperm nucleus is to deliver a genetically intact package of paternal DNA to the oocyte. In these experiments the DNA has been delivered intact to the oocyte. Furthermore, it is clear that the oocyte has access to the paternal DNA since 47–82% of the oocytes injected with sperm nuclei under all conditions tested develop at least to the 2-cell stage (Table 2), and virtually all the sperm nuclei injected formed pronuclei (data not shown). This demonstrates that the sperm DNA is completely unpackaged, and accessible to the developing embryo. As with the previous study, treatment of sperm nuclei with the ionic detergent ATAB did not prevent them from participating in development, but treatment with ATAB plus DTT did. DTT alone would not be expected to damage DNA. A recent study has suggested that pretreatment of spermatozoa with DTT actually improves the efficiency of fertilisation by sperm injection into human oocytes (Rho *et al.*, 1998). Thus, it is DTT in the presence of ATAB that deprived sperm nuclei of their competence to participate in embryogenesis, suggesting that a modification of a protein component, not DNA damage, is responsible for this loss of competence.

In this study we also demonstrated that the more subtle alteration in sperm protein structure that results from omission of the protease inhibitor PMSF renders sperm nuclei incompetent. PMSF is not necessary for

https://doi.org/10.1017/S0967199400000824 Published online by Cambridge University Press

Table 2. Development of mouse oocytes after sperm injection

Sperm extraction buffer	No. of oocytes injected	No. of oocytes survived	No. (%) development *in vitro* 72–76 h after sperm injection			Live offspring
			>8- cell	Morula	Blastocyst	
ATAB + PMSF	92	64 (70%)	3 (5%)	21 (33%)	40 (63%)	7 (11%)
ATAB	71	56 (79%)	46 (82%)	9 (16%)	1 (2%)	0
ATAB + PMSF + DTT	81	70 (86%)	52 (74%)	16 (23%)	2 (3%)	0
ATAB, DTT	150	120 (80%)	62 (52%)	51 (43%)	7 (6%)	0

Abbreviations as in Table 1.

the gross stability of the sperm nuclear matrix, as measured by the halo assay (Table 1), but it is necessary for sperm nuclei to participate fully in embryogenesis (Table 2). PMSF would be expected to protect nuclear proteins from autodigestion by endogenous proteases, thereby stabilising proteins, not DNA, so this is further evidence that a proteinaceous element is present in the mouse sperm nucleus that is important for keeping DNA intact for embryogenesis. This protein(s) is apparently not removed by extraction with the ionic detergent ATAB.

These results support the hypothesis that there are elements other than the DNA in the sperm nucleus that are essential for keeping the sperm nucleus competent to participate fully in embryogenesis.

Acknowledgements

This work was supported by NIH grants HD-34362 to R.Y. and HD-28501 to W.S.W.

References

Balhorn, R., Weston, S., Thomas, C. & Wyrobek, A.J. (1984). DNA packaging in mouse spermatids: synthesis of protamine variants and four transition proteins. *Exp. Cell Res.* **150**, 298–308.

Blow, J.J. & Laskey, R.A. (1986). Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of *Xenopus* eggs. *Cell* **47**, 577–87.

Bos-Mikich, A., Whittingham, D.G. & Jones, K.T. (1997). Meiotic and mitotic Ca^{2+} oscillations affect cell composition in resulting blastocysts. *Dev. Biol.* **182**, 172–9.

Chang, K.S., Fan, Y.H., Andreeff, M., Liu, J. & Mu, Z.M. (1995). The PML gene encodes a phosphoprotein associated with the nuclear matrix. *Blood* **85**, 3646–53.

Gerdes, M.G., Carter, K.C., Moen, P.T., Jr. & Lawrence, J.B. (1994). Dynamic changes in the higher-level chromatin organization of specific sequences revealed by *in situ* hybridization to nuclear halos. *J. Cell Biol.* **126**, 289–304.

Getzenberg, R.H. (1994). Nuclear matrix and the regulation of gene expression: tissue specificity. *J. Cell. Biochem.* **55**, 22–31.

Green, G.R., Balhorn, R., Poccia, D.L. & Hecht, N.B. (1994). Synthesis and processing of mammalian protamines and transition proteins. *Mol. Reprod. Dev.* **37**, 255–63.

Hogan, B., Beddington, R., Costantini, F. & Lacy, E. (1994). *Manipulating the Mouse Embryo,* pp. 173–81. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Kim, M.K., Lesoon-Wood, L.A., Weintraub, B. D. & Chung, J.H. (1996). A soluble transcription factor, Oct-1, is also found in the insoluble nuclear matrix and possesses silencing activity in its alanine-rich domain. *Mol. Cell. Biol.* **16**, 4366–77.

Kimura, Y. & Yanagimachi, R. (1995*a*). Development of normal mice from oocytes injected with secondary spermatocyte nuclei. *Biol. Reprod.* **53**, 855–62.

Kimura, Y. & Yanagimachi, R. (1995*b*). Mouse oocytes injected with testicular spermatozoa or round spermatids can develop into normal offspring. *Development* **121**, 2397–405.

Kimura, Y., Yanagimachi, R., Kuretake, S., Bortkiewicz, H., Perry, A.C. & Yanagimachi, H. (1998). Analysis of mouse oocyte activation suggests the involvement of sperm perinuclear material. *Biol. Reprod.* **58**, 1407–15.

Kuretake, S., Kimura, Y., Hoshi, K. & Yanagimachi, R. (1996). Fertilization and development of mouse oocytes injected with isolated sperm heads. *Biol. Reprod.* **55**, 789–95.

Laskey, R.A., Mills, A.D., Philpott, A., Leno, G.H., Dilworth, S.M. & Dingwall, C. (1993). The role of nucleoplasmin in chromatin assembly and disassembly. *Phil. Trans. R. Soc. Lond., Ser B.* **339**, 263–9; discussion 268–9.

Nadel, B., De Lara, J. & Ward, W.S. (1995). Structure of the rRNA genes in the hamster sperm nucleus. *J. Androl.* **16**, 517–22.

Ogura, A., Matsuda, J. & Yanagimachi, R. (1994). Birth of normal young after electrofusion of mouse oocytes with round spermatids. *Proc. Natl. Acad. Sci. USA* **91**, 7460–2.

Pardoll, D.M., Vogelstein, B. & Coffey, D.S. (1980). A fixed site of DNA replication in eucaryotic cells. *Cell* **19**, 527–36.

Perry, A.C., Wakayama, T. & Yanagimachi, R. (1999). A novel trans-complementation assay suggests full mammalian oocyte activation is coordinately initiated by multiple, submembrane sperm components. *Biol. Reprod.* **60**, 747–55.

Rho, G.J., Kawarsky, S., Johnson, W.H., Kochhar, K. & Bet-

https://doi.org/10.1017/S0967199400000824 Published online by Cambridge University Press

teridge, K. J. (1998). Sperm and oocyte treatments to improve the formation of male and female pronuclei and subsequent development following intracytoplasmic sperm injection into bovine oocytes. *Biol. Reprod.* **59**, 918–24.

Sato, Y., Miyazaki, S., Shikano, T., Mitsuhashi, N., Takeuchi, H., Mikoshiba, K. & Kuwabara, Y. (1998). Adenophostin, a potent agonist of the inositol 1,4,5-trisphosphate receptor, is useful for fertilization of mouse oocytes injected with round spermatids leading to normal offspring. *Biol. Reprod.* **58**, 867–73.

Vogelstein, B., Pardoll, D.M. & Coffey, D.S. (1980). Supercoiled loops and eucaryotic DNA replication. *Cell* **22**, 79–85.

Ward, W.S. & Coffey, D.S. (1989). Identification of a sperm nuclear annulus: a sperm DNA anchor. *Biol. Reprod.* **41**, 361–70.

Ward, W.S. & Coffey, D.S. (1991). DNA packaging & organization in mammalian spermatozoa: comparison with somatic cells. *Biol. Reprod.* **44**, 569–74.

Ward, W.S. & Zalensky, A.O. (1996). The unique, complex organization of the transcriptionally silent sperm chromatin. *Crit. Rev. Euk. Gene Exp.* **6**, 139–47.

Ward, W.S., Partin, A.W. & Coffey, D.S. (1989). DNA loop domains in mammalian spermatozoa. *Chromosoma* **98**, 153–9.

Ward, W.S., Kimura, Y. & Yanagimachi, R. (1999). An intact sperm nuclear matrix may be necessary for the mouse paternal genome to participate in embryonic development. *Biol. Reprod.* **60**, 702–6.

Yanagimachi, R. (1994). Mammalian fertilization. In *The Physiology of Reproduction*, ed. E. Knobil & J.D. Neill, pp. 189–317. New York: Raven Press.

https://doi.org/10.1017/S0967199400000824 Published online by Cambridge University Press