

Altered protamine expression and diminished spermatogenesis: what is the link?

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During the elongating spermatid stage of spermiogenesis, human sperm chromatin undergoes a complex transition in which histones are extensively replaced by protamines in a carefully regulated transition including histone modifications and intermediate and temporary replacement of the histones by sperm-specific transition proteins. The replacement of most histones by protamines 1 and 2 facilitates a high order of chromatin packaging necessary for normal sperm function and may also be necessary for DNA silencing and imprinting changes within the sperm cell. Protamines 1 and 2 are usually expressed in nearly equal quantities, but elevated or diminished protamine 1/protamine 2 ratios are observed in some infertile men and is often associated with severe spermatogenesis defects. Human and animal studies demonstrate that expression of the protamine proteins is uniquely regulated by transcription/translation factors, including storage of the mRNA in ribonucleoprotein (RNP) particles composed of the mRNA, transcription factors and a kinesin molecule necessary for transport of the RNP to the cytoplasm and removal of transcriptional activators from the nucleus. Recent studies indicate that most patients with abnormal protamine protein levels have elevated levels of protamine transcript in the mature sperm cell, indicating a possible defect in transcription or translation. The regulation of protamine expression is unique and includes several possible mechanisms which may be responsible for dysregulation of protamine expression and concurrent broad spectrum defects in spermatogenesis. We suggest two hypotheses: (i) that abnormal protamine expression is indicative of a generalized defect in mRNA storage and/or translation which affects other mRNA transcripts or (ii) that protamines may act as a checkpoint of spermatogenesis.

Key words: chromatin/gene expression/protamine/spermatogenesis/transition protein

Introduction

Sperm chromatin is a highly organized, compact structure consisting of DNA and heterogeneous nucleoproteins. The most abundant nucleoproteins in mature sperm are the protamines, positively charged molecules that replace histones during spermiogenesis. Protamines confer a higher order of DNA packaging in sperm than that found in somatic cells, and the condensed and insoluble nature of the highly condensed sperm chromatin protects the genetic integrity of the paternal genome during its transport through the male and female reproductive tracts (Gatewood *et al.*, 1987; Balhorn *et al.*, 1999; Brewer *et al.*, 2002). Protamine replacement may also be necessary for silencing of the paternal genome and reprogramming of the imprinting pattern of the gamete (Aoki and Carrell, 2003).

Humans express two protamines, protamine 1 (P1) and protamine 2 (P2), both of which are expressed in roughly equal quantities (Balhorn *et al.*, 1999; Corzett *et al.*, 2002). Protamines are highly basic sperm-specific nuclear proteins that are characterized

by an arginine-rich core and cysteine residues (Dixon *et al.*, 1986; Krawetz and Dixon, 1988). The high level of arginine causes a net positive charge that facilitates strong DNA binding (Balhorn *et al.*, 2000). The cysteine residues facilitate the formation of multiple inter and intra-protamine disulphide bonds that are essential for the high order of chromatin packaging necessary for normal sperm function (Courtens and Loir, 1981; Loir and Lanneau, 1984; Singh and Rao, 1988; Le Lannic *et al.*, 1993; Szczygiel and Ward, 2002).

During spermiogenesis, protamines progressively replace somatic histones in a stepwise manner (Dixon *et al.*, 1986). First, somatic histones are replaced by testis-specific histone variants, which are then replaced by transition proteins (TP1 and TP2) in a process that involves extensive DNA rearrangement and remodeling (Ward *et al.*, 1989). During the elongating spermatid stage, the transition proteins are replaced in the condensing chromatin by protamines. In humans, ~ 85% of the histones are replaced by protamines. (Hecht, 1989, 1990; Oliva and Dixon, 1990; Dadoune, 1995; Steger, 1999). This sequential process

facilitates molecular remodelling of the male genome within the differentiating spermatid nucleus (Figure 1) (Sassone-Corsi, 2002).

Previous studies have shown that the mean P1/P2 ratio in human sperm is approximately 1.0 (Balhorn *et al.*, 1999; Carrell and Liu, 2001; Oliva, 2006). Sperm from some infertile men have been shown to have altered P1/P2 ratios and/or non-detectable P2 in mature sperm, whereas the occurrence of protamine abnormalities in sperm from fertile men is extremely rare (no known cases have been reported) (Balhorn *et al.*, 1988; Chevallier *et al.*, 1987; Belokopytova *et al.*, 1993; de Yebra *et al.*, 1998; Aoki *et al.*, 2005a; Oliva, 2006). Additionally, transgenic mice with protamine haploinsufficiency have severely altered spermatogenesis and male infertility (Cho *et al.*, 2001). The link between abnormal protamine levels and infertility is intriguing because abnormal protamine expression has been associated with low sperm counts, decreased sperm motility and morphology,

diminished fertilization ability and increased sperm chromatin damage, some of which are not intuitively linked to abnormal chromatin structure (Carrell and Liu, 2001; Mengual *et al.*, 2003; Aoki *et al.*, 2005a).

A direct relationship between abnormal protamine expression and sperm count, motility, morphology or fertilization ability is not readily apparent. The reduction in P1 or P2 in these patients may be explained by reduced protamine transcription, altered translation of the transcript or failed post-translational modifications, but none of these scenarios would directly explain the associated decline in sperm counts and function unless the regulation of protamine exchange is linked to a broader control of spermatogenesis.

This review will briefly summarize the current understanding of protamine replacement of histones, the link between altered protamine replacement and male infertility, the regulation of protamine

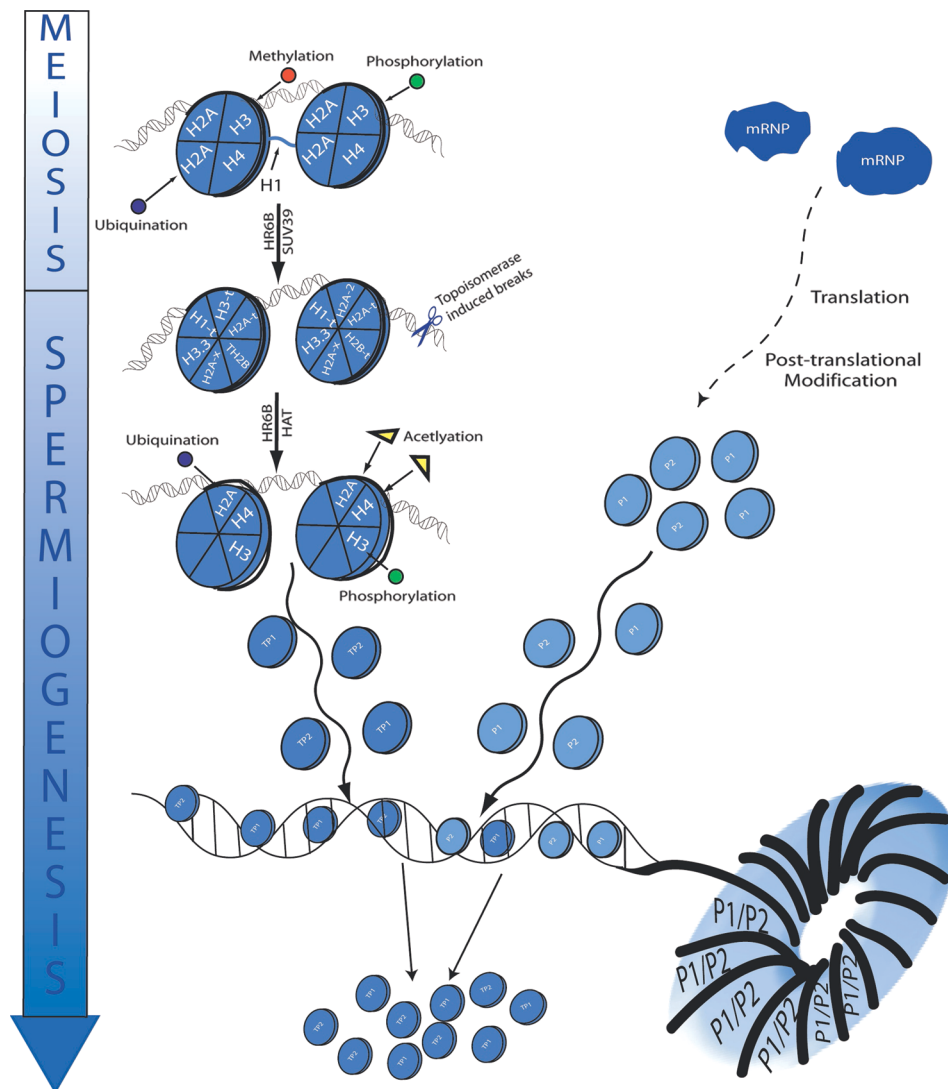


Figure 1. Diagram highlighting the key events in the transition of somatic histones to replacement by protamines. Somatic histones undergo site-specific methylation, phosphorylation and ubiquitination which facilitates their replacement by testis-specific histones (t) during meiosis. Hyperacetylation of H4-t is a key factor in relaxation of the DNA coil to facilitate replacement of the testis-specific histones by the transition proteins, whereas topoisomerase 1 relieves torsional stress by causing double-strand breaks which are subsequently re-ligated. Protamines 1 and 2, processed from a pool of RNP particles, undergo maturation before and during binding to the DNA and replacement of the transition proteins. HR6B, ubiquitin-conjugating enzyme E2B (UBE2B) (RAD6 homolog); HAT, histone acetyltransferase; Suv39, H3 Lys 9 histone methyltransferase.

expression during spermatogenesis and possible causes of altered protamine expression. Two possible models will be discussed regarding the link between abnormal protamine expression and aberrant spermatogenesis. The first hypothesis is that abnormal protamine expression is indicative of a general abnormality of spermatogenesis, possibly due to abnormal function of a transcriptional or translational regulator. Candidate regulatory factors will be discussed. The second hypothesis to be discussed is that the protamines may act as a checkpoint regulator of spermatogenesis and that abnormal protamine expression leads to induction of an apoptotic process that ends in severely diminished semen quality.

Clinical significance of abnormal protamine expression

Abnormal protamine expression is clearly associated with infertility, as recently thoroughly reviewed by Oliva (Oliva, 2006). Briefly, studies have identified males with undetectable P2, which has consistently been linked to severe male infertility (de Yebra *et al.*, 1993; Carrell and Liu, 2001). In mice, haploinsufficiency of the protamines has been shown to cause altered spermatogenesis, including lowered sperm counts and DNA damage (C. Cho *et al.*, 2001; Cho *et al.*, 2003). Numerous studies have demonstrated that an altered ratio of P1/P2, either increased or decreased, is associated with reduced fertility (de Yebra *et al.*, 1998; Carrell and Liu, 2001; Aoki *et al.*, 2006d; Oliva, 2006). Interestingly, no reports have been made of P1/P2 expression abnormalities in males of known fertility.

Initial studies suggested that the most common protamine abnormality in infertile men was an elevated P1/P2 ratio (Oliva, 2006). The elevated P1/P2 ratio is often the result of decreased P2 protein levels, concomitant with an increased level of P2 precursors (Carrell and Liu, 2001; de Yebra *et al.*, 1998; Aoki *et al.*, 2006d). Under expression of P2 accounts for the majority of the cases with high P1/P2 ratio, but subsequent studies have demonstrated that P1 dysregulation also accounts for some abnormalities (Aoki *et al.*, 2005a). However, P2 dysregulation is more common and this may be explained by the fact that the P2 gene is derived more recently than the P1 gene, which may suggest that the regulatory mechanisms governing P2 gene expression are not as stringent and more susceptible to variation than the P1 gene (Lewis *et al.*, 2003).

Human sperm protamine dysregulation is associated with diminished semen quality parameters, sperm functional ability and sperm DNA integrity (de Yebra *et al.*, 1993, 1998; Balhorn *et al.*, 1999; Carrell and Liu, 2001; Aoki *et al.*, 2005a). Aoki *et al.* (2005b) have shown that sperm concentration, motility and morphology are significantly reduced in patients with either a low or a high P1/P2 ratio when compared with patients with a normal P1/P2 ratio. In addition, an altered P1/P2 ratio is associated with decreased fertilization ability, although fertilization and pregnancy rates are not different when patients undergo intracytoplasmic sperm injection (ICSI) as opposed to standard in vitro fertilization (IVF) (Carrell and Liu, 2001; Nasr-Esfahani *et al.*, 2004; Aoki *et al.*, 2005b).

Protamines and DNA damage

One potential consequence of abnormal protamine expression is a susceptibility to DNA damage. Our laboratory has measured DNA

integrity using an assay similar to the sperm chromatin structure assay and compared the DNA fragmentation index with protamine levels in human sperm. Patients with low P1/P2 ratio had significantly elevated DNA fragmentation when compared with patients with normal and high P1/P2 ratios (Aoki *et al.*, 2005b). Moreover, patients who under-expressed P1, P2 or both P1 and P2 had significantly elevated levels of DNA fragmentation compared with patients normally expressing P1 and P2. Additionally, Torregrosa *et al.* (2006) have recently shown a positive correlation between TUNEL-positive sperm and the presence of P2 precursors. These studies emphasized the important role protamines play in protecting the genetic content of the mature sperm from nucleases.

A recent study evaluated the role of protamine abnormalities at an individual cell level by using fluorescence immunohistochemistry techniques to simultaneously evaluate protamine levels, cell viability and DNA damage as measured by the TUNEL assay (Aoki *et al.*, 2006c). Concurrently, global protamine levels were evaluated with a fraction of the semen sample that underwent standard nuclear protein extraction and electrophoresis. The data not only confirmed a close correlation between the mean protamine levels determined by fluorescence microscopy and the standard electrophoresis technique, but also showed that within a semen sample there is heterogeneity in protamine expression and a clear correlation between under-expression of protamines, DNA damage and lack of viability (Aoki *et al.*, 2006c). The intra-ejaculate protamine heterogeneity observed in this study is consistent with other reports using CMA3 and Aniline Blue staining to assess protamine quantity indirectly (Manicardi *et al.*, 1995; Hammadeh *et al.*, 2001), but novel in the direct link between protamine abnormalities in a given cell and DNA damage within the cell.

Possible mechanisms of DNA damage

DNA nicks may be induced through apoptotic processes (Cisternas and Moreno, 2006). Apoptosis regulates germ cell over proliferation and eliminates defective germ cells from the genetic pool (Hikim *et al.*, 1995; Rodriguez *et al.*, 1997; Pentikainen *et al.*, 1999). Apoptosis is characterized by DNA double-stranded breaks which occur as a result of activated endogenous DNA nucleases (Gorzcyca *et al.*, 1993). In somatic cells, the apoptotic cascade involves the formation of apoptotic body; however, in highly differentiated spermatozoa, the sequence of events may differ as a result of the highly condensed sperm nucleus (Hikim *et al.*, 1995). Spermatozoa that are marked for apoptotic degradation may have normal mitochondrial activity, high or low motility (Barroso *et al.*, 2000) as well as normal morphology (Host *et al.*, 2000a,b). Oosterhuis *et al.* (2000) reported that 20% of ejaculated spermatozoa showed DNA strand breaks and the apoptotic marker annexin V, whereas, Sakkas *et al.* (1999) reported that DNA strand breaks and apoptotic markers did not co-exist together in the same mature spermatozoa. Ejaculated spermatozoa with apoptotic markers appeared to have escaped programmed cell death in a process called abortive apoptosis (Sakkas *et al.*, 1999). Therefore, it will be important to distinguish between cells that show high levels of DNA strand breaks and cells that are positive for apoptotic markers. It is inappropriate to assume that strand breaks are synonymous with apoptotic degeneration.

DNA damage may also be increased if the DNA nicking and ligating activities of topoisomerase II are defective. The presence of higher than usual levels of topoisomerase II found during the elongating spermatid stage is associated with high levels of DNA nicks (Roca and Mezquita, 1989; McPherson and Longo, 1993), possibly needed to relieve torsional stress caused by the negative supercoiling associated with histone to protamine transition (Balhorn, 1982; Risley *et al.*, 1986; McPherson and Longo, 1993). These nicks are not usually harmful, since they are usually re-ligated prior to completion of spermiogenesis and ejaculation (McPherson and Longo, 1993). However, if the activity of topoisomerase is blocked or disrupted, then DNA nicks remain in mature sperm or are not repaired properly (Morse-Gaudio and Risley, 1994).

Shaman *et al.* have recently demonstrated that topoisomerase II likely acts in two ways. First, it relieves torsional stress by causing double strand breaks which are re-ligated (termed sperm chromatin fragmentation). Second, it acts in conjunction with an extracellular nuclease to cause regulated double-strand breaks in protamine-bound DNA at ~50 kb intervals, the DNA span of one loop bound to protamine (termed sperm DNA degradation) (Sotolongo *et al.*, 2005; Shaman *et al.*, 2006). In the absence of protamines, extensive degradation occurs. This topoisomerase/nuclease-induced DNA degradation may be a specialized apoptotic pathway in sperm, different from the normal function of topoisomerase in relieving torsional stress, followed by re-ligation of the DNA break (Shaman *et al.*, 2006).

Caron *et al.* (2001) suggested that the transient DNA nicks can be repaired by transition protein 1. Transition proteins have been found to have an undefined enzymatic activity that is responsible for repairing single-stranded breaks and UV-induced DNA lesions *in vivo*; therefore, the role of transition proteins extends beyond initiating DNA compaction to restoring transient DNA nicks. Evidence from the literature indicates that the disappearance of single-strand breaks during spermiogenesis is coincident with the presence of the transition proteins in elongating spermatid (Sakkas *et al.*, 1995; Kistler *et al.*, 1996; Smith and Haaf, 1998).

Reactive oxygen species and chromatin damage

In recent years, concern has been expressed about the generation of reactive oxygen species (ROS) in the male reproductive tract. High levels of ROS are toxic to sperm quality and function (Saleh and Agarwal, 2002). Elevated levels of ROS have been reported in 25–40% of the infertile patients (Padron *et al.*, 1997). Two factors that protect the DNA from oxidative stress are tight DNA packaging and antioxidants present in seminal plasma (Twigg *et al.*, 1998). Oxidative stress happens as a result of the imbalance between ROS generation and antioxidants scavenging activities (Sikka, 2001). Strong evidence suggests that the presence of single and double-stranded breaks observed in infertile patients is a result of ROS (Fraga *et al.*, 1996; Kodama *et al.*, 1997; Sun *et al.*, 1997; Aitken and Baker, 2004). The presence of 8-hydroxy-2-deoxyguanosine in seminal plasma has been used as a marker for oxidative DNA damage (Ames *et al.*, 1993). A significant positive correlation was established between DNA fragmentation and ROS (Barroso *et al.*, 2000). Furthermore, exposure of sperm to artificially produced ROS resulted

in a significant increase in DNA damage in the form of deletions, frame shifts, DNA cross-links and chromosomal rearrangements (Twigg *et al.*, 1998a,b; Kemal Duru *et al.*, 2000). However, direct studies on ROS-induced damage in protamine deficient sperm have not been performed.

Protamine abnormalities and assisted reproduction techniques

The intrasample heterogeneity of protamine content is clinically significant for patients undergoing assisted reproductive technology (ART) (Aoki *et al.*, 2006c). Protamine-deficient patients undergoing human IVF/ICSI have been shown to have normal embryo quality, implantation and pregnancy rates (Carrell and Liu, 2001; Nasr-Esfahani *et al.*, 2004; Aoki *et al.*, 2005b). This heterogeneity in protamine concentration in sperm of a given semen sample may explain how ICSI appears to overcome poor semen quality and DNA damage (Aoki *et al.*, 2006e). It is possible that selection of the most morphologically normal motile sperm for ICSI injection inherently selects for sperm with normal protamine expression, although studies evaluating sperm morphology and protamines in individual sperm have not been performed. Interestingly, the above findings are inconsistent with the data presented from the mouse protamine-deficient haploinsufficiency model, in which haploinsufficient mice were found to have a higher rate of embryo death when ICSI was performed (Cho *et al.*, 2001, 2003). This difference in embryo lethality, reflecting a more severe effect of abnormal protamine expression in this model, may be the result of a homogeneous pathology throughout the seminiferous tubule rather than the variable expression seen in infertile men with protamine expression defects or may be the result of a lack of effect on sperm morphology in mice.

The long-term consequences of ICSI with DNA-damaged sperm is still not clear (Silber, 1995; Ludwig, 2005; Verpoest and Tournaye, 2006). Animal studies have suggested a sperm DNA damage threshold below which a normal embryo can develop (Ahmadi and Ng, 1999). Others have demonstrated that the oocyte may have the DNA repair system that aids in 'repairing' altered chromatin (Ashwood-Smith and Edwards, 1996; Perry *et al.*, 1999). Another concern regarding the use of sperm with abnormal chromatin is the potential for improper gene imprinting, since protamines have been suggested to be a possible regulator of normal genomic imprinting (Aoki and Carrell, 2003; Oliva, 2006) and since imprinting errors have been suggested to be elevated in patients undergoing ART (Allen and Reardon, 2005; Chang *et al.*, 2005; Ludwig *et al.*, 2005). Hartman *et al.* (2006) have recently noted no increase in imprinting errors in men with severe spermatogenesis defects. Our laboratory has recently reported that defects in global methylation are not observed in men with known protamine abnormalities (Aoki *et al.*, 2006b). Clearly, there is a need for further studies to evaluate specific gene imprinting in those patients and other potential defects in sperm with abnormal protamine replacement and/or DNA damage.

Protamine replacement of histones

Although protamine replacement is often termed 'a two-step process' (histones replaced by transition proteins which are

replaced by protamines), more steps are involved, including the expression and incorporation of testis-specific histone variants, histone hyperacetylation, replacement of histones with transition proteins and protamine incorporation and phosphorylation (Aoki and Carrell, 2003; Churikov *et al.*, 2004b). Each of these steps is critical to proper progression of chromatin maturation and spermiogenesis.

Histone modifications

In somatic cells, nucleosomes are composed of two molecules of histones 2A, 2B, 3 and 4 (H2A, H2B, H3 and H4). Histone 1 (H1 or linker histone) links inter-nucleosomal DNA. In addition to the somatic-type histone variants, spermatogenic cells express testis-specific histones that replace somatic histones (Dadoune, 2003). Although termed 'testis-specific' histones, at least one testis variant, an H3 variant, has been shown to be expressed in somatic cells (Govin *et al.*, 2005).

The characterization of testis-specific histone variants is in the early stages, but several testicular variants have been identified in the human for both the nucleosome and linker histones (Churikov *et al.*, 2004b; Govin *et al.*, 2005). The differences observed between testis-specific histones and somatic histones include structural differences in the N-terminal region, the core region and the C-terminal region. Interestingly, some testis-specific histone variants do not undergo 3' polyadenylation and are translated early during spermatogenesis (Zalensky *et al.*, 2002; Churikov *et al.*, 2004a). Among those variants is an H2B variant which has been shown to localize in telomeres and may be important in meiosis (Gineitis *et al.*, 2000). Another key difference in the testis variant of H2B is the replacement of four prolines found in the N-terminal region of the somatic H2B with phosphorylatable amino acids, likely indicating that their function is regulated by phosphorylation (Churikov *et al.*, 2004a).

Recently, Zhang *et al.* (2006) have shown that increased levels of histone 2B in sperm is associated with lower levels of protamines. Although previous studies have demonstrated high levels of histones in the sperm of some infertility patients and an indirect link between histone retention and altered sperm protamine expression, this study is the first direct evidence of abnormal histone retention linked to altered protamine replacement. Future studies will likely focus on the further characterization of histone variants and whether abnormal expression of a testis-specific histone variant may be directly responsible for altered protamine replacement. In that regard, Tanaka *et al.* have recently evaluated the gene sequence of *HANP1* in infertile and fertile men. *HANP1* is the human orthologue of the mouse *Hanp1/Hit2* gene that encodes a testes variant of H1; homozygous disruptions of this gene in mice has previously been shown to cause male infertility (Tanaka *et al.*, 2005, 2006). Although five single nucleotide polymorphisms (SNPs) were identified for *HANP1* in their study population, the SNPs did not appear to be linked to male infertility (Tanaka *et al.*, 2006). Further studies are warranted to evaluate both the protein and the gene in males with known protamine abnormalities.

Hyperacetylation of the histones is critical for normal progression of spermatogenesis and is regulated by an interplay of histone acetyl transferases and histone deacetylases (Candido and Dixon, 1972; Grimes and Henderson, 1984; Meistrich *et al.*,

1992; Hazzouri *et al.*, 2000; Marcon and Boissonneault, 2004). Histone hyperacetylation reduces the binding between nucleosomes and DNA, leading to chromatin relaxation (Hong *et al.*, 1993), and is also associated with the activation of topoisomerases in inducing strand breaks. Species that retain histones throughout spermiogenesis have relatively low levels of acetylated histones (Kennedy and Davies, 1980, 1981). It has been suggested that hyperacetylation of core histones may facilitate their displacement by protamines (Oliva and Mezquita, 1982, 1986; Oliva *et al.*, 1987), and a double bromodomain containing testis-specific factor (BRDT) has been identified in mice as a possible key factor in the transition process. BRDT has been shown to be capable of condensing acetylated chromatin (Pivot-Pajot *et al.*, 2003) by recruiting a highly expressed chaperone protein, CIA-II, to mediate histone removal (Umehara and Horikoshi, 2003).

Sonnack *et al.* (2002) have demonstrated a relationship between decreased acetylation and abnormal spermatogenesis. They also observed increased acetylation in spermatocytes of testes exhibiting maturation arrest, indicating a possible relationship between premature hyperacetylation and maturation arrest. This same laboratory has also demonstrated that the administration of histone deacetylases results in severe infertility (Fenic *et al.*, 2004). Future studies will likely evaluate the degree of acetylation in protamine-deficient patients and experimental models with abnormal protamine expression.

Replacement of histones with transition proteins

DNA relaxation, as a result of hyperacetylation and topoisomerase activity, facilitates the exchange of histones with transition proteins which are proteins of intermediate basicity (Wilkins, 1956; Courtens and Loir, 1981; Luerssen *et al.*, 1989; Oliva and Dixon, 1991; Ward and Coffey, 1991; Kierszenbaum, 2001; Meistrich *et al.*, 2003). TP1 and TP2 mRNA are first seen in the post-meiotic, round spermatid stage in mice and are degraded around stages, 13–14. The proteins are observed in stages 12 and 13 and are removed by stage 14 when they are replaced with protamines (Figure 2) (Kistler *et al.*, 1996). There is significant overlap in

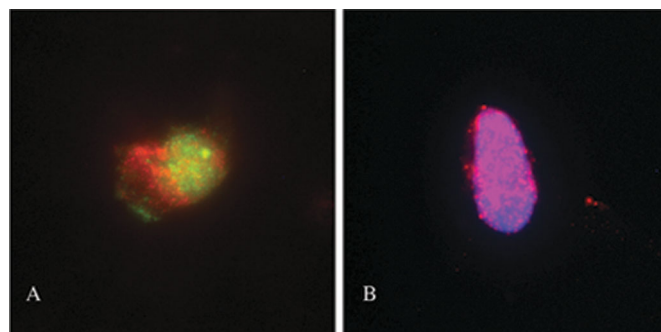


Figure 2. Immunofluorescence microscopy of the localization of DNA, transition protein 1 and protamine 1 in a round spermatid and elongating spermatid. (A) In the round spermatid, transition protein 1 is seen within the nucleus (green fluorescence) and protamine 1 is located in the cytoplasm (red fluorescence). (B) An elongating spermatid shows no staining for transition protein 1 within the nucleus, although mature protamine is observed throughout the nucleus (red fluorescence) (for methods, see Aoki *et al.*, 2006).

the expression of histones, transition proteins and protamines (Meistrich *et al.*, 2003). Expression of the proteins has been shown to have some overlap in human-elongating spermatids (Aoki *et al.*, 2006c).

TP1 is a 60 kDa protein with numerous basic amino acids distributed randomly throughout the molecule (Kistler *et al.*, 1975). TP1 has important DNA-destabilizing properties due to the presence of two tyrosine residues flanked by basic amino acids (Singh and Rao, 1988). TP2 is a 13 kDa protein that contains proline, serine, arginine and lysine residues (Grimes *et al.*, 1975). Two potential zinc finger domains exist in mouse and rat TP2 and may play an important role in the initiation of chromatin condensation and cessation of transcriptional activity during mammalian spermiogenesis (Baskaran and Rao, 1991). In transgenic mice, animals devoid of transition protein 2 had reduced amounts of processed P2 proteins and failed to complete chromatin compaction (Cho *et al.*, 2003; Meistrich *et al.*, 2003). In addition to incomplete chromatin condensation, sperm from TP2 null mice show an increase in DNA denaturation when compared with sperm from control mice (Zhao *et al.*, 2001). The increased denaturability of the DNA is believed to result from DNA strand breaks (Sailer *et al.*, 1995; Aravindan *et al.*, 1997).

Studies using double knock out mice for both TP1 and TP2 have shown that the absence of one transition protein does not affect the level of transcription or translation of the other transition protein or the protamines, but does affect the retention of the other transition protein through post-translational modifications (Shirley *et al.*, 2004; Zhao *et al.*, 2004). Although the redundancy is not complete, there is compensation for one transition protein by the other, as demonstrated by the fact that double heterozygous mice exhibit more severe sperm defects than do mice homozygous for a single mutation. Interestingly, sperm from transition protein-deficient mice are able to fertilize oocytes using ICSI if the sperm were isolated from the testis or caput epididymus, but are not capable of fertilization if isolated from the cauda epididymus (Suganuma *et al.*, 2005).

Replacement of transition proteins with protamines

Protamine 1 is translated as a mature protein of 50 amino acids, whereas protamine 2 is initially 103 amino acids and undergoes N-terminus cleavage to a mature protein of 57 amino acids (Figure 3) (Aoki and Carrell, 2003). Following translation, protamine 1 is immediately phosphorylated, primarily under the control of serine/arginine protein-specific kinase 1 (SRPK1) (Green *et al.*, 1994; Papoutsopoulou *et al.*, 1999). A protamine 2 intermediate protein is phosphorylated, largely under the control of Ca^{2+} /calmodulin-dependent protein kinase 4 (CAMK4). The phosphorylation of the P2 intermediate is requisite for binding of the protein to chromatin, which is required for final cleavage of the protein to its mature form of 57 amino acids.

Protamine phosphorylation is not only necessary for final processing of P2, but also for proper binding of the proteins to DNA. However, once bound to DNA, the protamines are de-phosphorylated. The de-phosphorylation appears to be essential for proper condensation of the chromatin, although some controversy exists (Gusse *et al.*, 1986; Aoki and Carrell, 2003). A recent study evaluating the effects of organophosphorous pesticides on sperm protamine phosphorylation showed that the

resulting sperm had abnormal chromatin condensation with subsequent DNA damage (Pina-Guzman *et al.*, 2005). Abnormal phosphorylation may be relevant to human exposure to organophosphorous pesticides (Sanchez-Pena *et al.*, 2004; Pina-Guzman *et al.*, 2005, 2006).

The replacement of transition proteins with protamines induces a conformational change in the packaging of the chromatin. The chromatin forms loop domains, which are less than half the size of somatic cell histone loops, then forms toroidal structures, which have a 6–20-fold increase in packaging compaction (Ward and Coffey, 1991; Balhorn *et al.*, 2000). The mechanism by which protamines induce the conformational changes is not well understood (D'Auria *et al.*, 1993; Bianchi *et al.*, 1994; Fuentes-Mascorro *et al.*, 2000; Aoki and Carrell, 2003). P1 and P2 may bind to the major and minor groove of DNA or to the DNA surface by interacting electrostatically with phosphate residues (D'Auria *et al.*, 1993; Bianchi *et al.*, 1994; Balhorn *et al.*, 1999; Fuentes-Mascorro *et al.*, 2000).

Protamines are currently thought to be necessary for (i) condensing the male genome to generate a more compact and hydrodynamic nucleus, (ii) protecting the genetic message from nucleases, mutagens or damage from ROS or other factors, (iii) epigenetic modification during spermiogenesis and (iv) removing transcription factors and proteins to help reset the imprinting code in the oocyte (Oliva, 2006). Altering the sperm protamine content can disrupt any of the functions listed above.

Regulation of protamine expression and potential causes of abnormalities

The human sperm haploid genome encodes a single copy of human P1 and P2 genes which maps to chromosome 16p13.3 (Domenjoud *et al.*, 1991). This locus also contains the TP2 gene. The P1–P2–TP2 locus spans a 28.5 kb region in which the three genes are arranged in a linear array, presumably facilitating concurrent or co-ordinated gene expression (Schluter *et al.*, 1992; Choudhary *et al.*, 1995). The P1 gene is present in all mammalian species, whereas P2 is present in mouse, hamster, rat, stallion and man (Calvin, 1976; McKay *et al.*, 1985, 1986; Poccia, 1986; Bower *et al.*, 1987; de Yebra *et al.*, 1993). However, in some species, the *protamine 2* gene is present but the protein is absent (Maier *et al.*, 1990). These findings suggest that *P1* is inherited from a common ancestor, since it is present in all species and that the *P2* gene may have been derived from the *P1* gene through divergent evolution. Alternatively, the *P1* and *P2* genes may have been inherited from a single common ancestor, but successive species have lost the ability to express *protamine 2* (Calvin, 1976; Oliva and Dixon, 1990).

Several factors have been postulated and studied as possible causes of P1/P2 deregulation. These factors are summarized in Table I and discussed in the following sections.

Gene polymorphisms

The protamine or transition protein genes could harbour mutations or polymorphisms that could induce conformational changes in the proteins, which could alter their incorporation into sperm chromatin. De Yebra *et al.* (1993) performed a preliminary mutational analysis of the protamine gene analysis in four patients with

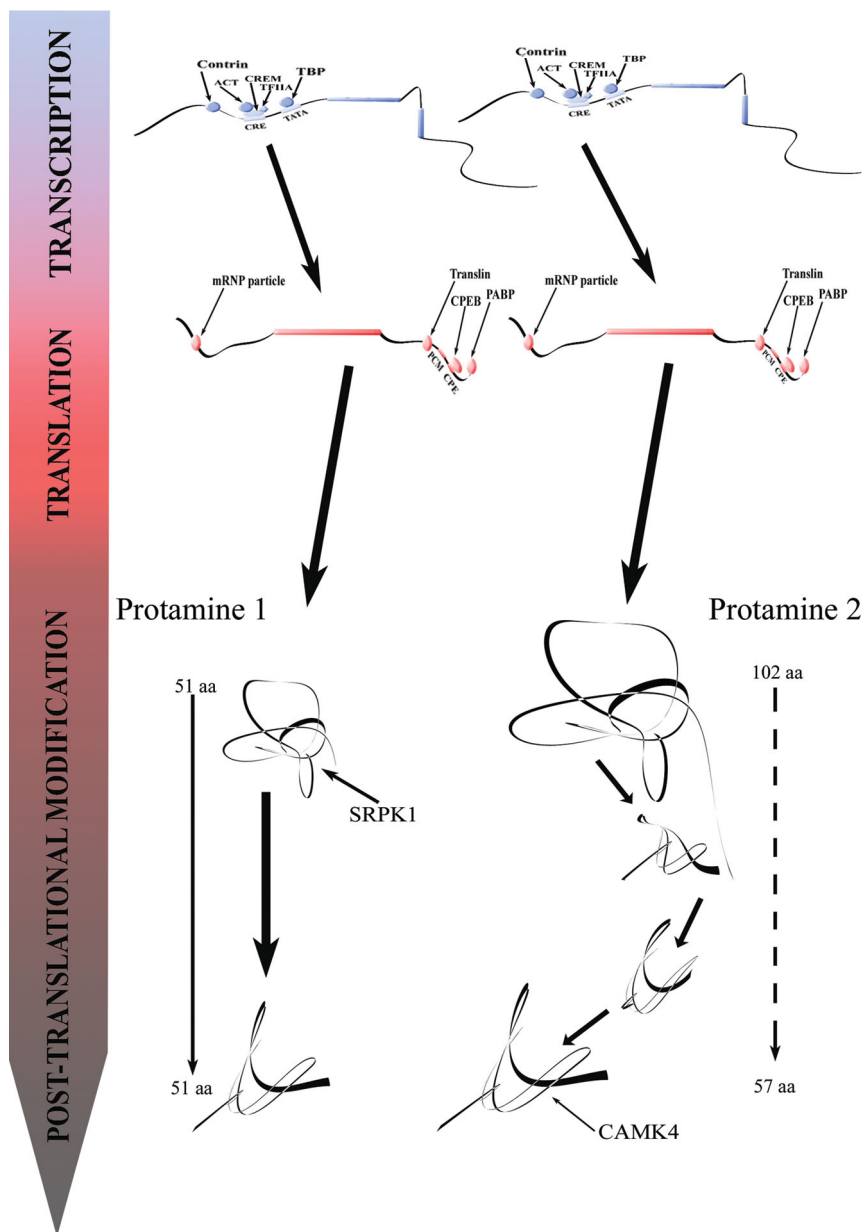


Figure 3. A schematic drawing of key regulators of expression of protamines 1 and 2 at the levels of genomic transcription, translation and post-translational modifications to yield the mature protamine products. The key regulating protein complexes are shown for each stage of protein maturation. CREM, cAMP response element modulator; ACT, activator of CREM in the testis; TFIIA, transcription factor II alpha; CRE, CREM response element; TBP, TATA box binding protein; PABP, poly-a binding protein; CPE, cytoplasmic polyadenylation element; CPEB, cytoplasmic polyadenylation element binding protein; PCM, polyadenylation consensus motif; SRPK1, serine/arginine protein-specific kinase-1; CAMK4, calcium/calmodulin-dependent protein kinase IV.

markedly altered P1/P2 ratios with no mutations observed. Subsequently, Schlicker *et al.* (1994) screened 36 infertile patients with chromatin anomalies, but he failed to identify any mutations in the genes encoding P1, P2 or TP1. Tanaka *et al.* (2003) reported four synonymous polymorphisms in P1 and one SNP in P2 that generated a premature stop codon. The SNP in the P2 gene that induces translation termination may result in male infertility due to haploinsufficiency of P2.

Iguchi *et al.* (2006) sequenced the protamine genes in men exhibiting semen quality defects consistent with protamine abnormalities (i.e. sperm DNA damage). In their study, a heterozygous SNP which altered a highly conserved arginine residue was found in

10% (3/30) of the patients studied, but not seen in controls. This SNP converts one of the highly conserved arginines to a serine residue, therefore creating an RS sequence which can serve as a potential phosphorylation site for the enzyme SRPK1. Improper phosphorylation can substantially alter both DNA binding and protamine-to-protamine interaction in the sperm nucleus.

Recently, a larger patient population with known abnormal protamine ratios was screened to identify SNPs in the protamine and transition protein genes potentially responsible for the patients' altered protamine expression (Aoki *et al.*, 2006a). Fifteen SNPs were identified in this study (three SNPs in P1, seven in P2, two

Table 1. Studies that have identified potential causes for P1/P2 dysregulation

Gene studies	
Protamines	Maier <i>et al.</i> (1990) Cho <i>et al.</i> (2003) Cho <i>et al.</i> (2001) Tanaka <i>et al.</i> (2003) Aoki <i>et al.</i> (2006) Iguchi <i>et al.</i> (2006) Queralt <i>et al.</i> (2003) Schnulle <i>et al.</i> (2004) Yu <i>et al.</i> (2000) Adham <i>et al.</i> (2001) Meistrich <i>et al.</i> (2003) Shirley <i>et al.</i> (2004) Suganuma <i>et al.</i> (2005) Zhao <i>et al.</i> (2004)
Transition proteins	Our laboratory's unpublished results De Jonckheere <i>et al.</i> (1994)
Protamine UTR	
Ancillary genes	
CAMK4/SRPK1	Wu <i>et al.</i> (2000)
AF5q31	Urano <i>et al.</i> (2005)
MARs	Wykes <i>et al.</i> (2003) Martins <i>et al.</i> (2004)
Transcription	
CREM	Steger (1999) Behr <i>et al.</i> (2000) Bleny <i>et al.</i> (1996) Weinbaur <i>et al.</i> (1998)
ACT /KIF17B	Macho <i>et al.</i> (2002) Kotaja <i>et al.</i> (2004)
MSY2	Yang <i>et al.</i> (2005b)
Storage	
Protamine 3' UTR	Lee <i>et al.</i> (1995) Fajardo <i>et al.</i> (1994, 1997) Kwon and Hecht (1993)
Translation	
PRBP/TRBP	Zhong <i>et al.</i> (1999) Braun <i>et al.</i> (2000)
ACT /KIF17B	Macho <i>et al.</i> (2002) Kotaja <i>et al.</i> (2004)

A summary of studies investigating potential abnormalities that may underlie aberrant P1 and P2 expression. These include mutations in the protamine genes or in ancillary genes, defects in transcriptional regulatory mechanisms, alterations in RNA binding proteins or RNA binding protein sites and aberrant translational regulatory factors.

in TP1 and three in TP2); however, the frequencies of these SNPs were similar in protamine-deficient patients, severely infertile patients without protamine defects and fertile controls. The SNPs identified in this study included differences from the SNPs reported by Tanaka *et al.* (2003). It is important to note that the differences may reflect differences in the populations studied (European ancestry versus Japanese ancestry). Also, it is important to note that the P1 SNP reported by Iguchi *et al.* was not elevated in the study by Aoki *et al.* (2006a) (Iguchi *et al.*, 2006). In summary, these studies indicate that gene SNPs in the protamine and transition protein genes are not likely to be a common cause of protamine abnormalities.

In addition to evaluating the coding gene sequences, it is important to consider possible mutations in the upstream and downstream non-coding regions of the gene. De Jonckheere *et al.* (1994) examined potential mutations in the untranslated regions (UTRs) of the protamine genes and identified a candidate mutation in the GA repeat upstream of the transcriptional start site of P2. Recently, our laboratory identified 14 SNPs in the UTRs of

P1 and P2 (Emery *et al.*, 2006, submitted for publication). Of greatest significance was a GC change 62 bp into the 3'-UTR of P2, which occurred in five infertility patients and in six of the Utah CEPH database patients. Interestingly, none of the men with confirmed normal protamine ratios, or the fertile sperm donors, carried this change. This G/C SNP was found in the sperm cDNAs and in the genomic sequence. We also noted the previously identified GA repeat in the genomic P2 5'-UTR. The variable length GA repeat length occurs with equal frequency in UGRP controls and in men with abnormal protamine expression. The most prevalent GA repeat lengths were 12, 15 and 18 bp.

Regulation of transcription

Protamines are expressed in the round spermatid stage, but protamine transcription and translation are temporarily uncoupled in the developing spermatid due to transcriptional and translational regulatory mechanisms (Calvin, 1976; Sassone-Corsi, 2002). Transcriptional regulation depends on potentiation of the genes via association with nuclear matrix attachment regions (MARs), and binding of *trans*-acting factors to the promoter region (Martins *et al.*, 2004; McCarrey *et al.*, 2005).

The 5' and 3' regions surrounding P1/P2/TP2 genes contain MARs which are *cis*-regulatory units involved in attachment of the DNA to the protein scaffolding of the nuclear matrix in an organized manner of loop domains which potentiates the genes for transcription dependent on other *trans*-regulatory factors (Martins *et al.*, 2004). The MARs are located at the linker sites of protamine toroids and contain repetitive elements which may, contrary to the usual paradigm, be highly methylated in the round spermatid stage in which the genes undergo transcription (Choi *et al.*, 1997; Shaman *et al.*, 2006). Kramer *et al.* (1997) scanned the P1–P2–TP2 locus in several oligozoospermic, infertile individuals and identified mutations in the sperm nuclear matrix of two of the five affected individuals. This laboratory later used transgenic analysis to evaluate the relationship between the nuclear matrix associations and the expression of protamine genes (Martins *et al.*, 2004). This study demonstrated that in the absence of the 5'-MAR and with the presence of only the 3'-MAR, protamine transcription was reduced but not ablated. This suggests that the 3'-MAR provides a protective role against silencing of the protamine genes and that synergy between the upstream and downstream MARS is required for the proper regulation of the protamine genes (Martins *et al.*, 2004).

Trans-regulatory factors of transcription act via the promoter region and include the TATA-box protein (TBP), cAMP response element modulator (CREM) and Y-box proteins (Steger *et al.*, 2000; Maclean and Wilkinson, 2005; Tanaka and Baba, 2005; DeJong, 2006). TBPs bind to the TATA sequence in conjunction with other factors to facilitate RNA polymerase 2 interaction with the gene sequence. TBP is not essential for gene expression, but is important in initiating transcription (Schmidt and Schibler, 1997; Kimmins *et al.*, 2004a). TBP over-expression occurs between 18 and 28 days in mice, which corresponds with the transcription of genes in haploid cells (Schmidt and Schibler, 1995). TBP-like factor (TLF) has a sequence similar to TBP and a similarly unique expression profile in the testis, but is unique in that it binds to TATA-less promoters such as pes-10 (Kaltenbach *et al.*, 2000). TLF is seen in the cytoplasm during

the early pachytene stage, then enters the nucleus and remains there throughout the round spermatid stage until it moves to the cytoplasm again in the elongating spermatid stage when transcription is terminated (Martianov *et al.*, 2002). TLF may act as both an activator and a repressor of transcription (Moore *et al.*, 1999). TLF null mice have abnormal heterochromatin organization, which may affect protamine replacement and chromatin condensation and induce apoptosis (Martianov *et al.*, 2001, 2002).

The transcription factor CREM is highly expressed in male germ cells (Delmas *et al.*, 1993) and is known to regulate the expression of several post-meiotic genes, such as the transition proteins and protamines, and is likely the key regulator of gene expression during spermatogenesis (Krausz and Sassone-Corsi, 2005; Hogeveen and Sassone-Corsi, 2006). Targeted disruption of the CREM gene blocks the differentiation program in the first step of spermiogenesis (Blendy *et al.*, 1996; Nantel *et al.*, 1996). These findings indicate a crucial role of CREM in post-meiotic germ cell differentiation, linking the action of hormonal stimuli to direct regulation of spermatogenesis genes (Sassone-Corsi, 1998).

The cAMP response element (CRE) is found in the promoter region of the protamine genes and it serves as a binding site for the transcriptional activator CREM tau in the testes. CREM tau is associated with the activator of CREM (ACT) and the testis-specific kinesin protein KIF 17B. In addition to serving as a molecular motor for mRNA transport, KIF 17B also acts as a co-factor to ACT, and hence as an activator for CREM-mediated transcription. Interestingly, all mRNAs isolated by co-immunoprecipitation with KIF 17B and testis-brain RNA-binding protein (TB RBP) are transcripts which are regulated through CREM activity (Chennathukuzhi *et al.*, 2003a). By transport of the ribonucleoprotein (RNP) to the cytoplasm, KIF 17B/ACT is not available in the nucleus, and hence CREM-regulated transcription is repressed (Kimmins *et al.*, 2004b; Krausz and Sassone-Corsi, 2005). The linkage of KIF 17B to regulation of both CREM-mediated transcription and temporal regulation of translation indicates the major role this protein plays in the regulation of the expression of protamines and other key spermiogenesis proteins (Nagamori *et al.*, 2006; Sassone-Corsi, 2005).

Male mice lacking a functional *CREM* gene are sterile due to maturation arrest at the round spermatid stage (Blendy *et al.*, 1996; Nantel *et al.*, 1996). Mice with selective ACT deletion display a drastic decrease in the number of mature sperm and exhibit major defects in sperm head morphology (chromatin compaction and acrosome defects) and tail morphology (Kotaja *et al.*, 2004). Our laboratory recently identified potentially significant polymorphisms of the *ACT* gene in men with severe infertility (Christensen *et al.*, 2006), but studies in patients with known protamine expression abnormalities have not yet been completed.

The role of Y-box proteins in the regulation of protamine expression

Y-box proteins are important regulators of protein expression during spermatogenesis. They bind both DNA and RNA and, generally, up-regulate transcription and down-regulate translation. Several Y-box proteins have been identified in the germ cells of mice and humans (Braun, 1990; Tafuri *et al.*, 1993; Yiu and

Hecht, 1997; Tekur *et al.*, 1999; Iuchi *et al.*, 2001) Among the Y-box proteins active during spermatogenesis is Contrin and its mouse orthologue MSY2, which are found in very high concentrations in male germ cells (Gu *et al.*, 1998; Tekur *et al.*, 1999). Two functions have been proposed for Contrin/MSY2 RNA-binding protein in regard to protamine expression (Yang *et al.*, 2005b). First, it serves as a co-activator for protamine transcription by binding to the Y box element sequence found in the gene promoter region. Second, it stabilizes the maternal and paternal transcripts in the cytoplasm to effect the temporal regulation of translation as described above. Yang *et al.* generated an MSY2 knockout model to define the function of MSY2 in mammalian development (Yang *et al.*, 2006). MSY2-null male mice were sterile, with severely amorphous and multinucleated spermatids. The sperm were observed in the seminiferous tubules, but not in the epididymus (Yang *et al.*, 2006).

Translin is another DNA/RNA-binding protein involved in translational regulation during spermatogenesis. There is a high degree of similarity between Translin and its mouse orthologue, TB RBP. Like Contrin, Translin binds to specific mRNAs in the testis, forming an RNP complex which is transported to the cytoplasm and adjacent cells through intercytoplasmic bridges (Morales *et al.*, 2002). TB RBP interacts with a protein termed Translin-associated factor X that has a nuclear localization signal, although TB RBP has a cytoplasmic localization signal. The ratio of the two proteins determines the actual localization of the complex (Cho *et al.*, 2004). KIF 17B is essential for proper movement of the Contrin and Translin-containing RNP particles to the cytoplasm and through adjacent cytoplasmic bridges (Chennathukuzhi *et al.*, 2003a). Mice lacking the TB RBP gene are able to sire offspring, but have reduced sperm production (Chennathukuzhi *et al.*, 2003b). The direct effect of TB RBP gene knock-out on protamine expression is not known (Kimmins *et al.*, 2004a; Sassone-Corsi, 2005).

Regulation of translation

Protamines 1 and 2 undergo translational control during spermiogenesis (Steger, 2001). Protamines are transcribed in the round spermatid stage, but the mRNAs are stored in translationally repressed RNPs in early haploid cells and activated in elongated spermatids (Kleene, 1989). This stage-specific pattern of gene expression is essential for correct sequential nucleoprotein exchange and complete differentiation of round spermatids into mature spermatozoa. Delaying mRNA translation prevents new mRNA synthesis in elongated spermatids, precocious chromatin condensation and infertility (Lee *et al.*, 1995).

The mechanism by which translational regulation is operating is not entirely understood; nevertheless, a few regulatory sites in the 3'-UTR have been identified to have profound effects on translation. The Y-box proteins Contrin and Translin and the kinesin KIF 17B are essential for transport of the RNP to the cytoplasm and for delayed translation (Kwon and Hecht, 1991, 1993; Murray *et al.*, 1992; Fajardo *et al.*, 1994; Aoki *et al.*, 1995; Fajardo *et al.*, 1997; Iuchi *et al.*, 2001). Poly-A-binding protein also has two important roles in regulating translation. First, it protects the mRNA transcript from degradation, thereby preserving the transcript until translational repression is removed. Second, it serves as a repressor protein (Bernstein *et al.*, 1989). Protamine

translation is initiated by covalent modification of the RNP, which leads to the release of the mRNA from the RNP and the shortening of the poly-A-tail (Kleene, 2003).

One interesting protein which may be involved in translational regulation of the protamines is protamine 1 RNA-binding protein (PRBP) and its human orthologue TAR RNA-binding protein (TRBP) (Lee *et al.*, 1996; Siffroi *et al.*, 2001). PRBP is highly expressed in the differentiating male germ cell and contains two copies of a double-stranded, RNA-binding domain which binds to the 3'-UTR of protamine 1 (Lee *et al.*, 1996). Male mice with PRBP deletion are infertile and severely oligozoospermic due to failure of protamine transcripts to undergo translation, which results in delayed replacement of the transition proteins and subsequent failure of spermatogenesis (Zhong *et al.*, 1999). TRBP is a 43 kDa protein found in the cytoplasm of elongating spermatids. No studies have evaluated this protein in men with known protamine defects (Siffroi *et al.*, 2001).

Post-translation modifications

P1 is translated as a mature protein, whereas P2 is synthesized as a 103 aa precursor that undergoes proteolytic cleavage at the N-terminus to generate the mature form (Meistrich *et al.*, 1992; Oliva and Mezquita, 1982; Balhorn *et al.*, 1999). P1 is rapidly phosphorylated by SRPK1 after translation, whereas CAMK4 phosphorylates P2 protein subsequent to its proteolytic modifications and its binding to the DNA (Green *et al.*, 1994; Papoutsoulou *et al.*, 1999). Phosphorylation of these proteins ensures their proper binding to DNA (Figure 3). Mutations or polymorphisms of SRPK1 or CAMK4 may be potential causes of abnormal protamine expression, but have yet to be evaluated. Targeted mutations in the CAMK4 gene in male mice resulted in infertility, a specific loss of P2 and retention of TP2, which suggests that the mature, phosphorylated P2 protein must interact with chromatin to displace TP2 (Wu *et al.*, 2000). Yoshii *et al.* (2005) have recently performed 2D electrophoresis and identified five bands for variants of protamine 1 and six bands for protamine 2 variants. This technique may be valuable in identifying post-translational defects of protamines in patients with known defects of protamine expression.

The relationship between abnormal protamine expression and spermatogenesis

Two caveats are relevant in considering the relationship between abnormal protamine expression and spermatogenesis. First, abnormal protamine expression is relatively common in male infertility patients, but rare in men with known fertility (Carrell and Liu, 2001). Second, many patients with abnormal protamine expression exhibit severe defects of semen quality, including oligozoospermia (Carrell and Liu, 2001; Aoki and Carrell, 2003; Aoki *et al.*, 2005a). The link between abnormal protamine expression and severely altered spermatogenesis is not intuitively obvious, but animal studies have also shown that spermatogenesis is severely altered when protamine expression is experimentally reduced (Zhong *et al.*, 1999).

One possible explanation for the possible link between altered protamine expression and severely reduced spermatogenesis is the hypothesis that protamine expression may act as a 'checkpoint' during spermiogenesis and that abnormal protamine expression

leads to an increased level of apoptosis. This hypothesis is supported by the fact that protamine haploinsufficiency causes severe disturbances of spermatogenesis in the mouse and the fact that protamine alterations are associated with DNA damage which may result in the initiation of an apoptosis pathway (Cho *et al.*, 2001, 2003). Differences in microenvironments within the seminiferous tubules or incomplete (abortive) apoptosis may allow some sperm survival.

An alternative hypothesis may be that abnormal protamine expression is usually the result of an abnormal functioning of a regulator of transcription, translation or post-translational modifications that affects not only the protamines, but also a broad range of genes involved in spermatogenesis. Candidate regulators may include ACT, CREM, Translin, Contrin and KIF17B. Particularly attractive targets in this regard may be the Y-box proteins Translin and Contrin, and the associated kinesin, KIF 17B, because they are involved not only in transcriptional regulation, but also in transport to the cytoplasm and translational regulation, since the data indicate that patients with abnormal protamine expression often have higher than normal levels of transcript in mature sperm (Aoki *et al.*, 2006d). Other key regulators, such as phosphorylation mechanisms, could be responsible for a broad defect affecting spermatogenesis.

Conclusions

From studies performed to date, it is clear that the presence of an altered P1/P2 ratio is clinically relevant and portends a reduced fertility. Semen samples with altered P1/P2 ratios generally have other abnormalities, such as increased DNA damage, low sperm counts and reduced fertilizing capacity (Aoki *et al.*, 2006e; Oliva, 2006). Two over-riding questions should be addressed in future studies. First, what is the clinical relevance, beyond low sperm quality, of abnormal sperm protamine expression? and, second, what is the cause of abnormal protamine expression?

When undergoing IVF with ICSI, men with abnormal protamine expression have fertilization, implantation and pregnancy rates equal to patients undergoing IVF for other diagnoses, including obstructive azoospermia (Aoki *et al.*, 2006e). However, given the role of protamines in silencing and resetting the paternal genome, and given the controversial, but possible relationship between increased imprinting errors in offspring and ICSI, future studies are needed to evaluate the potential risks of undergoing ART with sperm of known protamine abnormalities (Allen and Reardon, 2005; Chang *et al.*, 2005; Ludwig, 2005). Those studies will likely include an evaluation of offspring derived from animal models and also include further human sperm studies, such as the imprinting status of specific genes. Additionally, preliminary studies indicate that the P1/P2 ratio may be a more sensitive and accurate predictor of sperm functional ability than other available sperm function assays (Aoki *et al.*, 2006e). This concept should be further analysed.

Second, abnormal protamine expression is an intriguing pathology due to its relationship with altered spermatogenesis. Future studies will include analysis of regulators of transcription and translation and post-translational modifications, areas that will likely yield an increased understanding of the cause of abnormal protamine expression, and may also provide important

information regarding spermatogenesis in general. Protamines may be an important marker in better understanding the key regulatory pathways of spermatogenesis, especially if abnormal protamine expression reflects a aberrant function of a key transcription of translation regulator or acts as a crucial part of a checkpoint pathway.

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