Transcriptional dynamics of the sex chromosomes and the search for offspring sex-specific antigens in sperm

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Abstract

The ability to pre-select offspring sex via separation of X- and Y-bearing sperm would have profound ramifications for the animal husbandry industry. No fully satisfactory method is as yet available for any species, although flow sorting is commercially viable for cattle. The discovery of antigens that distinguish X- and Y-bearing sperm, i.e. offspring sex-specific antigens (OSSAs), would allow for batched immunological separation of sperm and thus enable a safer, more widely applicable and high-throughput means of sperm sorting. This review addresses the basic processes of spermatogenesis that have complicated the search for OSSAs, in particular the syncytial development of male germ cells, and the transcriptional dynamics of the sex chromosomes during and after meiosis. We survey the various approaches taken to discover OSSA and propose that a whole-genome transcriptional approach to the problem is the most promising avenue for future research in the field.

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Introduction

In mammalian spermatogenesis, Mendelian segregation of sex chromosomes during meiosis leads to the production of a 50:50 ratio of X-bearing sperm (leading to female offspring) and Y-bearing sperm (giving rise to male offspring). The ability to sort X from Y sperm and thus to pre-select the sex of offspring has profound implications for the efficiency and sustainability of livestock production, for example preferential production of female calves in the dairy industry or male calves in the beef industry. There is also an approved role for sex selection in human medicine to avoid the transmission of sex-linked diseases.

Sperm sorting requires the existence of detectable differences between X and Y sperm: to date, the only widely accepted example of such a difference is that Y sperm have lower DNA content due to the smaller size of its chromosome. Various physical parameters based on this difference have been proposed as a basis for sperm sorting, for example Percoll gradient centrifugation based on hypothetical differential density of X and Y sperm (Kaneko *et al.* 1983) or albumin gradient separation based on hypothetical differential motility of the 'lighter' Y sperm (Beernink *et al.* 1993). Disappointingly, in most cases, follow-up studies failed to confirm the results of these studies. For example, albumin

separation does not enrich for Y-bearing sperm (Chen *et al.* 1997), there are no differences in dimensions or dimensional distributions between human X and Y sperm or spermatids (Hossain *et al.* 2001), and bull Y sperm do not swim faster than X sperm (Penfold *et al.* 1998). Thus, the only unequivocally proven method for sperm sorting is direct detection of DNA content by staining with a DNA-binding dye such as Hoechst 33342 followed by flow cytometric separation of X and Y sperm. This technique has become commercially viable in cattle despite requiring expensive equipment for the complex separation procedure; however, the number of sexed sperm produced per unit time is limited (Seidel 2003). Moreover, results are highly variable between individual bulls, likely due to the damage caused by staining and/or subsequent u.v. scanning (Rath *et al.* 2009).

There is, therefore, considerable interest in developing simpler and more efficient methods to separate X from Y sperm. Identification of a specific cell surface marker for immunoselection would represent a major advance in this field by a) allowing for batch selection and b) reducing the safety issues associated with DNA damage from the intercalating dye/u.v. detection methods currently used. It should be noted that while previous studies (e.g. Howes *et al.* 1997, Sang *et al.* 2011) have used the term 'sex-specific antigen' (SSA) or 'sex-specific

protein' to refer to such markers, this terminology has the potential for ambiguity. The term 'SSA' has historically been used to refer to transplantation antigens differing between male and female individuals. While it was initially surmised that such SSAs would prove useful in distinguishing X- and Y-bearing sperm, this has not turned out to be the case due to transcript sharing (see Syncytial development of male germ cells). We, therefore, propose the term offspring SSA (OSSA), specifically referring to markers that distinguish the two categories of sperm and are therefore indicative of offspring sex.

The term 'sex chromosome-specific protein' (SCSP; Blecher *et al.* 1999), while useful, is not appropriate as OSSAs need not necessarily be encoded by the sex chromosomes (see Box 1).

This review discusses the processes through which OSSAs arise, including transcription, compartmentalisation and translation of sex-linked genes in spermatids, and surveys past and future research avenues in the ongoing search for OSSAs. Several previous reviews (e.g. Gledhill 1988, Bradley 1989, Hendriksen 1999) are available that provide valuable historical context.

Box 1 Models of OSSA formation

There are several potential routes by which individual spermatozoa may become distinguishable from each other. For clarity, we here illustrate the development of X and Y sperm from a pair of linked spermatids, with the X sperm bearing an offspring sex-specific antigen (OSSA) on the surface. In models A through C, the OSSA arises via compartmentalisation of a sex-linked transcript and/or its encoded protein product. In model A, transcription and translation occur in early stage spermatids, hence both transcript and protein must remain compartmentalised, for example by binding to the cytoskeleton (mRNA) or immediate insertion into the cell membrane (protein). Alternatively, in model B, the transcript is expressed in early spermatids and compartmentalised; however, translation is delayed until later stages of development, after the breakage of the cytoplasmic bridges. Transcriptional delay is a hallmark of many spermatid-expressed genes (reviewed in Kleene (2003)). Finally, in model C, both transcription and translation occur after bridge breakage. This model is the least likely, given the condensed nature of the sperm genome at this point and the greatly reduced potential for transcription (see Transcriptional and translational activity in spermatozoa).

In models D through F, a non-compartmentalised gene nevertheless generates an OSSA via differential protein localisation (D), degradation (E), or post-translational modification (F) in X- and Y-bearing cells. These routes could potentially lead to autosomally encoded OSSAs as well as sex-linked OSSAs. However, these latter three models present a bootstrapping problem: if there is a difference in (e.g.) translational activity between X and Y spermatids, how does this difference itself arise? Ultimately, any antigenic difference must be rooted in a difference at the nucleic acid level. We therefore conclude that if OSSAs exist, there will be a detectable transcriptional difference between X- and Y-bearing spermatids or sperm due to compartmentalisation of one or more sex-linked genes. However, the downstream consequences of this (e.g. from compartmentalisation of a sex-linked transcription factor) need not be restricted to the sex chromosomes and thus this should be taken into account in any candidate gene approach to OSSA discovery.

Figure 1 Schematic showing the syncytial development of sperm and the transcriptional status of the X (pink) and Y (blue) chromosomes at each stage. Sharing of transcripts across the bridges leads to the presence of both X- and Y-derived mRNAs in germ cells (purple).

Syncytial development of male germ cells

Spermatogenesis is a complex physiological process occurring in the seminiferous tubules of the testis (Fig. 1), in which germ cells proliferate and differentiate to form the mature haploid spermatozoa. This process can be divided into three phases: a) mitotic proliferation of spermatogonial stem cells to generate progressively more differentiated progeny, b) meiotic divisions generating haploid spermatids, and c) spermiogenesis, during which the spermatids elongate, shed most of their cytoplasm, undergo nuclear condensation and develop sperm-specific organelles such as the acrosome and flagellum.

During spermatogenesis, cytokinesis is incomplete during mitotic and meiotic divisions. Germ cells that arise from the same undifferentiated A₁ spermatogonium remain connected to each other by intercellular bridges (0.1–2 µm), which persist until late spermiogenesis (Dym & Fawcett 1971, Guo & Zheng 2004). The precise point at which these bridges are broken is unclear. Some studies indicate that they persist until the residual bodies are shed, while others indicate that they are broken before this point and connection between residual bodies is rare (Breucker *et al.* 1985). This may reflect species differences in post-meiotic development.

Several studies have provided strong evidence that spermatids share transcripts and/or gene products across the cytoplasmic bridges during spermiogenesis. Transgenic mice hemizygous for a spermatid-specific chimeric gene consisting of the mouse protamine 1 (*mP1*) transcriptional regulatory sequence fused with the human GH (*hGH*) structural gene carry the hGH protein in nearly all (~90%) epididymal spermatozoa, despite the fact that the transgene is only present in 50% of spermatids. This indicates that the transcript, the gene product or both is shared (Braun *et al.* 1989). Analysis of mice carrying Robertsonian translocations of chromosome 16 shows that sharing occurs at the transcript level for protamine (Caldwell & Handel

1991). Further indirect evidence is provided by the existence of essential spermatid-specific genes on the sex chromosomes (see Sex chromosome transcriptional activity in spermatids), which must be shared in order to allow spermatogenesis to continue. This syncytial development is a significant hurdle to immunological sperm separation: if all transcripts and/or gene products are shared evenly, then there can be no antigenic means to distinguish X from Y sperm. Box 1 presents a number of models showing how sperm become non-equivalent despite the presence of cytoplasmic bridges: this may potentially lead to sex-encoded OSSAs given a non-shared, sex-linked transcript that encodes a cell surface protein or even to autosomally encoded OSSAs given a non-shared, sex-linked transcript that encodes an enzyme involved in post-translational modification of autosomally encoded cell surface proteins.

Sex chromosome transcriptional activity in spermatids

The sex chromosomes undergo widespread radical changes in transcriptional status during spermatogenesis owing to the fact that their axes remain largely unsynapsed during meiosis. During meiosis, they become transcriptionally silenced and migrate to a peripheral location within the nucleus, remaining silent throughout diplotene (Kierszenbaum & Tres 1974; reviewed by McKee & Handel (1993)). This phenomenon, originally known as meiotic sex chromosome inactivation, is now known to be part of a more general meiotic silencing of unsynapsed chromatin (MSUC), which has been suggested to be a defence against selfish elements such as retroposons (Turner *et al.* 2005). No protein-coding genes are known to escape MSUC, although X-linked genes that are transcribed into miRNAs appear not to be silenced (Song *et al.* 2009).

After meiosis, sex chromosomal transcriptional repression is partially maintained, and the sex chromosomes

Table 1 Sex-linked genes expressed in spermatids. References are given for the first published reports of expression in spermatids and (where different) for the first report of X/Y linkage.

Gene	Species	Map	Copies	References
1700003E24Rik	M	X	3	Ellis <i>et al.</i> (2005) and Mueller <i>et al.</i> (2008)
1700010D01Rik	M	X	2	Ellis <i>et al.</i> (2005) and Mueller <i>et al.</i> (2008)
1700020N15Rik	M	X	2	Mueller <i>et al.</i> (2008)
1700042B14Rik	M	X	4	Mueller <i>et al.</i> (2008)
4921511M17Rik	M	X	4	Mueller <i>et al.</i> (2008)
4930453H23Rik	M	X	3	Ellis <i>et al.</i> (2005)
4930524N10Rik	M	X	5	Mueller <i>et al.</i> (2008)
4930567H17Rik	M	X	2	Mueller <i>et al.</i> (2008)
4933434C23Rik	M	X	3	Mueller <i>et al.</i> (2008)
AK006152	M	Y	2+	Ellis <i>et al.</i> (2005) and Ferguson <i>et al.</i> (2009)
AKAP4	H, M	X	1	Carrera <i>et al.</i> (1994) and Moss <i>et al.</i> (1997)
Asty	M	Y	Many	Touré <i>et al.</i> (2005)
Bex1	M	X	1	Brown & Kay (1999) and Yang <i>et al.</i> (2002)
Cdy	C, H	Y		Lahn & Page (1999), Kleiman <i>et al.</i> (2003), and Wang <i>et al.</i> (2008)
Cypt genes	M	Mostly X	15	Ellis <i>et al.</i> (2005) and Hansen <i>et al.</i> (2006)
Daz	H	Y	4	Reijo <i>et al.</i> (1995) and Habermann <i>et al.</i> (1998)
E330016L19Rik	M	X	21+	Mueller <i>et al.</i> (2008)
EG245347	M	X	1	Ellis <i>et al.</i> (2005)
EG434797	M	X	2	Mueller <i>et al.</i> (2008)
EG546347	M	X	1	Ellis <i>et al.</i> (2005)
Esx1	M	X	1	Branford <i>et al.</i> (1997) and Li <i>et al.</i> (1997)
Gmcl11	M	X	21+	Ellis <i>et al.</i> (2005)
H2a11	M	X	14+	Ellis <i>et al.</i> (2005), Govin <i>et al.</i> (2007), and Ferguson <i>et al.</i> (2009)
H2a12y	M	Y	2	Ellis <i>et al.</i> (2005) and Ferguson <i>et al.</i> (2009)
LOC245376	M	X	3	Mueller <i>et al.</i> (2008)
LOC278181	M	X	3	Mueller <i>et al.</i> (2008)
LOC207318	M	X	2+	Mueller <i>et al.</i> (2008)
LOC665542	M	X	9+	Mueller <i>et al.</i> (2008)
Magea5	M	X	8	Rogner <i>et al.</i> (1995) and Wang <i>et al.</i> (2005)
MGC58426	M	X	2	Mueller <i>et al.</i> 2008
Nxf2	M	X	1	Herold <i>et al.</i> (2000) and Wang <i>et al.</i> (2005)
Orly	M	Y	Many	Ellis <i>et al.</i> (2007)
Ott	M	X	12	Kerr <i>et al.</i> (1996) and Mueller <i>et al.</i> (2008)
Pramel3	M	X	1	Wang <i>et al.</i> (2005)
Pramey	C	Y	10+	Chang <i>et al.</i> (2011)
Sat11	M	X	1	Ellis <i>et al.</i> (2005)
Slx11	M	X	14+	Ellis <i>et al.</i> (2005) and Reynard <i>et al.</i> (2007)
Slx	M	X	25+	Calenda <i>et al.</i> (1994), Ellis <i>et al.</i> (2005), and Reynard <i>et al.</i> (2007)
Sly	M	Y	Many	Ellis <i>et al.</i> (2005), Touré <i>et al.</i> (2005), and Reynard <i>et al.</i> (2009)
Spanx	H	X	10	Westbrook <i>et al.</i> 2000
Sry	H, M	Y	1	Sinclair <i>et al.</i> (1990) and Koopman <i>et al.</i> (1990)
Ssty1/2	M	Y	Many	Conway <i>et al.</i> (1994) and Touré <i>et al.</i> (2004)
Ssxb	M	X	13+	Chen <i>et al.</i> (2003) and Mueller <i>et al.</i> (2008)
Taf7l	M	X	1	Wang <i>et al.</i> (2001, 2005)
Tex11	M	X	1	Wang <i>et al.</i> (2001, 2005)
Tex16	M	X	1	Wang <i>et al.</i> (2001, 2005)
Tgif2lx1	M	X	2	Blanco-Arias <i>et al.</i> (2002), Ellis <i>et al.</i> (2005), and Mueller <i>et al.</i> (2008)
Tsga8	M	X	1	Taketo <i>et al.</i> (1997) and Uchida <i>et al.</i> (2000)
Tspy	R	Y	1	Arnemann <i>et al.</i> (1991) and Kido & Lau (2006)
Ube2a	M	X	1	Koken <i>et al.</i> (1992) and Hendriksen <i>et al.</i> (1995)
Ubelx	M	X	1	Imai <i>et al.</i> (1992) and Hendriksen <i>et al.</i> (1995)
Ubely	M	Y	1	Mitchell <i>et al.</i> (1991), Kay <i>et al.</i> (1991), and Hendriksen <i>et al.</i> (1995)
Usp9y	M	Y	1	Brown <i>et al.</i> (1998) and Wang <i>et al.</i> (2005)
Vcy	H	Y	2	Lahn & Page (2000) and Tse <i>et al.</i> (2003)
Vsig1	M	X	1	Ellis <i>et al.</i> (2005)
Zfy	M	Y	2	Page <i>et al.</i> (1987) and Nagamine <i>et al.</i> (1990)
Znf280by-as	C	Y	130+	Yang <i>et al.</i> (2011)

C, cow; H, human; M, mouse; R, rat.

assume a conformation called post-meiotic sex chromatin (PMSC; Turner *et al.* 2006), which shares some of the same repressive histone marks, such as histone H3 dimethylated at lysine-9 (dimH3K9; Khalil *et al.* 2004). In contrast to the complete meiotic silencing of sex chromosome genes during MSUC, post-meiotic

repression is incomplete (Mueller *et al.* 2008). The RNA polymerase excluded from the XY body at pachytene re-engages the X and Y chromosomes in round spermatids (Khalil *et al.* 2004), and multiple studies have reported the transcription of sex-linked genes in spermatids. Initially, sex-linked spermatid genes

were identified as a by-product of other investigations, e.g. *Zfy* and *Sry* during the search for the sex-determining gene; *Usp9y*, *Tsyp*, *Daz*, and *Vcy* during the characterisation of Y-linked genes conserved between human and rodents; and *Akap4* and *Spanx* during the investigation of sperm proteins (Table 1). Hendriksen *et al.* (1995) carried out the first targeted investigation of sex chromosome transcription in spermatids, showing that the X-linked genes *Ubelx* and *MHR6A* (now known as *Ube2a*) and the Y-linked genes *Ubely* and *Sry* were highly transcribed in this cell type. *Ube2a* was also confirmed by northern blotting.

Recently, more systematic approaches have begun to unpick the full diversity of the post-meiotic sex chromosomal transcriptome. The investigation of mice with deletions on chromosome arm Yq (see Evidence for functional differences between haploid cells) identified the genetic complement of this region: *Ssty1*, *Ssty2*, *Sly*, *Asty*, and *Orly*, all of which are spermatid specific. A marked up-regulation of X-linked spermatid transcripts is also seen in the testes of these males, enabling the identification of several such genes (Ellis *et al.* 2005). Wang *et al.* (2005) examined a panel of 14 sex-linked genes known to be expressed in spermatogonia and showed that 13 of these genes showed some degree of post-meiotic reactivation. In particular, *Tex11*, *Tex16*, *Taf7l*, *Pramel3*, *Nxf2*, *Magea5*, *Ube1y*, and *Usp9y* showed the same mRNA levels in post-meiotic cells as in pre-meiotic cells. Most recently, Mueller *et al.* (2008) reported that, in mice, a collection of 33 multicopy X-linked gene families, representing ~273 individual gene copies, are expressed predominantly in spermatids. In addition, they showed low-level reactivation of many X-linked single-copy genes in round spermatids.

Collectively, the data indicate that for single-copy X genes, post-meiotic repression is 'leaky'. This leakage appears to be context dependent, with single-copy genes showing reactivation in a variable proportion of round spermatids (up to 18% for *Zfx*). In contrast, the majority of multi-copy X genes are active in the majority of X spermatids. Mueller *et al.* (2008) propose that the copy number amplification of these genes is a downstream response to post-meiotic repression, allowing the transcription of critical post-meiotic sex-linked genes. An alternative hypothesis was formulated by Ellis *et al.* (2005) that the gene amplification on the mouse X was a consequence of a genomic conflict between X and Y chromosomes (see also Evidence for functional differences between haploid cells). In support of this, the most highly ampliconic genes on the mouse became amplified shortly after *Sly* arose in the Y chromosome (Ellis *et al.* 2007, 2011). These hypotheses are not mutually exclusive: *Sly*, one of the participants in the proposed genomic conflict is believed to act via modulation of PMSC (Cocquet *et al.* 2009). The mechanism by which increased gene copy number leads to increased post-meiotic leakage is currently unclear.

It may be simply stochastic, with a higher copy number meaning a higher probability of at least one gene copy reactivating; alternatively, the repeat regions may fold into specialised structures such as hairpins or cruciforms and thereby evade the full extent of PMSC and the preceding MSUC.

The genes that have been confirmed by non-array methods as being transcribed post-meiotically are listed in Table 1. The criteria for inclusion in this list are either direct visualisation of nascent transcripts in spermatids by RNA FISH; or an increase in transcript expression in spermatids compared with spermatocytes, seen by RNA *in situ* hybridisation to testis slices or RT-PCR on separated cells; or increased transcription in testes with spermatids compared with testes without spermatids. This is thus a conservative list that explicitly excludes genes such as *Hprt1* in which the transcript is present in the spermatids solely due to the presence of long-lived mRNAs transcribed pre-meiotically, but which is not actively transcribed in spermatids. Also excluded are genes such as *Dby*, *Rps4y*, and *Smcy*, in which the transcript and/or protein have been observed in haploid cells, but retention of pre-meiotic transcripts has not been excluded.

Confirmation of post-meiotic expression is largely confined to the mouse at present, as while many sex-linked genes are known to be germ cell specific in human, few human studies have investigated the stage-specific localisation of X/Y transcripts, and still fewer studies have been performed in farm animal species. The functions of the genes that escape post-meiotic repression are largely unknown. *Akap4* is a flagellar protein expressed solely in post-meiotic spermatid cells and is essential for the development of all sperm as a component of the fibrous sheath and as a scaffolding protein for the regulatory proteins in the flagella of spermatozoa (Miki *et al.* 2002). *Cypt* and *Spanx* genes may have roles in acrosomal development, and *Sly* is a regulator of PMSC. *Slx*, the multi-copy X-linked homologue of *Sly*, is exclusively expressed in spermatids as confirmed by RNA FISH (Reynard *et al.* 2007), and siRNA knockdown of *Slx* family members has significant effects on sperm head morphology (Cocquet *et al.* 2010).

Evidence for functional differences between haploid cells

Compelling evidence for biochemical differences between individual sperm is provided by certain examples of transmission ratio distortion (TRD), in which there is a non-Mendelian inheritance pattern in the offspring of males heterozygous for a given genetic variant. In principle, TRD may arise meiotically (affecting chromosome segregation), post-meiotically (affecting spermatid maturation and fertilising ability), or post-fertilisation (affecting embryo viability). Distorter

genes falling into the second category exert their effects by producing a functional difference between haploid cells with and without the distorter: the gene products encoded by such distorters must, therefore, either be shared unevenly between sister spermatids or interact with other factors that are not shared. Such distorters are most commonly studied in non-mammalian model species such as *Drosophila* (Tao *et al.* 2007); however, there are three known mammalian examples. In each, normal and mutant sperm are produced in equal ratios and normal/mutant embryo survival is unaffected, but there is nevertheless skewed inheritance, demonstrating that the TRD must be due to functional differences between normal and mutant sperm.

Genes in the *t* complex are the most well-known and best characterised distorters in mammals. The *t* complex is a variant region of mouse chromosome 17 characterised by large-scale inversions that suppress recombination between *t* and normal haplotypes, thus preserving an association between several distorter genes and a single responder gene, which collaboratively lead to TRD (Lyon 2003). The identities of the distorter genes are unknown; however, the responder has been identified as an aberrant kinase fusion gene, *Tcr^{Smok}* (Herrmann *et al.* 1999). Another example of TRD involving sperm functional heterogeneity concerns Robertsonian translocations involving mouse chromosome 6. Males heterozygous for Rb(6.16) and Rb(6.15) translocations transmit almost exclusively euploid gametes resulting from alternate segregation, with very little transmission of aneuploid adjacent segregants. Within the euploid gamete population, the normal (non-translocated) chromosome complement transmits at an increased rate relative to the balanced translocation during natural mating. If aged sperm is used for fertilisation by housing the male apart from the female for 14 days, the TRD is abolished, thus indicating that equal numbers of balanced/normal sperm are produced and that their relative fertilising ability depends on the intercoital interval (Aranha & Martin-DeLeon 1995). There is also no TRD when IVF is performed using sperm from carrier males. More interestingly, these mouse models also exhibit a sex ratio skew in favour of males during natural mating, which is also abolished in aged sperm and in IVF. This first indicates that there must be some functional asymmetry between X and Y sperm which, if identified, could form the basis for sperm selection; and secondly that this X/Y asymmetry interacts with the chromosome 6 complement of any given sperm.

Most interesting of all with regard to sperm selection is the sex ratio skewing seen in the offspring of mice bearing partial deletions on the long arm of the Y chromosome (Conway *et al.* 1994). Males with smaller deletions on Yq (~2/3 of the long arm deleted) show mild sperm head abnormalities and a sex ratio skew in favour of females, while larger deletions lead to more severe head shape abnormalities and sterility (Touré

et al. 2004). ICSI using sperm from Yq-deleted males yields a balanced sex ratio, indicating that the skew does not show decreased differential gamete production or increased embryonic death, but must result from differential fertilising ability of X and Y sperm (Ward & Burgoyne 2006). In this model, Yq deletion leads to decreased transcript levels of multi-copy Yq-linked genes and increased transcription of X-linked and Yp-linked genes (Ellis *et al.* 2005, Touré *et al.* 2005). It has recently been shown that *Sly*, one of the Yq-linked genes affected by the deletion, is a regulator of PMSC (see Sex chromosome transcriptional activity in spermatids), and *Sly* deficiency leads to the up-regulation of other sex-linked genes in the spermatids of affected males (Cocquet *et al.* 2009). The association of increased X transcription with increased X transmission is strongly suggestive of a genomic conflict between X and Y chromosomes, i.e. one or more genes on the X chromosome constitutes the distorter gene(s) responsible for increased X chromosome transmission, and *Sly* acts to repress the distorter(s) and restore a normal sex ratio.

As discussed in Box 1, the functional differences between the sperm that underlie TRD depend on a lack of transcript sharing between sister spermatids. This has been observed for both the *t* complex, in which the *Tcr^{Smok}* responder was recently shown not to be shared between syncytial spermatids (Véron *et al.* 2009), and the chromosome 6 models, in which the transmission ratio skew appears to be due to non-sharing of allelically distinct variants of the *Spam1* hyaluronidase gene (Zheng *et al.* 2001, Martin-DeLeon *et al.* 2005). The genes responsible for the sex ratio skew in Yq-deleted males and/or the sex ratio skew in the Robertsonian fusion models remain to be identified; however, they are predicted to be encoded by the sex chromosomes and to escape transcript sharing. In addition to compartmentalisation of the transcripts, the resulting protein products must also escape sharing, for example by immediate insertion into the cell membrane (e.g. *Spam1*) or incorporation into a non-diffusible protein complex (e.g. insertion of *Tcr^{Smok}* into the flagellum).

Transcriptional and translational activity in spermatozoa

Unlike sperm mitochondria, which remain transcriptionally competent, mature sperm nuclei have historically been believed to be transcriptionally inert due to the repackaging of chromatin with protamines, leaving it inaccessible for transcription. Despite this, it is increasingly clear that some regions of the sperm genome remain packaged by histones rather than proteins (reviewed in Miller *et al.* (2010)), either as conventional nucleosomes or in novel nucleosome-like structures that organise non-standard lengths of DNA (Govin *et al.* 2007). It is likely that this less-condensed portion of the

sperm genome, the nucleohistone fraction, primes specific genes for early reactivation during embryogenesis, but it is also possible that it is permissive for transcription in sperm. No nuclear gene has yet been shown to be transcribed in sperm; however, RNA polymerase II is known to be present and associated with the *Hspa1b* promoter in mouse epididymal sperm (Wilkerson & Sarge 2009).

A further factor affecting transcriptional competence of sperm chromatin is the activity of 'selfish' genomic elements such as retroposons. The sperm nucleohistone fraction is enriched for retroposons (Pittoggi *et al.* 1999), reverse transcriptase activity encoded by such retroposons has been observed in sperm nuclei of several species (e.g. Fuster *et al.* 1977), and reverse transcriptase molecules can be directly visualised in association with the sperm nuclear scaffold by immunogold staining (Giordano *et al.* 2000). Moreover, the nucleohistone fraction is preferentially targeted for incorporation of exogenous DNA during sperm-mediated gene transfer (Smith & Spadafora 2005). Collectively, these results imply active transcription of retroposons in sperm and that these parasitic genomic elements are able to maintain regions of the sperm genome in a transcriptionally competent state in order to facilitate their own transmission during late spermatogenesis. In this light, it is interesting to note that the X chromosome is enriched for full-length actively proliferating LINE elements and is thus a potential location for continued transcription in mature sperm.

Transcription in sperm cannot lead to an antigenic difference unless the transcripts are translated. A radiolabeling study has indicated that spermatozoa are indeed translationally active and incorporate labelled amino acids ($[^{35}\text{S}]$ methionine, $[^{35}\text{S}]$ cysteine and BODIPY-lysine-tRNA^{Lys}) into newly synthesised polypeptides during capacitation (Gur & Breitbart 2006). The incorporation is completely inhibited by mitochondrial translation inhibitor D-chloramphenicol (CP) but not by the cytoplasmic translation inhibitor cycloheximide. Moreover, 55S mitochondrial ribosomes were present in polysomal fractions and 80S cytoplasmic ribosomes were not, indicating that translation in sperm utilises mitochondrial ribosomes. Intriguingly, CP treatment lowered the levels of a number of nuclear-encoded proteins in sperm, suggesting that the mitochondrial ribosomes translate nuclear transcripts in addition to mitochondrial transcripts. This translation is most likely to utilise stored spermatozoal RNAs retained following cytoplasmic shedding, for which there is now a large and growing body of evidence (reviewed in Lalancette *et al.* (2008) and Dadoune (2009)). The current belief is that the stored mRNA either represents non-functional remnants from earlier spermatid stages or a paternal contribution to early embryonic events. Evidence for the latter is given by a recent study showing that *Dby* mRNA is retained in mature mouse sperm and that injection of

an interfering antisense RNA into the male pronucleus (but not the female pronucleus) disrupts zygotic development (Yao *et al.* 2010). Evidence for a potential role in sperm function is given by Gur & Breitbart (2006), which showed that CP treatment significantly altered sperm parameters including motility, capacitation and fertilisation rate: this may alternatively reflect disruption of sperm mitochondrial function rather than dependence on ongoing translation of nuclear genes by nuclear ribosomes.

The ongoing search for OSSAs

Current searches for OSSAs have tended to follow one of three approaches: they take a single-transcript approach and test to see whether an expression of a selected sex-linked gene is specific to X or Y sperm, a proteomic approach screening for differentially expressed proteins, or the immunological approach of raising antibodies to mixed sperm proteins and testing for specificity. To date, all the three approaches have failed: the first due to insufficient throughput and the probability in selecting the correct candidate gene and the latter two due to the signal/noise ratio trying to discern a single OSSA from hundreds if not thousands of antigenic sperm surface proteins.

Initial candidate gene work focused on the H-Y antigens, a collection of male-specific minor histocompatibility antigens encoded by Y chromosomal proteins. H-Y epitopes encoded by *Smcy* have been shown to be present on the plasma membrane over the sperm head and midpiece (Bradley *et al.* 1987). Subsequent work shows that the levels on X and Y sperm are similar and that this gene is not a candidate for immunoselection (Sills *et al.* 1998). As subsequent work elucidated the gene content of the Y chromosome gene content, various studies (Table 1) investigated the expression of Y-borne genes and their X homologues in spermatids; however, none has as yet been found to be differentially expressed. Very few other X-linked genes, i.e. those without Y homologues, have been tested as candidate OSSAs. In the Ellis *et al.* (2005) study, RNA *in situ* for *H2a1*, *Gmcl1*, *Slx*, *Slx1*, and *Vsig1* showed a signal in all round spermatids at a given tubule stage, but this does not rule out more subtle dosage differences, translational differences or continuation of transcription into late-stage spermatids after breakage of the cytoplasmic bridges. Yeh *et al.* (2005) claim that ESX1 antiserum stains approximately half the sperm population, whereas SRY antiserum shows a mutually exclusive staining pattern. Since transcripts of *Esx1* are known to be present in all spermatids (Branford *et al.* 1997), this is compatible with models B–F for OSSA generation (see Box 1). While the study is suggestive that ESX1 and SRY may constitute OSSAs, it did not directly test whether the ESX-positive sperm carry an X chromosome or whether the SRY-positive sperm carry a Y chromosome. Thus, it is

possible that ESX1 and SRY are markers for different sperm populations but that this does not correlate with their X/Y-bearing status. Moreover, the translational status of SRY in mouse germ cells is highly disputed, since the spermatid transcripts are circular (Dolci *et al.* 1997) and hence lack necessary translational signals. It is thus likely that SRY antiserum used in this study is detecting some other antigen in sperm. If the findings can be confirmed and ESX1 does prove to be a true OSSA, it is nevertheless unlikely to be amenable for use in sperm separation because its epitope is within the nucleus rather than in the plasma membrane. Nevertheless, this provides an interesting demonstration of functional heterogeneity in the sperm population that opens the way for a more systematic search for OSSAs.

Two proteomic studies have used flow-sorted X and Y sperm populations to look for protein differences using a range of techniques, also with limited success. Two-dimensional electrophoresis of pig sperm proteins did not reveal any qualitative or quantitative difference in protein spots between X and Y sperm (Hendriksen *et al.* 1996). The same negative result was found for bull X and Y sperm surface membranes using one-dimensional SDS-PAGE (Howes *et al.* 1997). Encouragingly, the latter study did detect a group of proteins at ~40 kDa, 5.3 pl using two-dimensional electrophoresis that appeared to be enriched in X-bearing sperm. These proteins were detected in whole sperm extract but not in a membrane-enriched sample, suggesting that they are not surface antigens and thus unsuitable for sperm sorting. Proteomic screening studies are usually limited to the abundant proteins present in any given sample, although the advent of mass screening via mass spectrometry is changing this, and are also subjected to other considerations such as solubility, proteolysis, etc.

The immunological approach was first used by Blecher *et al.* (1999), who focused on SCSPs, i.e. proteins encoded by sex-linked transcripts. They argued that Ohno's law (Ohno 1967) dictates that SCSPs should be more highly conserved than other proteins, and that therefore: a) immunisation of rabbits with same-sex mixed bovine tissue would not raise antibodies to SCSPs since these would be conserved enough to be recognised as 'self', allowing immunopurification of putative SCSPs; and b) subsequent immunisation of male rabbits with the putative X-linked SCSPs and female rabbits with the putative Y-linked SCSPs would generate X- and Y-specific antisera. The underlying rationale is flawed on two grounds: first, both reproductive and Y-linked genes are known to evolve much faster than other categories of gene, undermining the 'SCSP conservation' hypothesis. Secondly, male rabbits (XY) still possess an X chromosome and will, therefore, not selectively raise anti-X-antisera. Nevertheless, this study is noteworthy for directly testing whether it was possible to manipulate offspring sex by this method, reporting a partially successful enrichment for male IVF embryos in antimalle

antibody-selected (66% male, 6% female, and 28% untypeable) relative to control sperm samples (55% male, 28% female, and 17% untypeable). This suggests that there may be conserved cell surface proteins on bovine Y spermatozoa that are immunogenic in female rabbits but not in male rabbits. More recently, phage selection was used to generate a library of monoclonal antibodies recognising sperm surface proteins (Soares & Barbosa 2008). Twenty-four clones that each bound 40–60% of spermatozoa were then tested for their ability to bind male and female leukocytes, and one clone was found to bind only to male cells. Collectively, these results demonstrate functional heterogeneity in the sperm population, but as with Yeh *et al.* (2005), these findings once again fall short of demonstrating that the sperm recognised by the putative male-specific antigen are in fact Y-bearing sperm. Most recently of all, Sang *et al.* (2011), carried out a more rigorous version of the Blecher approach, generating rabbit antisera against flow-separated X and Y bovine sperm and subsequently using immunoprecipitation to remove cross-reacting antibodies. This study reports the presence of an ~30 kDa OSSA in X-bearing bovine sperm: it will be interesting to determine whether this is related to the group of ~40 kDa OSSAs observed by Howes *et al.* (1997).

Conclusion: where next?

Haploid spermatids harbour a diverse transcriptome and remain translationally competent throughout the mature sperm stage. This diversity is not uniformly expressed but is variable between individual cells and categories of cells, as shown by various functional/immunological studies of sperm antigens. There are consistent functional differences between X and Y sperm, as shown by examples of sex ratio skewing in the offspring of mutant males, and the mechanisms by which transcript compartmentalisation contributes to sperm functional heterogeneity are beginning to be investigated. To date, the search for OSSAs associated with these differences has been fruitless, although the immunological approach has shown some promise.

As we argue above in Box 1, we believe that any functional difference between X and Y sperm will ultimately be traceable to a difference in their transcriptional content. DNA microarray technology is now a sufficiently mature technology that robust, genome-wide screening can be performed on very small amounts of starting material such as flow-sorted cells. A full investigation of transcriptional differences between flow-sorted X- and Y-bearing round spermatids, elongating spermatids, condensing spermatids and mature sperm would potentially allow the selection of strong candidate genes for subsequent immunological testing as suitable antigens for sperm sorting. These could then be prioritised based on their protein domain structure and the likelihood that they will be exposed on the

plasma membrane. Additionally, as proteomic technologies continue to improve and more complete coverage of the proteome is obtained, studies on separated X- and Y-bearing cells will continue to be of great interest in this regard. Another promising avenue for investigation would be a systematic chromatin immunoprecipitation analysis of the regions bound by RNA polymerase II in mature spermatozoa: these regions may potentially remain transcriptionally competent following the breakage of the cytoplasmic bridges. Finally, continued analysis of known sex ratio distortion phenotypes in mouse and other animal models will reveal naturally occurring modifiers of sex ratio, which may in turn be amenable to commercial development.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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