Towards a better understanding of RNA carriage by ejaculate spermatozoa

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Research on spermatozoal RNA has made considerable progress since the original reports on its presence appeared in the late 1950s and early 1960s. Through the use of stringent procedures aimed at eliminating contamination artefacts, we now appreciate that a complex cohort of mRNAs persists in the ejaculate cell but that 80S (cytoplasmic) ribosomal complexes are not present in sufficient quantities to support cytoplasmic mRNA translation. Despite this, under certain conditions, at least some cytoplasmic mRNAs can apparently be translated de novo, possibly on mitochondrial polysomes. The detection of mRNA translation by mature spermatozoa essentially supports the earliest research reports on spermatozoal gene expression although the suggested relationship with protein turnover and capacitation is wholly unexpected. We also examine some alternative explanations and roles for RNA carriage, including the RNAs passive retention as a consequence of nuclear shutdown and a more active role in chromatin repackaging, genomic imprinting, gene silencing and post-fertilization requirements of essential paternal RNAs. The recent report of an RNA-mediated epigenetic alteration to phenotype that is likely to be sperm derived is of particular interest in this regard. We finally show that regardless of the biological role(s) of spermatozoal RNA, its utility in infertility studies, particularly when coupled with modern techniques in gene-expression analysis (e.g. microarrays), is obvious. As a wholly non-invasive proxy for the testis, this RNA offers considerable potential as a marker for fertility status and the genetic and environmental influences that could make all the difference between a fertile and an infertile phenotype.

Key words: fertilization/gene expression/imprinting/spermatozoal chromatin/spermatozoal RNA

Introduction

The spermatozoon is a highly differentiated and specialized cell, which until recently was thought only to transport the paternal genome to the oocyte. However, other components of the spermatozoon that are essential for normal syngamy in all investigated mammalian orders (with the notable exception of some rodents) have also come to light in recent years. These include an oocyte-activating factor (a member of the phospholipase group of hydrolytic enzymes) (Parrington et al., 2002) and an essential component of the zygote’s microtubule-organizing centre (the spermatozoal centrosome) (Schatten, 1994). An increasing number of reports have also appeared over the past 15 years, describing and cataloguing the presence of various RNAs in the male gametes of many species including gymnosperms and angiosperms (recently reviewed in Krawetz, 2005; Miller et al., 2005). These reports have reawakened interest in RNA carriage by spermatozoa because it is an anomalous component of this ‘quiescent’ specialized cell. Indeed, nuclear gene expression in mature spermatozoa is progressively shutdown during spermiogenesis (the haploid phase of spermatogenesis) to allow substitution of histones by the highly charged and physically smaller protamines, facilitating further compaction of the haploid genome (Balhorn et al., 1999).

To overcome the shutdown of nuclear transcription, developing spermatids rely on translational control of stored mRNAs to produce the proteins necessary for chromatin re-compaction and to finalize the differentiation of the spermatozoon (Gold et al., 1983; Braun et al., 1989; Gu and Hecht, 1996; Brevini-Gandolfi et al., 1999; Steger, 2001). Mature spermatozoa do not, however, contain sufficient 28S or 18S rRNAs (and hence 80S ribosomal complexes) to support translation, indicating their removal or breakdown during spermatozoal maturation and, by extension, the ‘selective’ retention of mRNA species (Miller et al., 1999; D. Dix, unpublished data). The absence of essential components necessary to support a (cytoplasmic) translational machinery begs the question of whether spermatozoal mRNA can be translated de novo and, if not, what alternative roles it might have in the sperm itself.
and perhaps beyond fertilization. This review examines the phenomenon of spermatozoal RNA through the twin lenses of established analysis and suspected or speculative functions. In the former context, RNA in spermatozoa and indeed the existence of gene expression in these cells were the topic of some heated debate 50 years ago. Interest in the phenomenon re-emerged in recent years, primarily through the development of modern molecular analytical techniques and a need to look beyond the testsis for less invasive assessments of male infertility. Indeed, one of the implied clinical uses that sparked our earliest interest in spermatozoal RNA was as a convenient and sensitive marker of successful (or unsuccessful) human vasectomy (Miller, 1997). A brief comparison with gene expression in the oocyte is also warranted because the female gamete builds up significant stores of mRNA for later translation following a shutdown of transcription (Paynton and Bachvarova, 1994; Briggs et al., 1999; de Moor and Richter, 2001; Dai et al., 2005).

Indeed, these stored mRNAs (and perhaps delivered spermatozoal RNAs) are essential for establishing the gene-expression programme in zygotes before the activation of the embryonic genome. Finally, the (potential) clinical utility of spermatozoal RNA is worth examining because testis biopsy as a route to infertility investigation is generally only undertaken as a last resort. If spermatozoa can provide equivalent information on what underlies a subfertile or infertile phenotype, then a non-invasively obtained semen sample is surely a preferable and more widely acceptable option.

Historical context

The metabolism of spermatozoa has always been a significant area of andrological research because it underpins male fertility. It is of particular concern to animal husbandry where semen quality is an essential economic factor (Foote, 1989) as well as to clinicians investigating the infertile human couple (Balen and Jacobs, 2003). Much of the earliest basic research that looked into the metabolism of spermatozoa was carried out on bovine spermatozoa that were shown to be either capable (Bhargava, 1957; Abraham and Bhargava, 1963) or incapable (White et al., 1953; Martin and Brachet, 1959; Markewitz et al., 1967) of incorporating radiolabelled substrates into acid-precipitable RNA or protein. The earlier experiments (Mauritzen et al., 1952) used RNA detection methods based on relatively insensitive colorimetric procedures. When reports on spermatozoal RNA synthesis began appearing using the more sensitive radio-tracing techniques, the possibility that the metabolically active components of semen were not spermatozoa was correctly raised (Martin and Brachet, 1959). Semen, of course, is a good medium for bacteriological activity, and it took the detailed and concurrent works of MacLaughlin and Terner (1973) and Premkumar and Bhargava (1972) to convincingly demonstrate that mature spermatozoa are indeed metabolically active with respect to transcription but that this was restricted to the mitochondrion. Translation of (mitochondrial) RNAs was inferred rather than demonstrated at the time. The later work of Hecht and Williams (1978), however, demonstrated the apparent presence of RNA synthesis in spermatozoal nuclei although this could have been due to experimental ‘switching on’ of a normally silent RNA polymerase (see section ‘The dynamic sperm’).

In general, the prevailing consensus is that although RNA and protein synthesis does occur in mature spermatozoa, they are confined to the cells’ mitochondria. Betlach and Erickson (1976) reported 28S and 18S RNAs in murine epididymal spermatozoa, indicating that although the nucleus cannot normally transcribe RNA, stored mRNAs could potentially be translated allowing nuclear protein synthesis to occur, possibly directed at mitochondrial components (Alcivar et al., 1989). However, this scenario seemed unlikely because (i) the condensed mature spermatozoon is virtually devoid of cytoplasm and (ii) there is an insufficiency of cytoplasmic ribosomes capable of supporting de-novo translation. Recently, however, de novo translation of spermatozoal RNAs has been convincingly demonstrated in human and bovine spermatozoa using the uptake of 35S-methionine-35S-lysine and BODIPY lysine tRNA as visualized by autoradiography and fluorescence microscopy, respectively (Gur and Breitbart, 2006). Surprisingly, mitochondrial but not cytoplasmic translation inhibitors prevented translation.

One explanation for the apparent contradiction in these findings is the insufficient removal of contaminating somatic cells or the presence of immature spermatids in the sampling sources. Contamination became a contentious issue in sperm RNA research in general because of the assumption that with transcriptional and subsequent translational shutdown, there was no clear physiological or mechanistic reason for the retention of cytoplasmic mRNA. Hence, much of the earlier caution that followed the deployment of molecular detection of mRNAs in ejaculate spermatozoa by RT-PCR. Questions arose over whether these exquisitely sensitive methods were detecting low levels of residual spermatogenic RNA (possibly cytoplasmic droplets) or even RNA sourced from a somatic (or possibly round germ cell) population contaminating the semen samples. Stringent preparative precautions have effectively eliminated these possibilities, and indeed, it is now the virtual absence of detectable 28S and 18S rRNAs that is a ‘feature’ of a pure spermatozoal RNA (Miller et al., 1999; Ostermeier et al., 2002). Furthermore, the residual droplet (at least of human spermatozoa) is highly labile and normally lost during semen processing (Cooper and Yeung, 2003; Cooper, 2005). The spermatozoon’s mitochondrial transcription-translational machinery, however, is active; hence the work demonstrating translation de novo essentially confirms the conclusions drawn ~50 years ago in this regard (Premkumar and Bhargava, 1972; MacLaughlin and Terner, 1973; Gur and Breitbart, 2006). What is entirely novel about the most recent work is the evidence that polysomal complexes containing mitochondrial ribosomes may support the translation of nuclear-encoded cytoplasmic mRNAs, a controversial conclusion in its own right.

Composition and location of spermatozoal RNA

RNA in the spermatozoid nucleus of the fern Scolopendrion was the first to be described histologically using colloidal gold and electron microscopy (Rejon et al., 1988). Subsequently, both rat and human spermatozoa were examined histologically and ultrastructurally (Pessot et al., 1989; Concha et al., 1993), and RNA was localized to the nucleus using a similar assay. When subjected to acrylamide electrophoresis, the RNA described by Pessot et al. (1989) was found to resolve into discrete bands that included 5.8S and 5S RNAs as well as tRNAs. This and the later report by Concha et al. (1993) localizing U1 and U2 small nuclear RNAs were the first to provide visual information on the localization of (human) spermatozoal RNA to the nucleus. Moreover, the work of Rejon...
et al. (1988) indicated that the gold-labelled RNA observed in the
fern nucleus was mRNA based on their use of polyuridine probes.
Subsequently, Kumar et al. (1993) reported the presence of c-myc
mRNA in the principal piece of human spermatozoa, and a few
years later, two independent reports indicated the presence of pro-
tamine 2 mRNA by RT–PCR and in situ hybridization, with the
latter also localizing the RNA to the nucleus (Miller, 1997; Wykes
et al., 1997). The list of (human) spermatozoal mRNA species has
grown steadily over the intervening years by target-directed RT–PCR
and now includes HLA, t-type calcium channels, N-cadherin,
estrogen receptors, cyclic-nucleotide phosphodiesterases (PDE),
inTEGRINS, aromatase and nitric oxide synthase (NOS) to name a
few (see Miller et al., 2005, for a more detailed listing). As indicated
above, the list also extends to cover the male gametes from other
animals and from non-animal sources such as the already described
fern (Rejon et al., 1988) and maize [an angiosperm species (Engel
et al., 2003)]. These reports generally support the localization of
spermatozoal RNA in or around the nucleus proper rather than any
other component of the cell. See Figure 1 for illustrations of these
alternative possibilities.

Target-independent strategies such as cloning and sequencing
of randomly primed amplicons hinted at a heterogeneous population
of mRNAs (Miller et al., 1999). However, the overall complexity
of the mRNA population (in human spermatozoa) was only realized
when array-based tests were applied for the first time (Ostermeier
et al., 2002). This study compared testis-derived RNA with the
RNA isolated from a pool of nine individual ejaculate samples and
a single individual ejaculate sample and identified 7157, 3281 and
2780 transcripts, respectively. Interestingly, both spermatozoal
RNA populations were present in the testis population, and all but
four of the individual ejaculate sample mRNAs were found in the
pooled ejaculate population.

Figure 1. Localization of spermatozoal RNAs. Mounting evidence supports the view that spermatozoa contain a complex repertoire of RNAs. However, little
information is available to indicate where this RNA is stored within these streamlined cells. Panel A highlights the four main segments of a mature spermatozoon.
A longitudinal section of the spermatozoon head is shown in panel B. As suggested by various studies (see main text), we have highlighted in grey the nucleus
itself, the perinuclear theca and the post-acrosomal sheath as possible areas for spermatozoal RNA repositories. Additionally, Kumar et al. (1993) have localized
transcripts to within the midpiece. As such, the centrosome and the mitochondria are highlighted in panels C and D, respectively, as probable additional sites.
Cross-sectional diagrams of the principal piece and end piece are shown in panels E and F, respectively. Again, the fibrous sheath and axoneme are highlighted in
grey, indicating that they too may carry spermatozoal RNA.
These findings indicated some variation in the mRNA populations of individual ejaculates, possibly indicative of spermatozoa quality and/or fertility status. Independent verification of the data has been established using an alternative array platform and indicates that ‘normal’ ejaculate spermatozoa retain ~5000 mRNA types but with a degree of inter-sample variability estimated at ±10% (D. Dix, personal communication). More recently, a serial analysis of gene expression (SAGE) tag map of human spermatozoal RNA has been reported that identified 2712 and 2459 unique transcripts from pooled and individual ejaculate samples, respectively (Zhao et al., 2006), again providing evidence for inter-sample variation. SAGE, however, also permits more accurate estimates of the relative abundance of mRNAs in any sample than is normally possible with array-based analyses. Reassuringly, the 30 most abundant SAGE tags (mRNAs) were identical in both sample populations examined in this study. Moreover, the most abundant tag, GA17 (Unigene ID 502244), is a clear candidate for a fusogenic protein, possibly involved in sperm–oocyte interaction. Interestingly, COX5B, a subunit of the terminal mitochondrial respiratory transport enzyme, and transcription factor A-mitochondrial (TFAM), a mitochondrial transcription factor, were the fourth and fifth most abundant SAGE tags, respectively. This observation is interesting because although both proteins are mitochondrially located, their respective genes are nuclear encoded. That stored cytoplasmic mRNAs may be translated in the spermatozoon’s mitochondria or possibly intra-nuclearly is also intriguing in this respect (Gur and Breitbart, 2006).Small RNA-binding proteins were also present in the ‘most abundant tag’ list, which supports a much earlier report describing similar small RNP polypeptides in spermatozoal nuclei by electron microscopic (EM) immunohistochemistry (Brito et al., 1992). Irrespective of their identities, the evidence to date indicates that spermatozoal mRNAs are fully processed, in that they contain 3′-poly-A tails and lack intronic sequences (Concha et al., 1993; Miller, 1997; Ostermeier et al., 2002).

Spermatozoa also contain the antisense equivalents of several protein-encoding sense mRNAs as well as some putative siRNA candidates (Ostermeier et al., 2005). The latter are now understood to be very important mediators of gene-expression control in other cell types (Lim et al., 2003) and have been adapted as ‘knock-down’ reagents in the laboratory [oocytes included (Yu et al., 2004)]. Full-length non-coding antisense RNAs may reflect the peculiar transcriptional promiscuity of the testis (Schmidt, 1996) which in itself may be a particularly good example of the generalized expansion of the human transcriptome described recently (Kapranov et al., 2005). However, the reported presence of several developmentally important candidates among them is particularly noteworthy. Among these candidates, insulin-like growth factor (IGF2R) is interesting because its ligand is known to be maternally imprinted and hence normally expressed only from the paternally derived allele (Bartolomei et al., 1993). IGF2R itself is known to be maternally expressed in mouse and hence paternally imprinted. In humans, biallelic expression of IGF2R is thought to be the norm; hence, the presence of antisense RNA for IGF2R in human spermatozoa is intriguing. Antisense mRNAs could mark genes for uniparental down-regulation or repression in a system that normally displays biallelic expression. Perhaps such a mechanism serves as an alternative pathway for down-regulation of alleles that are not imprinted per se (Vu et al., 2004).

![Figure 2](image)

**Where does spermatozoal RNA come from and why is it retained?**

**Passive versus active origins and roles**

On the basis of the reports mentioned above, it is clear that spermatozoal RNA comprises a subset of testis RNA. But why should a terminally differentiated and essentially ‘silent’ cell (with respect to nuclear gene expression) retain mRNA? One view is that this RNA is residual, reflecting the shutdown of transcription during spermiogenesis (Figures 2 and 3). According to this hypothesis, because of the requirement for translational control of gene expression, developing spermatids rely for long periods on stored mRNAs and, as a consequence, may lose the capacity to eliminate it from the maturing cell. Indeed, a general block on RNA elimination pathways favours the equitable sharing of gene products by developing spermatids, a process that is supported by the continued presence of cytoplasmic bridges interconnecting spermatids (Caldwell and Handel, 1991). Sharing maximizes the production of spermatozoa by eliminating any disequilibrium resulting from the random inheritance of a ‘defective’ allele during meiosis (spermatozoa ‘co-operate’ in their ‘siblings’ production). However, this
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additional RNA load. Delivery of this RNA somehow dysregulates c-Kit expression leading to the observed coat colour anomaly. These experiments demonstrated for the first time, a clear epigenetic paramutation introduced by the spermatozoon and operating independently of genotype. Although the phenotype is only marginally relevant to developmental function, the model shows the potential for the spermatozoon to effect developmental processes outside the ‘remit’ of the delivered paternal genome. Interestingly, one unique aspect of sperm delivery to the oocyte in most mammals, the primary zygotic centrosome, is maternally derived in mice. In this species at least, delivery of spermatozonal RNA cannot be associated with or controlled by centrosomal entry (an otherwise compelling explanation for this organelle’s usually paternal inheritance). The known involvement of this organelle in the partitioning of mRNAs during early embryonic development could be equally facilitated by paternally or maternally derived centrioles (Lambert and Nagy, 2002). The work of Rassoulzadegan et al. (2006) therefore supports the delivery of sperm RNA to the oocyte as a general phenomenon functioning independently of centrosomal origin.

**Dynamic molecular interactions**

Reorganizational aspects of spermatozonal chromatin have also been put forward to explain RNA retention (Miller et al., 2005). During spermiogenesis, somatic histones are progressively replaced by transition proteins and then by protamines (Balhorn et al., 1999). However, a proportion of sperm DNA is packaged by histones (up to 15% in human spermatozoa), and this histone-bound compartment appears to be associated with the nuclear envelope or matrix, possibly on the nuclear periphery, forming a nucleohistone shell (Gatewood et al., 1987; Pittoggi et al., 1999). Because RNA is a known component of the envelope (Jackson, 1997), it is possible that spermatozonal RNA acts to stabilize an interaction between the envelope and histone-bound DNA. This could be a purely passive structural requirement of the RNA, or the RNA may actively delineate or mark those DNA sequences for packaging by histones (Figure 3). It is not known whether histone-bound DNA sequences are protected from protamination in spermiogenesis (i.e. are marked in some way to avoid being repackaged) or result from a selective association with newly synthesized histones. Regarding imprinted genes, it is notable that IGF2 (which is paternally expressed) appears to be (at least partially) packaged by histones (Wykes and Krawetz, 2003) and is in a nuclease-sensitive compartment in spermatozonal chromatin (Banerjee and Smallwood, 1998). Considering that even the compact DNA in spermatozoa includes domains that are apparently in a transcriptionally ‘ready’ state (essentially a state that is primed for expression by virtue of being in a DNAse sensitive and hence more open conformation (Kramer et al., 1998)), it is tempting to speculate that genes active in the paternal pronucleus of pre-cleavage-stage zygotes (Latham and Schultz, 2001) will likewise reside in more open regions and, furthermore, that such regions may be packaged by histones. Could sperm RNA play a role in establishing these regions (Figure 3)? Again, the study of Rassoulzadegan et al. (2006) strengthens the notion that distinct chromatin domains in spermatozoa may be related somehow to spermatozonal RNA carriage and subsequent gene expression in the zygote. In any case, it would be interesting to fully characterize DNA sequences that are histone bound and look for relationships between those sequences and the isolated mRNA population.

**Comparison with oogenesis**

The retention of spermatozonal RNA bears some comparison with the equivalent process in oogenesis, where large stores of mRNA are built up until transcription ceases by the time germinal vesicle breakdown occurs (Briggs et al., 1999). From this point on, maturing oocytes (and early embryos) rely on translated stores of maternal mRNAs to support protein synthesis. The control of translation in both gametes is also very similar, relying on equivalent changes in poly-A tail length in coordination with dynamic associations between untranslated regions of RNA and appropriate RNA-binding proteins (see Kleene, 1996; Hecht, 1998; DeJong, 2006, for recent reviews). On the one hand, it could be argued that storage and controlled translation of RNA is an essential but mutually exclusive requisite for spermatid and oocyte maturation. On the other hand, because we know that many maternally derived RNAs are required to support early embryonic development, there remains the intriguing possibility that some paternal RNAs are also required for this purpose. Perhaps there exist two populations of mRNA in ejaculate spermatozoa that perform exclusive functions: a nuclear population supporting either translational replacement of ‘spent’ proteins or chromatin repackaging and a centriolar population supporting an embryonic and/or developmental role (Figure 3). A number of possible candidates that are delivered to the oocyte have already been highlighted in this respect (Ostermeier et al., 2004) and await further investigation.

**The dynamic sperm**

Although it is now widely accepted that under normal circumstances, spermatozoa are transcriptionally silent, recent evidence indicates that the spermatozonal nucleus is itself a more dynamic organelle than was originally considered. In addition to the new evidence for active translation of stored mRNAs (Gur and Breitbart, 2006), spermatozoa have been shown to be capable of ‘taking up’ foreign DNA and RNA, and this phenomenon has been used to generate transgenic animals (Spadafora, 1998). Although the mechanism for this process is rather poorly understood (like DNA microinjection into fertilized oocytes, it is not target directed), it is thought to involve the activation of normally dormant enzymes that may be ordinarily used to disable any spermatozoa compromised by contact with exogenous nucleic acids (such as viral and bacterial genomes). This apoptosis-like phenomenon brings about the autodigestion of spermatozonal chromatin (Maione et al., 1997; Sotolongo et al., 2003), and there is good evidence suggesting that the initial target of this digestion is the cells’ more loosely packaged histone-bound DNA (Pittoggi et al., 1999). Regardless of the intended outcome of the enzymes’ activation, one unintentional outcome may be the transcription of fresh RNA from competent DNA templates that are likely to be histone bound. This is a possibility because (i) spermatozoa contain RNA polymerase (Hecht and Williams, 1978), (ii) there are abundant transcription factors present (Pittoggi et al., 2001) and (iii) if RNA can be converted to DNA by an enzyme-mediated process in these cells (Sciannanna et al., 2003), then why not DNA to RNA? The recent experiments of Grunewald et al. (2005) looked for evidence of incorporation of radiolabelled uridine in ejaculate human spermatozoa but failed to find any. Although reassuring from the perspective of infertility research (See section *Spermatozonal RNA in fertility research*) because incubations were carried out under conditions that would
not have favoured the up-regulation of a dormant transcriptional capacity, this study did not exclude the possibility.

**Spermatozoal RNA in fertility research**

Assuming that spermatozoal RNA offers us a snapshot of the spermatogenic potential of the testis, there is now every reason to exploit this resource for toxicology (Ostermeier and Krawetz, 2006) and infertility research. The principal justification for this assertion is that the vast majority of human male factor infertility has no clearly identifiable cause. There are many reasons for this situation, of which the most important is the comparative difficulty in studying infertile men where, in general, testicular biopsies are only occasionally justified (on either clinical or ethical grounds). Moreover, almost all research tends to follow paths that are most likely to give the best returns on investment, and as the vast majority of non-obstructive male infertility (>90%) encompasses a wide range of causation, it remains resistant to systematic investigation. As an example of the unintentional bias inherent in concentrating on obvious phenotypes, a considerable amount of research effort has been expended on Y-chromosomal microdeletions over the years, showing the great importance of genes on this chromosome for spermatogenesis (Skaletsky et al., 2003). Although in no way belittling this finding, it is worth remembering that such deletions probably afflict <15% of men with complete azoospermia or severe oligozoospermia (Silber et al., 1998), which in themselves comprise a very small fraction of male factor infertility. Considering the misery that human infertility can cause, research into molecular mechanisms underpinning an infertile genotype is clearly lacking and warranted (Balen and Jacobs, 2003). The murine model has provided us with a collective wealth of knowledge that simply would not be available otherwise (Cooke and Saunders, 2002). However, to the best of our knowledge and notwithstanding studies looking at DNA packaging anomalies (Steger et al., 2003; Aoki et al., 2005), few reports identifying a mutation or deletion in any autosomal or X-linked gene involved in murine spermatogenesis have appeared in a corresponding human study (Roest et al., 1996; Grootegoed et al., 1998; Hagaman et al., 1998; Adham et al., 2001; Cho et al., 2001; Miki et al., 2002; Steger et al., 2003; Aoki et al., 2005). Perhaps more intractable is the observation that an infertile phenotype affecting the semen profile is rarely obvious, given the natural heterogeneity of human semen profiles and single gene defects that may cause it are unlikely to have any clear effect unless the genotype is homozygous. Such events are likely to be exceptional, hence the understandable lack of available testicular samples for molecular investigation in the vast majority of clinical cases.

Would ~5000 spermatozoal transcripts containing a record of molecular events underpinning spermatogenesis and, by extension, male fertility (Ostermeier et al., 2002) be of interest to toxicology and infertility researchers? If so, how could such a useful and promising research tool be more fully exploited (Figure 4)? On the gross assessment end of the spectrum, we indicated some years ago that sperm RNA was a useful diagnostic for successful vasectomy (Miller, 1994). On a finer scale, microarray, SAGE and other assays compatible with differential analysis offer considerable promise for characterizing spermatozoal RNAs and facilitating comparisons between fertile (non-affected) and infertile (affected) men (Wang et al., 2004). Some reports have already appeared aimed at testing the utility of spermatozoal RNA as a molecular resource for infertility investigation. Spermatozoal motility, e.g. which is usually a reliable indicator of male fertility, appears to carry a molecular signature that can be detected using simple RT–PCR-based tests. Lambard et al. (2004) showed that the quantity of protamine 1 (PRM1) in the poorly motile subpopulation of spermatozoa harvested from the interface of a discontinuous Percoll gradient was higher than in cells obtained from the pellet (where the most motile population is normally found). Similar effects were noted for both endothelial (eNOS) and neuronal (nNOS) mRNAs. A similar study using microarray-based data coupled with real-time PCR detected quantitative changes in the presence of several spermatozoal mRNAs relating to the motility of the sampled population (Wang et al., 2004). These reports highlight two important findings. The first is that spermatozoal RNA appears to reflect the inter-sample non-affected versus affected phenotype (at least for motility). The second is that intra-sample variations in transcript presence associate with spermatozoal morphology on the basis of buoyant density. This latter observation is interesting because it suggests that spermatozoal RNA could provide molecular information on what drives the heterogeneity in human semen and hence the differences in buoyant density that underlie the separation of spermatozoal subpopulations in density gradient media, including those commonly used in the assisted conception setting (Figure 5). Although this may not at first sight appear to be significant, it could provide deeper insight into the testicular microenvironments that give rise to subpopulations of spermatozoa and inform our understanding of how these environments may be affected by pathological and environmental perturbations. Because environmental aspects of male infertility are much in the news these days, particularly the decline in male infertility and the concomitant rise in testicular cancer (Skakkebaek et al., 1998; Virtanen et al., 2005), spermatozoal RNA may be the best opportunity we have for understanding these effects at the molecular level.

**New perspectives on spermatozoal function**

**Ensuring transmission of the paternal genome**

If, as indicated above, spermatozoal RNA contains components that are critical for embryogenesis, then it follows that without these components, couples will be unable to reproduce, irrespective of the quality of the male partner’s genome. What explanation, therefore, can be made of the experimental observation that the entire paternal contribution is potentially as dispensable for mammalian reproduction as it frequently is for reproduction in other animal classes (Kono et al., 2004). The most compelling aspect of this report on gynogenetic mice shows how it is possible, by manipulation of the ‘perceived’ imprint, to ‘fool’ an oocyte into ‘thinking’ that it has received a spermatozoon and permitting ‘normal’ development. This feat was achieved by injecting a murine oocyte nucleus carrying a deleted h19 gene into a normal oocyte. Because h19 is paternally imprinted (expressed from the maternal allele), syngamy in at least a small number of these gynogenetic constructs led to viable offspring. As h19 and IGF2 are reciprocally imprinted in male and female gametes (Bartolomei et al., 1993), the elimination of h19 in the introduced nucleus essentially mimicked the presence of the male genome. As with
somatic cell nuclear transfer, however, the efficiency of the process was poor, showing that the establishment of an epigenetic balance in the zygote is not readily accomplished.

These data suggest that a likely role for spermatozoal RNA (aside from supporting the replacement of degraded proteins) including antisense RNA and possibly siRNA (Ostermeier et al., 2005) lies in the oocyte’s response to fertilization through reprogramming of the male genome (Miller et al., 2005). In this scenario, some spermatozoal RNAs may act to regulate or even co-ordinate the rapid series of events that precedes pronuclear formation including the de-protamination of the spermatozoal nucleus by maternal factors. In this respect, the post-fertilization

Figure 4. Clinical application of spermatozoal RNA. Spermatozoal RNAs are transcribed in the testes during the production of spermatozoa. (A) As indicated by spermatogenic and Leydig cells in highlighted colours, perturbations in spermatogenic gene expression may occur. This altered gene expression can lead to the production of abnormal spermatozoa as shown in panel B. Changes in spermatogenic gene expression leading to subfertility or infertility should be detected by variations in spermatozoal fingerprints utilizing in this example, microarrays (panel C).

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demethylation of the sperm genome with the exception of imprinted loci and the early, pre-zygotic gene activation transcription reported from the murine male pronucleus are possible outcomes of this process (Adenot et al., 1997; Aoki et al., 1997; Trasler, 1998; Latham and Schultz, 2001). However, imprinting events and the timing of zygotic genome activation (ZGA) are not the same in different mammalian species [e.g. ovine spermatozoa do not undergo genome-wide post-fertilization demethylation (Young and Beaujean, 2004)], and ZGA occurs at the 2-cell or 4-cell stages in mouse and human zygotes, respectively. Hence, different species may have adopted different strategies to ensure bi-parental syngamy, and the utilization of spermatozoal RNA in this respect need not be universal.

Nevertheless, under normal physiological conditions, parental allelic imprinting may help to ensure the continued requirement for a male contribution to the zygote and hence the preservation of male-derived genes. Indeed, the general dependency on the paternal centrosome for syngamy is likely to be evolutionarily driven by this imperative. One possible side effect of this ‘self-preservation’ phenomenon might be the rare gestation of molar pregnancies where duplication of the paternal genome occurs. Hence, different species may have adopted different strategies to ensure bi-parental syngamy, and the utilization of spermatozoal RNA in this respect need not be universal.

Figure 5. (A) Inter-sample comparisons to define the shared, fertile (core) set of transcripts using microarrays. (i) The same sample probed twice by an arbitrary microarray illustrating procedural noise; (ii) two samples illustrating shared and variant sets; (iii) the more comparisons we make, the higher the resolution obtained for defining the core set of transcripts panel; (B) (iv–vi) intra-sample comparisons resolved on discontinuous density gradients showing (iv), normozoospermic sample with three sperm population subsets comprising the pellet, cells of abnormal morphology and cells with poor motility (recovered from the fractionated semen). The same subsets are indicated for asthenozoospermic (v) and teratozoospermic (vi) samples showing expected variations in their respective sizes.

Conclusions

Spermatozoal RNA research began >50 years ago, and now that the controversy over its presence has abated, we are nearer a consensus regarding its likely utility in fertility studies. Perhaps the most significant breakthrough in this respect is the recognition that spermatozoal RNA is essentially a proxy for the germ cell component of the testis. Hence, investigations that hitherto would have required a biopsy of the testis can now obtain spermatozoal RNA profiles as a viable alternative. This is a very attractive alternative considering that (i) it is wholly non-invasive and (ii) the great majority of male factor infertility does not clear spermatozoa from the ejaculate. The authors are aware of fresh studies awaiting public disclosure with regard to the utility of spermatozoal RNA in fertility research, and we await these reports with anticipation. With respect to the role(s) of spermatozoal RNA, we have discussed the evidence suggesting that it is not residual to spermatogenesis. Possible functions include de-novo translational replacement of degraded proteins (now demonstrated), structural (repackaging of chromatin), post-fertilization (delivering some essential RNAs to the oocyte) and epigenetic (helping establish and/or maintain the parental imprint as suggested by the paramutated mouse model). Perhaps the most exciting development to date is the inference of naturally occurring spermatozoal RNA-mediated epigenetic effects on the zygote, but detecting these effects remains a challenge for future research. Certainly, the renewed interest in the male gamete is both welcoming and timely. Far from being the quiescent cell whose sole job is delivery of the paternal genome, we have seen that spermatozoa are capable of
surprising intra-nuclear dynamics that includes renewed activity of endogenous endonucleases and possibly polymerases and potentially novel uses for mRNA stores. The male gamete can no longer endogenous endonucleases and possibly polymerases and potentially novel uses for mRNA stores. The male gamete can no longer

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The RNA of ejaculate spermatozoa


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