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Sperm head shaping in ratites: New insights, yet more questions

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ABSTRACT

Head shaping in mammalian sperm is regulated by a number of factors including acrosome formation, nuclear condensation and the action of the microtubular manchette. A role has also been suggested for the attendant Sertoli cells and the perinuclear theca (PT). In comparison, relatively little information is available on this topic in birds and the presence of a PT *per se* has not been described in this vertebrate order. This study revealed that a similar combination of factors contributed to head shaping in the ostrich, emu and rhea, although the Sertoli cells seem to play a limited role in ratites. A fibro-granular structure analogous to the mammalian PT was identified, consisting of sub- and post-acrosomal components. The latter was characterized by stage-specific finger-like projections that appeared to emanate from the cytoplasmic face of the nuclear envelope. They were particularly obvious beneath the base of the acrosome, and closely aligned, but not connected to, the manchette microtubules. During the final stages of chromatin condensation and elongation of the sperm head the projections abruptly disappeared. They appear to play a role in stabilizing the shape of the sperm head during the caudal translocation of the spermatid cytoplasm.

1. Introduction

Formation and shaping of the sperm head has been studied in numerous vertebrate and invertebrate species (Fawcett et al., 1971; Phillips, 1974; Toshimori and Ito, 2003), particularly in mammals, and a link established between defective head formation/structure and compromised fertility (Barth and Oko, 1989; Dadoune, 1995, 2003; Zamboni, 1987; Zhuang et al., 2014). Although conflicting evidence has been presented in the literature, morphological data supplied by conventional transmission electron microscopy, supplemented by the use of animal models, have implicated various cellular components and processes in nuclear (head) morphogenesis (Fawcett et al., 1971; Hermo et al., 2010; Kierszenbaum et al., 2003, 2007; Kierszenbaum and Tres, 2004; Meistrich, 1993). These include the role of the forming acrosome (Kierszenbaum et al., 2003, 2007; Kierszenbaum and Tres, 2004; Russell et al., 1991), inherent properties of the chromatin condensation process (Fawcett et al., 1971; Prisant et al., 2007; Nozawa et al., 2014; Zhuang et al., 2014) and the involvement of the manchette microtubules (Kierszenbaum, 2002; Meistrich, 1993; Meistrich et al., 1990; Nozawa et al., 2014; Russell et al., 1991, 1994; Toshimori and Ito, 2003). In addition, it has been proposed that the ectoplasmic specializations of the Sertoli cells surrounding the sperm head are instrumental in effecting remodeling of the spermatid nucleus (Kierszenbaum et al., 2007; Russell et al., 1994). The interactive nature of the above factors has also been noted (Tovich et al., 2004).

Recent studies based primarily on the immunocytochemical localization of a range of proteins have identified two key components involved in mammalian head morphogenesis, namely, the perinuclear theca (PT) (Mújica et al., 2003; Toshimori and Ito, 2003) and acroplaxome (Kierszenbaum et al., 2003, 2007; Toshimori and Ito, 2003). The PT has been described as the major cytoskeletal component of the sperm head and is credited with binding of the acrosome to the nucleus (Oko, 1995; Oko and Sutovsky, 2009) as well as maintaining sperm head shape by acting as a mechanical scaffold (Escalier, 1990; Mújica et al., 2003). The acroplaxome, effectively representing the sub-acrosomal component of the PT (Kierszenbaum et al., 2003) described in other mammals, serves to anchor the acrosome to the nuclear envelope and also guides nuclear elongation (through the interaction of the acroplaxome-manchette perinuclear rings) by providing "a mechanical planar scaffold modulating external clutching forces generated by a stack of Sertoli cell F-actin-containing hoops encircling the elongating spermatid nucleus" (Kierszenbaum et al., 2003, 2007).

Compared to the volume and sophistication of the work carried out on sperm head shaping in mammals, surprisingly little infor-

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mation on this phenomenon has been presented in birds. Based on circumstantial morphological evidence, the circular manchette (CM), which manifests during the earlier stages of avian spermiogenesis, has been implicated in effecting nuclear morphogenesis in a number of non-passerine species (Gunawardana and Scott, 1977; Lin and Jones, 1993; McIntosh and Porter, 1967; Okamura and Nishiyama, 1976; Soley, 1996, 1997). The pattern of chromatin condensation (Fawcett et al., 1971), as well as the limited role of the forming acrosome (Soley, 1996, 1997), have also been proposed as factors determining nuclear shaping in birds. The possible involvement of a PT, or indeed the existence of this structure, has not been reported in birds, although the translucent layer beneath the forming acrosome and the peri-nuclear fibro-granular layer posterior to the acrosome (Aire, 2007, 2014; Fawcett et al., 1971) described in some species would suggest the existence of a PT in this vertebrate order. The recent identification of a unique, transient cytoplasmic structure that manifests during spermiogenesis around the post-acrosomal region of the sperm nucleus in ratites (Du Plessis and Soley, 2013) provides compelling evidence for the presence of an analogous entity, at least in ratites.

This paper documents the morphological events relevant to head shaping that occur during spermiogenesis in three ratite species, the ostrich (*Struthio camelus*), emu (*Dromaius novaehollandiae*) and rhea (*Rhea americana albisceus*). The work supplements previous observations on this topic (Soley, 1994, 1996, 1997) and also reports for the first time on the presence, and stage-specific elaboration, of material analogous to the mammalian PT in the post-acrosomal region of the developing spermatid.

2. Materials and methods

The testes of 10 sexually mature and active emus (*Dromaius novaehollandiae*) and ostriches (*Struthio camelus*), respectively, were collected during the breeding season following slaughter at commercial abattoirs. Processed, resin-embedded testes samples of a rhea (*Rhea americana albisceus*) were kindly supplied by Dr. David M. Phillips.

Small blocks of tissue were removed from the emu and ostrich testes and immediately fixed for 24 h at 4 °C in 4% glutaraldehyde buffered with 0.13 M Millonig's phosphate buffer, pH 7.4. Samples were post-fixed for 1 h in 1% similarly buffered osmium tetroxide and routinely prepared for transmission electron microscopy (TEM) (Soley, 1997; Du Plessis and Soley, 2012). Ultrathin sections were viewed in a Philips CM10 transmission electron microscope (Philips Electron Optical Division, Eindhoven, The Netherlands) operated at 80 kV. The resin samples of rhea testes had been fixed and processed as previously described (Phillips and Asa, 1989) and were sectioned and stained as indicated above.

In order to establish the sequence of cellular events characterizing sperm head development on a comparative basis, spermatids were grouped into four categories based on nuclear structure (shape and degree of chromatin condensation) as proposed by Gunawardana and Scott (1977) and applied to spermiogenesis in the ostrich (Soley, 1994, 1996, 1997). Phase I spermatids presented with round nuclei; phase II spermatids with irregularly shaped nuclei; phase III spermatids displayed elongated nuclei containing coarse granular chromatin and phase IV spermatids elongated nuclei with dense, homogeneous chromatin (Fig. 1).

3. Results

3.1. Phase I: spermatids with round nuclei

Early round spermatids displayed a prominent, centrally positioned, round nucleus containing scattered clumps of heterochromatin most of which were arranged peripherally against the inner nuclear membrane (Figs. 1 -Ia,b, 2 a). The intervening clear areas contained fine flocculent material. The cytoplasm displayed numerous mitochondria, as well as smooth and granular endoplasmic reticulum, a Golgi apparatus, chromatoid bodies, the occasional lipid droplet and multivesicular bodies. Most of the organelles were concentrated around the centriolar complex (CC) composed of proximal and distal centrioles. At this early stage, only occasional microtubules were present in the cytoplasm. However, small, regularly spaced deposits of moderately electron-dense granular material appeared close to the outer nuclear membrane in the position later occupied by the microtubules of the CM.

The first obvious sign of acrosome formation was the appearance of typical pro-acrosomal vesicles which coalesced to form a large, round acrosome vesicle filled with homogenous material of moderate electron density. This structure was positioned close to the nuclear membrane in the vicinity of the CC and was generally associated with the Golgi apparatus. The acrosomal vesicle subsequently made contact with the outer nuclear membrane, nestling in a shallow concavity formed by the nuclear envelope (Figs. 1 -Ib, 2 a,b). At the point of attachment the nuclear envelope narrowed and, as a result, this part of the nuclear envelope appeared electron-dense. A thin, translucent layer of cytoplasm remained trapped between the acrosomal vesicle and the nuclear membrane (Fig. 2b). Patches of heterochromatin were always associated with the nuclear aspect of the concavity. In later phase I spermatids the acrosome vesicle was located at a variable distance from the now attached CC, eventually approaching the future cranial pole of the nucleus relative to the forming neck region. The acrosome vesicle remained round during this phase of development with signs of collapse (indicated by ruffling of the membrane) towards the end of this phase (Fig. 2b).

Based on the above observations it was clear that in phase I spermatids the formation of the acrosome was initiated as well as placement of the CC and associated development of the flagellum. No obvious signs of head-shaping were apparent although the sub-acrosomal component (the translucent layer) of the PT (see below) was established.

3.2. Phase II: spermatids with irregularly shaped nuclei

The onset of phase II was characterized by a change in the shape of the nucleus from round to pear-shaped, dumbbell-shaped or scalloped in longitudinal profile (Fig. 1-IIa). Associated with this change in nuclear profile was the appearance of circumferentially disposed microtubules in the cytoplasm close to the outer nuclear membrane, and which were present in greater numbers at the points of nuclear constriction (Fig. 3a). Chromatin clumping gradually became less obvious and the nucleus was filled with fine granular material with intervening clear spaces (Fig. 3c,d). Changes in the appearance of the chromatin were accompanied by an increase in the number of microtubules located at the nuclear perimeter and which collectively constituted the forming CM. The CM ran the entire length of the nucleus, starting just below the base of the acrosome and ending at the proximal part of the midpiece. In the rhea, the CM extended anteriorly beyond the base of the acrosome within a cytoplasmic collar (Fig. 3b), a phenomenon only occasionally observed in the ostrich but not in the emu. In transverse sections of phase II spermatids the microtubules appeared as loosely arranged rings wrapped around the nucleus (Fig. 4a), while in longitudinal sections they were visible as a single row of microtubules closely aligned to the outer nuclear membrane (Fig. 3b). The deposits of granular material observed during later phase I spermatids were intimately associated with the manchette microtubules (see Fig. 3a). During subsequent nuclear elongation and condensation the chromatin displayed localized separation from