REPRODUCTION
REVIEW

The sperm nucleus: chromatin, RNA, and the nuclear matrix

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Abstract

Within the sperm nucleus, the paternal genome remains functionally inert and protected following protamination. This is marked by a structural morphogenesis that is heralded by a striking reduction in nuclear volume. Despite these changes, both human and mouse spermatozoa maintain low levels of nucleosomes that appear non-randomly distributed throughout the genome. These regions may be necessary for organizing higher order genomic structure through interactions with the nuclear matrix. The promoters of this transcriptionally quiescent genome are differentially marked by modified histones that may poise downstream epigenetic effects. This notion is supported by increasing evidence that the embryo inherits these differing levels of chromatin organization. In concert with the suite of RNAs retained in the mature sperm, they may synergistically interact to direct early embryonic gene expression. Irrespective, these features reflect the transcriptional history of spermatogenic differentiation. As such, they may soon be utilized as clinical markers of male fertility. In this review, we explore and discuss how this may be orchestrated.

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Introduction

Unlike the vast size of the oocyte, the diminutive sperm may have initially seemed unlikely to carry information in excess of its genomic cargo. Indeed, our ability to appreciate the contrary only began to gradually develop over the last two decades. This has been due to several factors, primarily reflecting the distinct nuclear environment of the mature spermatozoon. The sperm genome is repackaged into a near crystalline state, which has proven resistant to dissection often likened to a 'tough nut to crack'. This extensive remodeling both protects the paternal genome and is requisite for the characteristic reduction in nuclear volume which occurs as the head takes on a unique shape (reviewed in Braun (2001) and Balhorn (2007)). The assumption that sperm occupy a limited developmental role compared to oocytes has in part been due to these physical constraints and the appropriate enabling physical, chemical, and biological technologies (Kierszenbaum & Tres 1975).

Despite the near complete packaging of the sperm genome as protamine (PRM)-associated DNA, it is increasingly clear that specific regions retain a somatic-like structure (reviewed in Miller *et al.* (2010)). In some cases, these regions are differentially marked by modified histones in a manner reminiscent of the epigenetic states observed in somatic or stem cells (Hammoud *et al.* 2009, Brykczynska *et al.* 2010). This feature of sperm chromatin has been suggested to influence the order that genes are repackaged into a nucleosomal bound state and/or expressed following fertilization (reviewed in Rousseaux *et al.* (2008)). Additionally, sites of histone retention are likely to provide insight into the transcriptional history of spermatogenesis.

RNAs produced during this prior window of transcription are retained in sperm and delivered to the oocyte. The biological role of these transcripts post-fertilization remains a subject of debate. Regardless of their function, several of these molecules are currently being developed as biomarkers of male fertility (Depa-Martynow *et al.* 2007, Jedrzejczak *et al.* 2007, Lalancette *et al.* 2009). Importantly, the notion of a sperm enriched in RNAs continues to expand with the isolation and characterization of a complement of male gamete small non-coding RNAs (sncRNAs; C Lalancette, AE Platts, MP Diamond & SA Krawetz 2010, unpublished observations).

A subset of sperm RNAs may also serve to structurally support the nuclear matrix. This proteinaceous network present in most cells functionally organizes the genome by binding discreet regions of DNA at sequences termed scaffold/matrix attachment regions (S/MARs). S/MAR binding partitions the genome into cell type-specific

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loop domains, which range in size from 30 to 110 kb in somatic cells (Vogelstein *et al*. 1980, Linnemann *et al*. 2009, Drennan *et al*. 2010) and from 20 to 50 kb in sperm (Ward *et al*. 1989, Barone *et al*. 1994, Nadel *et al*. 1995). Nucleosome-bound DNA maintained in mature sperm has been proposed to mark sites of nuclear matrix attachment in these cells. These structural markers likely correspond to the S/MARs anchoring the decondensed DNA loops of prior cell types and may serve to recapitulate paternal nuclear architecture in the zygote (Ward 2010).

The notion that the male gamete merely delivers paternal DNA to the oocyte is falling by the wayside. This reflects several developments pertaining to the interacting function of the three main structural genetic elements of the sperm nucleus: chromatin, RNA, and the nuclear matrix. In a manner accessible to all reproductive biologists, this review explores and discusses how this unique nuclear symphony may be conducted. As such, when appropriate, a role for paternal chromatin, RNA, and the nuclear matrix beyond the interior of the sperm nucleus is discussed in terms of potential impact on embryonic development. While not the primary focus of this review, one is also referred to several timely reviews discussing paternal imprinting, the transgenerational effects of germline mutations (Butler 2009, Nadeau 2009, de Boer *et al*. 2010) providing additional perspectives.

Sperm chromatin

Spermatogenesis is characterized by ordered histone replacement. As spermatogonia commit to this differentiative pathway, they have already begun to incorporate testis-specific histone variants into their chromatin (Meistrich *et al*. 1985, van Roijen *et al*. 1998). Synthesis and deposition of these proteins peak during meiosis (Kimmins & Sassone-Corsi 2005). Supported by the action of testis-specific histone variants, in round spermatids, the majority of histones are replaced first by the transition proteins (TNPs) and subsequently by PRMs. Some histone variants, as well as canonical histones, are maintained throughout the remaining stages of spermatogenesis (Shires *et al*. 1976, Seyedin & Kistler 1980, Gatewood *et al*. 1987, 1990, Witt *et al*. 1996, Chadwick & Willard 2001, Zalensky *et al*. 2002, Yan *et al*. 2003, Churikov *et al*. 2004*a*, reviewed in Churikov *et al*. (2004*b*), Tanaka *et al*. (2005) and Govin *et al*. (2007)).

Chromatin remodeling requires regulated post-translational modifications of histones including acetylation (Oliva & Mezquita 1982, Christensen *et al*. 1984, Grimes & Henderson 1984, Meistrich *et al*. 1992, Marcon & Boissonneault 2004), ubiquitination (Chen *et al*. 1998, Baarends *et al*. 1999, Lu *et al*. 2010), methylation (Godmann *et al*. 2007), and phosphorylation (Meyer-Ficca *et al*. 2005, Krishnamoorthy *et al*. 2006, Leduc *et al*. 2008*a*), and has been recently reviewed in the context of spermatogenesis (Rousseaux & Ferro 2009). Among these modifications, the best characterized to date is the global hyperacetylation of histones. Incorporation of these marks destabilizes nucleosomes in preparation for their replacement by the TNPs and ultimately by the PRMs (Pivot-Pajot *et al*. 2003, Kurtz *et al*. 2007).

Hyperacetylation is essential in mice and men as perturbation is correlated with defective spermatogenesis (Sonnack *et al*. 2002, Fenic *et al*. 2004). This is supported by the observation that species maintaining chromatin in a somatic-like state do not exhibit elevated levels of histone acetylation in sperm (Christensen *et al*. 1984). For example, trout spermiogenesis spans several weeks during which spermatids exhibit high steady state levels of hyperacetylation. Extended maintenance of this modification in the absence of protamination suggests that additional factors are needed to complete nuclear remodeling (Christensen *et al*. 1984, Csordas 1990). Even precocious hyperacetylation in *Drosophila* does not prematurely induce the histone to PRM spermatid transition (Awe & Renkawitz-Pohl 2010). There are several potential pathways regulating initiation of chromatin remodeling. However, inhibition of the ubiquitin–proteasome pathway by loss of an ubiquitin ligase can block global histone acetylation, degradation, and PRM deposition, resulting in sterility (Lawrence 1994, Roest *et al*. 1996, Lu *et al*. 2010). In these studies, mature spermatozoa were low in number and exhibited altered morphologies, reminiscent of teratozoospermia. Indeed, microarray analysis of sperm RNAs from teratozoospermic patients presents a severe disruption of the ubiquitination pathway (Platts *et al*. 2007).

During murine and human protamination, histones are replaced first by the TNPs then subsequently displaced by the PRMs (Balhorn *et al*. 1984). Binding of these small arginine-rich proteins to the negatively charged phosphodiester backbone of the double helix abolishes the electrostatic repulsion between the proximal chromatin strands resulting in the formation of a toroid loop (Hud *et al*. 1993). Containing ~50 kb of DNA, these donut-shaped structures are further stabilized by inter- and intramolecular disulfide bridges compressing the genome into a semi-crystalline state as the spermatozoon transits through the epididymis (Golan *et al*. 1996). The resulting mature human sperm nucleus is now condensed to 1/13th the size of that of the oocyte (Martins & Krawetz 2007*b*).

Despite compaction, the restructured paternal chromatin retains a hierarchical layer of genomic organization (Zalensky & Zalenskaya 2007). Reminiscent of somatic cells, individual chromosomes are not randomly positioned, but occupy rather distinct territories preferentially localized within the nucleus with respect to one another (Hazzouri *et al*. 2000, Zalenskaya & Zalensky 2004). The positioning of chromosome territories in porcine spermatozoa is first observed in spermatids. Preceding meiosis, their relative position resembles that seen in somatic cells (Foster *et al*. 2005). It has

been proposed that within sperm, each chromosome territory generally adopts a 'looped hairpin' conformation orienting its centromere towards the nuclear interior and distal telomeres towards the periphery (Mudrak *et al*. 2005).

Nuclear remodeling has been proposed to serve three functions (Braun 2001). First, the reduced size and shape of the sperm nucleus yields a hydrodynamic structure that is predictive of fertility in bulls and red deer (Ostermeier *et al*. 2001, Malo *et al*. 2006, Gomendio *et al*. 2007). Second, protamination renders the majority of the sperm genome resistant to nuclease attack, irradiation, and shearing forces (Kuretake *et al*. 1996, Wykes & Krawetz 2003, Rathke *et al*. 2010). Presumably, both features were evolutionarily optimized to protect the paternal genome while traversing the female reproductive tract en route to the oocyte. Third, although a subject of debate, the selective post-meiotic retention of histones provides the zygote a dichotomous chromatin package that could serve to preferentially poise regions for early use (Gatewood *et al*. 1987, Hammoud *et al*. 2009, Brykczynska *et al*. 2010).

Murine spermatozoa organize about 1–2% of their genome with nucleosomes (Balhorn *et al*. 1977, Brykczynska *et al*. 2010), whereas up to 15% of human sperm DNA is packaged in this manner (Tanphaichitr *et al*. 1978, Gusse *et al*. 1986, Gatewood *et al*. 1990). Interrogation of isolated nucleosome-associated sequences demonstrated that some of these genomic regions included imprinted regions (Banerjee & Smallwood 1998), telomeres (Pittoggi *et al*. 1999, Zalenskaya *et al*. 2000), retroposon DNA (Pittoggi *et al*. 1999), and specific gene loci (Gardiner-Garden *et al*. 1998, Pittoggi *et al*. 1999, Wykes & Krawetz 2003). Lacking comparable nucleosomal enrichment, the centromeric and pericentromeric regions of mammalian sperm present a mix of nucleosomes and PRMs (Wykes & Krawetz 2003). Specifically, these regions retain modified histones such as H3K9me3 as well as the histone variants CENPA and H2A.Z (Palmer *et al*. 1990, Zalensky *et al*. 1993, Hammoud *et al*. 2009). Together these observations led to the hypothesis that the maintenance of nucleosomes at specific sites may prime discreet regions for use shortly after fertilization. Initial support for this premise came from the finding that, in human sperm, histones bind DNA in a sequence-specific manner around gene regulatory regions (Gatewood *et al*. 1987, Wykes & Krawetz 2003).

Studies reporting the *in situ* localization of nucleosome-associated genomic regions in the sperm should be met with caution. The compact nuclear environment of the spermatozoa cannot be accurately interrogated by immunofluorescence without prior membrane destabilization and chromatin decondensation. Treatment may skew interpretations as decondensation alters the position of nuclear elements (van Roijen *et al*. 1998). With this caveat, in human spermatozoa, core histones as well as testes-specific histone variants have been observed within the basal portion of the nucleus proximal to the tail (Zalensky *et al*. 2002, Li *et al*. 2008). In contrast, histone H2B as well as nucleosome-associated telomeric regions exhibits a partially overlapping punctuate pattern throughout the nucleus (Gineitis *et al*. 2000, Zalensky *et al*. 2002). In the mouse, telomeres are bound by linker H1, which is absent in human sperm, and appear localized to the periphery (Gatewood *et al*. 1990, Pittoggi *et al*. 1999). It cannot be excluded that these results primarily reflect nuclear access. As an additional point of comparison, the canonical histones found in spermatozoa of the evolutionarily distant marsupial, *Sminthopsis crassicaudata*, are also peripherally located (Soon *et al*. 1997). Regardless of the limitations inherent to these studies, it is generally agreed that the nucleoprotamine and nucleohistone components in sperm are discreetly partitioned (van der Heijden *et al*. 2006, Li *et al*. 2008).

Recent advances in methods of genome-wide analysis now allow for the detection of histone-enriched regions at the primary sequence level. Using CGH tiling arrays, it was established that histone-bound DNA is associated with gene-dense regions and enriched for developmentally regulated promoters as well as CTCF binding sites (Arpanahi *et al*. 2009). In parallel, next generation sequencing (NGS) provided a significantly higher resolution analysis (Hammoud *et al*. 2009). Nucleosome-associated sequences exhibited a modest enrichment within the promoters of developmentally important genes including embryonic transcription factors and signaling pathway components, as well as microRNA (miRNA) and imprinted gene clusters. Independent analysis has demonstrated that internal exons also display significantly greater histone enrichment than flanking intronic sequences (Nahkuri *et al*. 2009). Outside of promoters, histones were found to be distributed, at low levels, throughout the genome. This pattern of nucleosome retention has recently been confirmed using similar NGS technologies (Brykczynska *et al*. 2010).

Combining chromatin immunoprecipitation (ChIP) and NGS (i.e. ChIP-seq) revealed that developmentally regulated promoters may be bivalently marked by H3K4me2/3 and H3K27me3 (Hammoud *et al*. 2009, Brykczynska *et al*. 2010). The bivalent promoter is a hallmark of developmentally regulated stem cell genes and has recently been observed in zebrafish blastomeres (Vastenhouw *et al*. 2010). In addition to harboring sites of both active and repressive histone modifications, bivalent promoters are often bound by RNA polymerase and are therefore poised for expression. To date, this correlation has not been established in mature sperm. The coordinated removal of repressive H3K27me3 throughout differentiation permits the initiation of transcription, providing temporal and spatial

control of gene expression. Bivalent promoters might reflect the male contribution to early gene expression (Petronis 2010).

Alternatively, differential enrichment of histone modifications within specific ontological categories of promoters, and not bivalency, may regulate early embryonic gene expression (Brykczynska *et al*. 2010). In human sperm, H3K4me2 marked promoters of genes associated with spermatogenic and housekeeping processes, whereas H3K27me3 was enriched within the promoters of developmentally regulated genes expressed following implantation or in differentiated cells. Furthermore, the degree to which a promoter was occupied by H3K27me3 positively correlated with repression of the corresponding gene during early mouse embryonic development. Together these results argue that the retention of the repressive H3K27me3 modification at specific promoters in human sperm may provide a paternal and possibly transgenerational mark (Petronis 2010).

The two modes of paternally derived epigenetic promoter regulation introduced above, bivalency and differential enrichment of modified histones, are likely present in sperm of both mice and men. As illustrated in Fig. 1, the use of one mechanism in lieu of the other would be expected to hinge on shared spermatogenic transcriptional requirements and the species-specific timing of zygotic genome activation (ZGA).

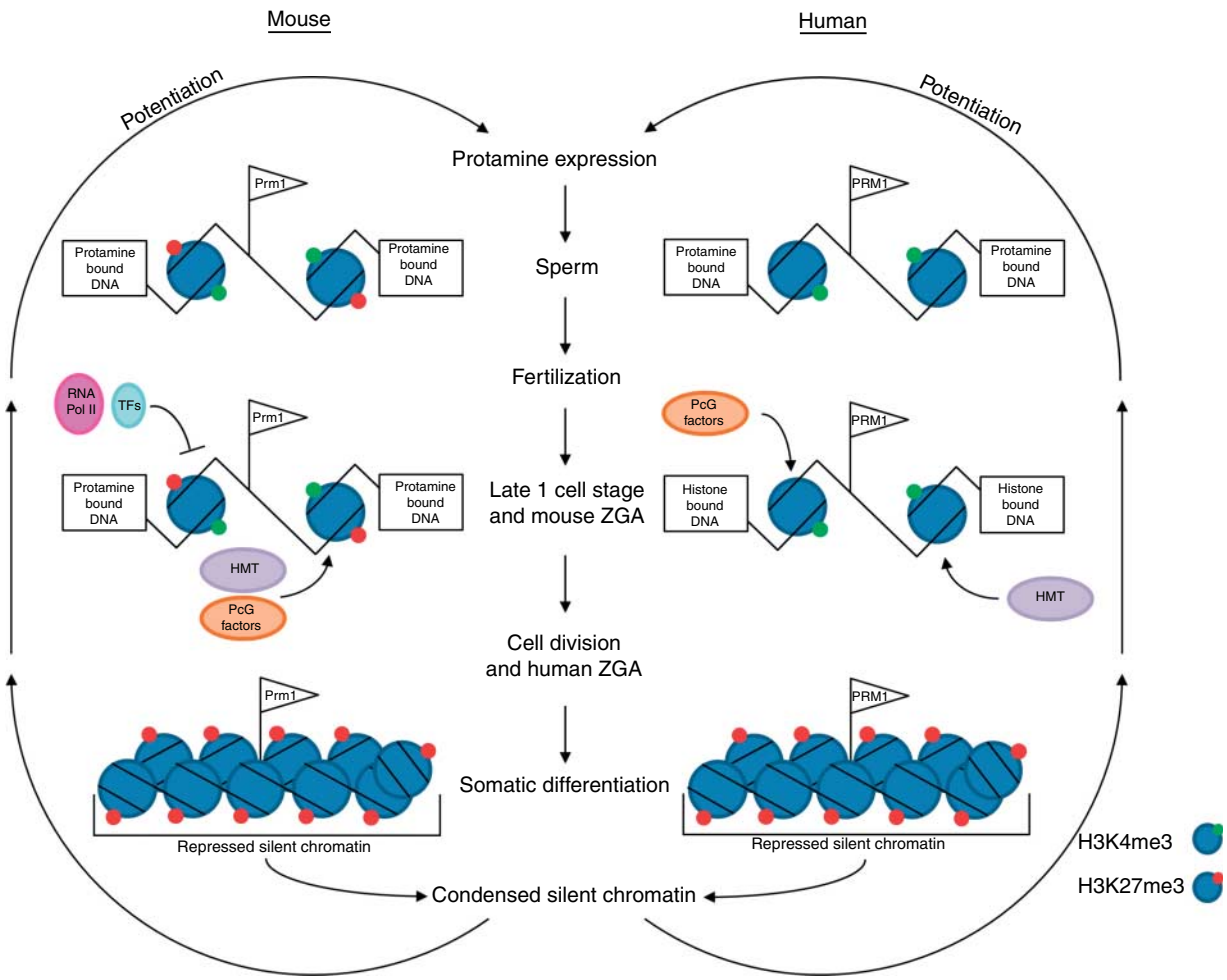

Figure 1 The potential influence of zygotic genome activation on paternal chromatin structure. In mouse and human sperm, the protamine genes are bound by nucleosomes residing within a potentiated DNase I-sensitive domain. These regions are differentially marked by modified histones in each species. In mouse, the bivalently marked spermatogenic promoters may reflect the early initiation of zygotic expression at the late one-cell stage. Recruitment of transcriptional machinery (RNA polymerase, RNA Pol II; transcription factors, TFs) is coincident with the activation of silencing pathways (histone methyltransferases, HMTs; polycomb factors, PcG). The retention of the silencing H3K27me3 mark in promoters may prevent detrimental expression prior to gene silencing. In comparison, human zygotic genome activation occurs at the four- or eight-cell stage. This affords the embryo time to silence these genes, which in sperm are marked with the active H3K4me3 modification lacking the repressive mark. In both species, the protamine domain remains silenced throughout differentiation by adopting a highly condensed chromatin conformation. During male gametogenesis, this region becomes potentiated in spermatocytes prior to its expression in round spermatids.

Whereas promoters of potent developmental regulators in sperm from both species are primarily associated with repressive histone modifications, spermatogenic genes are bivalently marked in murine but not in human sperm (Brykczynska *et al*. 2010). The former reflects a shared need for early repression of developmental gene expression. The presence of active modifications in mouse and human spermatogenic promoters likely corresponds to the transcriptional history of these silent cells. In mouse, these regions are marked by repressive histone modifications to ensure their appropriate regulation following fertilization. Mice initiate ZGA late in the one-cell embryo (Schultz 2002, Minami *et al*. 2007), concurrent with DNA replication (Aoki *et al*. 1997). This is paralleled by an increase in the levels of H3K27me3 within the paternal pronuclei through the activity of polycomb group (PcG) proteins (Santos *et al*. 2005). Prior to this, H3K27me3 cannot be microscopically detected in paternal chromatin of the one-cell fertilized oocyte (Santos *et al*. 2005, van der Heijden *et al*. 2005, Puschendorf *et al*. 2008). Methylated sperm histones are expected to remain reflecting the lack of histone demethylase activity in either the oocyte or the zygote (Puschendorf *et al*. 2008). This is likely essential to ensure proper transcriptional regulation from the paternal chromatin during this initial wave of ZGA. Concomitantly, the male pronucleus exhibits a higher level of transcriptional activity (Aoki *et al*. 1997), an increased concentration of transcription factors (Worrad *et al*. 1994), and a more transcriptionally permissive chromatin structure compared to the female pronucleus (Adenot *et al*. 1997, Schultz 2002). It is reasonable to assume that the presence of sperm-derived H3K27me3 within the bivalent promoters of the paternal spermatogenic genes enables the propagation of the polycomb repressive mark preventing their transcription (Margueron *et al*. 2009, Brykczynska *et al*. 2010). This would be expected to block transcription factor recruitment and subsequent expression. Repression of these genes is necessary as expression of PRM1, which is bivalently marked in mouse sperm, would likely perturb further development (Lee *et al*. 1995). Indeed, mutant mice lacking the methyltransferase activity (required to propagate H3K27me3) do not progress past early development (O'Carroll *et al*. 2001). Though undoubtedly this mutation is responsible for a wide range of developmental defects (Erhardt *et al*. 2003, Puschendorf *et al*. 2008), it would be informative to probe these late zygotic mutants for expression of those spermatogenic genes marked by a bivalent promoter in wild-type sperm. Comparatively, the delayed ZGA of humans (Braude *et al*. 1988) should permit PcG-mediated repression of orthologous spermatogenic promoters altering the paternally derived poised chromatin structure. The inability to detect trimethylated paternal H3K27 in G2 tripronuclear zygotes suggests that deposition of this modification occurs sometime after the first cleavage event but before the start of embryonic gene expression at the four- to eight-cell stage (van der Heijden *et al*. 2009).

The number of histone variants and associated secondary modifications found in mammalian sperm has greatly increased in the last two decades (reviewed in Rousseaux & Ferro (2009) and Carrell & Hammoud (2010)). Detection of these proteins following fertilization has proven challenging for several reasons. First, the amount of histone-associated chromatin in sperm is limited, ranging from 1 to 15% in mice and men respectively. Secondly, epitopes may be inaccessible prior to decondensation limiting detection. Thirdly, deposition of maternal histones, which are virtually indistinguishable from their paternally derived counterparts, directly coincides with sperm chromatin decondensation (van der Heijden *et al*. 2005, 2008). This is best exemplified by the replication-independent histone variant H3.3. Though, present in mature sperm (Gatewood *et al*. 1990), H3.3 is not microscopically detectable in paternal chromatin until maternally derived histones are deposited at the start of decondensation (van der Heijden *et al*. 2005, Torres-Padilla *et al*. 2006), the prevalence of this variant in paternal chromatin is conserved and likely essential to remodeling as mutation of the HIRA chaperone blocks H3.3 incorporation precluding decondensation in *Drosophila* zygotes (Loppin *et al*. 2005, Ooi & Henikoff 2007).

Despite the difficulty in detecting nucleosome-bound DNA delivered by sperm, some paternally derived modified histones and histone variants have been observed following fertilization. These include both H4K8ac and H4K12ac (van der Heijden *et al*. 2006) as well as the testis-specific variants H2AL1 and H2AL2. First detected in the centromeres of spermatids, these variants remain enriched in heterochromatin until displaced from paternal DNA shortly after fertilization (Wu *et al*. 2008). In contrast to histone, H3 replication-dependent variants H3.1 and H3.2 (Tagami *et al*. 2004) are detected following fertilization in decondensed sperm chromatin prior to DNA synthesis, though in much lower abundance than in maternal chromatin (van der Heijden *et al*. 2005, 2008). These sperm-derived proteins are detected until the zygotic S phase initiates, at which point they become indistinguishable from their newly incorporated maternal counterparts (van der Heijden *et al*. 2008).

As described above, many sites of histone enrichment likely have no impact on the zygote and simply reflect the transcriptional history of these silent cells. Indeed, this has been hypothesized to be the role of H3K4me2 in human sperm (Brykczynska *et al*. 2010). A comparison of the genic regions, which remain associated with nucleosomes following spermiogenesis to those RNAs retained in sperm, may help identify this population of promoters.

RNA in sperm

It is now accepted that mature spermatozoa harbor a distinct population of RNAs. The biological role of these transcripts largely remains unknown. Undoubtedly, some of the transcripts retained in sperm represent products expressed in various spermatogenic cells. The proposed functions of others include the regulation of early embryonic gene expression and stabilization of the nuclear matrix.

Owing to the observation that mature mammalian sperm are transcriptionally quiescent (Kierszenbaum & Tres 1975), the presence of mRNAs in these cells was originally thought to represent incomplete expulsion of cytoplasmic elements during nuclear condensation. Indeed, sperm do contain remnants of their developmental expression profile, which seemingly serve no purpose in the mature gamete. Furthermore, some of these RNAs are highly abundant in sperm and expected to be detrimental to the embryo (Lee *et al*. 1995). In this regard, the PRM transcripts are the most conspicuous. Following their transcription in round spermatids, these RNAs are translationally repressed and stored as inactive messenger ribonucleoprotein particles prior to remodeling (Kleene 1989, Kwon & Hecht 1993). Loss of this repression causes premature PRM translation in these cells. The subsequent developmental arrest is likely due to precocious PRM-dependent nuclear condensation. Nuclei from these cells, such as those from mature spermatozoa, are sonication resistant (Lee *et al*. 1995, Kuretake *et al*. 1996). The affinity of PRMs for DNA coupled with the enduring abundance of these transcriptionally repressed RNAs in sperm presents a potentially precarious situation to the zygote. However, failure to detect these transcripts soon after fertilization by ICSI or round spermatid injection despite the persistence of other sperm RNAs (Ziyyat & Lefevre 2001, Avendano *et al*. 2009) suggests that the zygote has evolved mechanisms and pathways to cope with this consequence of paternal genome compaction.

An evolutionarily distant precedent for such a mechanism has recently been observed in *Arabidopsis* (Bayer *et al*. 2009). Expressed during male gametogenesis, short suspensor (*SSP*) transcripts are translationally repressed and stored in pollen. Following fertilization, repression is relieved, and the *SSP* gene product undergoes zygotic translation. Sufficient accumulation of this protein in the seed activates a MAP kinase signaling cascade prompting the first cell division. In this model, embryo patterning is temporally linked to fertilization by a paternally contributed mRNA. Whether such regulation exists in other species is the subject of intense debate. It should be noted that parthenogenetic mice survive to adulthood and produce offspring in the absence of a paternal factor (Kono *et al*. 2004, Kawahara *et al*. 2007). However, efficient generation of these embryos requires the deletions of both copies of two paternally methylated imprinting control regions. Furthermore, the possibility that transgenerational affects may present must be considered.

Regardless of species, if paternally derived mRNAs are to impact embryogenesis, they must, like *SSP*, first be selectively stored in sperm. Aiding in the detection of transcripts that fulfill this prerequisite has been the development of high throughput technologies. Accordingly, the use of microarrays to screen RNA profiles from human sperm and preceding cell types provided the first evidence for the existence of a sperm-specific transcript (Ostermeier *et al*. 2002). Interestingly, in the bull, despite a high percentage (~37%) of transcripts being shared between cell types, the majority of mRNAs (59%) present in round spermatids are absent in the mature gamete (Gilbert *et al*. 2007). In addition to the selective loss of transcripts, ~120 RNAs were enriched in sperm compared to spermatids.

Comparing transcripts retained in sperm from pooled and individual human ejaculates suggested the existence of a common spermatozoal mRNA fingerprint (Ostermeier *et al*. 2002). Intriguingly, the RNA profile shared among these fertile donors included transcripts implicated in fertilization and development (Ostermeier *et al*. 2002). Some of these mRNAs are absent in human and hamster oocytes but are present in embryos (Kocabas *et al*. 2006, Avendano *et al*. 2009). Several laboratories have since independently observed these RNAs in zygotes following heterologous fertilization (Ostermeier *et al*. 2004, Avendano *et al*. 2009). These findings suggest that in a species-specific manner, some mRNAs are selectively retained in mature spermatozoa, delivered to the oocyte, and persist in the zygote.

Early investigations comparing sperm RNAs from pooled and individual fertile donors identified few, if any, differences between samples (Ostermeier *et al*. 2002). However, recent technological advances have resolved their variability (Lalancette *et al*. 2009). This may be due to the inherent heterogeneity of sperm (Lefievre *et al*. 2007, Lewis 2007), as evidenced by the normalization of transcript profiles following sperm selection (Garcia-Herrero *et al*. 2010). For example, when sperm mRNA profiles from 24 fertile individuals (Lalancette *et al*. 2009) were clustered using standard microarray comparative techniques, groups of samples clustered to differing degrees. However, a total of 453 transcripts were detected above background in all 24 samples. Of these, 30 'transcript pairs' were identified on the basis that although the signal intensity of the transcripts changed from one sample to another, this change occurs in parallel, such that the signal ratio of two transcripts in a pair was relatively stable across all 24 samples. This method of microarray analysis has since been utilized to evaluate tumor gene networks for diagnosis and prognosis, which also exhibit considerable variability between individual transcript profiles (Platts *et al*. 2010). Interestingly, transcripts known to be translationally repressed in mature spermatozoa were

detected, though none formed 'stable pairs'. Whether the paired transcripts are also translationally repressed and by what mechanism(s) remains to be elucidated. Irrespectively, the non-random enrichment of RNAs in sperm suggests that these RNAs are not solely remnants of transcription. Though some paternal transcripts may function in the early embryo, it seems unlikely that all of the selectively retained mRNAs stored by the male gamete should impact development. What other functions can be ascribed to these transcripts?

With the exception of PLC zeta (Parrington *et al*. 1999), it is not known whether the proteins corresponding to the majority of these retained transcripts are also present in mature spermatozoa and what proteins survive delivery to the oocyte. Comparing these mRNAs to the still developing sperm proteome (Baker *et al*. 2008, Oliva *et al*. 2008, Baker & Aitken 2009, Nixon *et al*. 2009) would help guide future investigations concerning the functional significance of the sperm-retained transcripts. This approach was recently used to demonstrate the selective retention of mRNAs expressed from the non-recombining region of the human Y chromosome (Yao *et al*. 2010).

Analysis of the sperm transcripts cannot be confined solely to mRNA. Acceptance of RNA in sperm was well timed with the discovery of RNAi (RNA interference) and the subsequent appreciation for the biological role of sncRNAs and their initial identification in spermatozoa (Moldenhauer *et al*. 2003). sncRNAs are approximately between 18 and 30 nucleotides in size, and classified in families according to their biogenesis (Moazed 2009). In somatic cells, these transcripts contribute to gene regulation and chromatin structure, and inhibit transposition. Two of the most studied classes of sncRNAs are the small interfering RNA (siRNA) and the miRNA families. These molecules of 20–24 nucleotides are processed from hairpins through pathways involving DICER, an endoribonuclease of the RNase III family. Data pertaining to these male germline transcripts in testis have recently been reviewed (Papaioannou & Nef 2010). However, they remain largely uncharacterized in mature sperm (C Lalancette, AE Platts, MP Diamond & SA Krawetz 2010, unpublished observations).

In addition to siRNAs and miRNAs, the testis expresses piwi-interacting RNAs (piRNAs). These transcripts of 26–30 nucleotides are produced in a DICER-independent manner that does not require double-stranded RNA folding (reviewed in Klattenhoff & Theurkauf (2008) and Ghildiyal & Zamore (2009)). Complementary to transposons, these RNAs repress the rate of transposition, thereby protecting the genome from mobile elements. Currently, the presence of these small RNAs has been demonstrated in spermatogenic cells (reviewed in Lau (2010)) where their function is essential to spermatogenesis (Deng & Lin 2002, Kuramochi-Miyagawa *et al*. 2004). Though assumed to be absent from the mature gamete, a restricted set of piRNAs may be retained in human spermatozoa (C Lalancette, AE Platts, MP Diamond & SA Krawetz 2010, unpublished observations).

The demonstration that miRNAs, and other small RNAs, are retained in the mammalian sperm nucleus and similar to mRNAs delivered to the zygote has ignited much debate (Ostermeier *et al*. 2005, Amanai *et al*. 2006, Yan *et al*. 2008, Curry *et al*. 2009). The absence of transcriptional activity in sperm has prompted the hypothesis that paternally contributed miRNAs may regulate early embryonic expression influencing offspring phenotype (Rassoulzadegan *et al*. 2006, Grandjean *et al*. 2009). However, the current pace at which novel sncRNAs can be identified by high throughput sequencing technologies far surpasses the ability to determine their biological role, if any. A detailed catalog and analysis of the sperm RNA are wanting.

Towards this end, a recent study has provided the first glimpse of the complexity of this component of the sperm transcriptome (C Lalancette, AE Platts, MP Diamond & SA Krawetz 2010, unpublished observations). Small sperm RNAs (<200 bp) purified from single ejaculates from three fertile donors were subjected to high throughput sequencing. Isolated sncRNAs comprised ~3 of the 10–20 fg of the RNA found in an individual sperm (Krawetz 2005). The average length of these transcripts was 18 bp. Sequenced reads were classified as either aligning uniquely or to multiple locations (two to ten sites) throughout the genome. Greater than half of the RNAs (58%) mapped to multiple locations in the genome. The majority (70%) of uniquely mapped reads correspond to novel sncRNAs primarily derived from intronic and intergenic regions. The miRNAs were a small percentage (3%) of the known sncRNAs in those that uniquely aligned to the genome as well as those that aligned to multiple locations.

Though miRNAs were the first class of sncRNAs observed in mammalian sperm, they account for relatively few of the sncRNAs shared between donors. However, there may only be limited opportunities for post-transcriptional regulation of early development by miRNAs. Indeed, recent reports have established that this pathway is strongly down-regulated during oocyte maturation and not required for preimplantation development (Ma *et al*. 2010, Suh *et al*. 2010). Perhaps, paternal miRNAs and other short RNA species delivered to the zygote bypass their canonical regulatory pathway altogether. In somatic cells, sncRNAs and short RNAs (~50–200 nt) bind to complimentary promoter regions silencing gene transcription through the recruitment of PcG proteins and repressive histone marks (Kim *et al*. 2008, Kanhere *et al*. 2010). The majority of miRNAs identified in sperm (C Lalancette, AE Platts, MP Diamond & SA Krawetz 2010, unpublished observations) originate from promoter regions. These transcripts may bind to paternal DNA during nuclear remodeling such that they are delivered to the oocyte in association with their targeted *cis* sequences presumably influencing their local chromatin structure.

The sperm nuclear matrix

As discussed above, appreciation that the mature spermatozoon is more than a vehicle for the delivery of inert DNA has evolved with the acceptance that distinct regions of the paternal genome remain nucleosome-bound (Gardiner-Garden *et al*. 1998, Wykes & Krawetz 2003, Arpanahi *et al*. 2009, Hammoud *et al*. 2009). Complementing this development was the discovery that sperm also deliver a suite of RNAs to the oocyte (Ostermeier *et al*. 2004). Both have contributed to expanding the post-fertilization genetic influence of the male gamete. Our understanding of how these elements coalesce to potentially influence embryonic development would not be complete without considering the RNA containing nuclear matrix (Malyavantham *et al*. 2008).

In most cells, DNA is functionally organized by a proteinaceous network termed the nuclear matrix (Cook & Brazell 1975, Ward *et al*. 1989, Choudhary *et al*. 1995, Kramer & Krawetz 1996, Heng *et al*. 2004, Linnemann & Krawetz 2009*a*, 2009*b*, Ward 2010). When isolated and viewed by electron microscopy, this ultrastructure resembles the fibrous architecture of the cytoskeleton (Comings & Okada 1976, Berezney & Coffey 1977, Fey *et al*. 1984). The list of proteins comprising the nuclear matrix is vast and to some degree cell type-dependent (reviewed in Mika & Rost (2005) and Albrethsen *et al*. (2009)). Associated with the sperm nuclear matrix are various structural proteins such as actin, myosin, and lamin B, as well as transcription factors and chromatin modifiers such as the topoisomerases (Moss *et al*. 1993, Carrey *et al*. 2002, Ocampo *et al*. 2005, Har-Vardi *et al*. 2007). Only recently, spermatozoa have, similar to somatic cells, been shown to contain a population of RNAs that associate with the nuclear matrix (reviewed in Lalancette *et al*. (2008)). Perhaps these transcripts fulfill a structural role.

The ordered positioning of chromatin within the nucleus results from attachment of discrete S/MAR sequences to this network of proteins and RNAs. Chromatin anchored to the matrix by S/MARs forms cell type-specific loop domains within interphase nuclei. Differential matrix attachment has been shown to coincide with DNA synthesis (Adom & Richard-Foy 1991, Anachkova *et al*. 2005, Courbet *et al*. 2008) and contribute to cell type-specific gene expression (Heng *et al*. 2004, Linnemann & Krawetz 2009*a*, 2009*b*). Despite the absence of replication and transcription in sperm, evidence suggests that the nuclear matrix both structurally orders and imparts function to the paternal genome.

Studies investigating the role of the sperm nuclear matrix commonly require chromatin to be relieved of PRM compaction. Treating sperm with alkali or high concentrations of buffered salts in the presence of a reducing agent such as dithiothreitol (DTT) displaces PRMs and the remaining histones. However, the strong interactions between DNA and nuclear matrix appear preserved (Ward *et al*. 1989). Once decondensed, the otherwise unconstrained DNA loops radiate out from the matrix forming a diffuse weakly staining halo around a brightly staining central region. The strong fluorescent signal corresponds to chromatin at the bases of the DNA loop domains which remain associated with the nuclear matrix (Kramer & Krawetz 1996). Similar extraction protocols are commonly used with somatic cells; though due to the absence of disulfide bonds, reducing agents are not required (Berezney & Coffey 1977, Linnemann *et al*. 2009, Drennan *et al*. 2010).

Studies of sperm nuclear halos have yielded estimates of the length of individual DNA loops (20–50 kb), which approximately correspond to the amount of DNA within an individual toroid (Ward *et al*. 1989, Hud *et al*. 1993, Barone *et al*. 1994, Nadel *et al*. 1995). This observation has prompted the notion that these discrete subunits of DNA are directly related (Ward 1993). It was proposed that during spermiogenesis, individual DNA loop domains condense to form single toroid structures (Ward 2010). Each toroid is then tethered to the nuclear matrix by adjacent nuclease-sensitive linker regions. These regions are expected to correspond to the S/MARs flanking DNA loop domains. Nuclease sensitivity would be ensured if these sequences escaped protamination. Accordingly, following sperm chromatin decondensation, these linker regions may be used to recapitulate the paternal DNA structure (Ward 2010).

Support for this model comes from the observation that spermatozoa possess endogenous nuclease activity that releases 50 kb DNA fragments (Sotolongo *et al*. 2005). Unlike the proposed nuclease-sensitive linker regions, the PRM-bound sequences would be shielded from degradation. Preferential digestion of the chromatin tethers would release the toroids, each of which contains a DNA sequence of approximately uniform length.

In addition to partitioning the sperm genome, the nuclear matrix may serve as a platform for the transgenerational inheritance of paternal chromatin structure. The proposal that matrix-associated linker regions in sperm may be recycled as embryonic S/MARs (Ward 2010) demarcating the initial embryonic replicons is broadly evidenced by the chromatin architecture of embryonic stem cells (ES cells). Unconstrained DNA loops in mammalian sperm and ES cells are reduced in size compared to those present in the liver or brain (Klaus *et al*. 2001, Ward 2010). The large widely spaced chromatin loops of differentiated mammalian cells are also observed in *Xenopus* erythrocytes. Nuclei from these cells incubated with M-phase egg extract remodel their chromatin structure to resemble the condensed narrowly space DNA loops of sperm and early embryonic cells. Once remodeled, these nuclei replicate their DNA at an efficiency and rate similar to that of the undifferentiated cells (Lemaitre *et al*. 2005). This activity is dependent on TOP2 as well as acetylated H3/4 (Adenot *et al*. 1997, Shaman *et al*. 2006). These results

suggest that the ordered positioning of chromatin domains by the sperm nuclear matrix persists in the early embryo and directs initial DNA synthesis.

Additional evidence for the inheritance of sperm DNA architecture has been garnered. Experimental disruption of the sperm nuclear matrix by treatment with detergent precludes embryogenesis following ICSI (Ward *et al*. 1999). Injection of intact sperm nuclear halos into oocytes supports the formation of male pronuclei capable of DNA replication. Similar results are achieved after restricted endonuclease digestion of extracted loop domains prior to ICSI. Maintenance of MAR sequences in conjunction with an intact nuclear matrix was sufficient to support the formation of the male pronucleus and subsequent paternal DNA replication. However, neither occurred when oocytes were injected with isolated DNA, DNase I-digested nuclear matrices, or both in parallel (Shaman *et al*. 2007). The necessity of the interaction between MARs and the nuclear matrix was confirmed by inducing TOP2-mediated cleavage presumably at toroid linker regions prior to ICSI. Loss of this association resulted in irreversible degradation of paternal DNA by as yet unidentified factors (Shaman *et al*. 2006). Several reports suggest a role for TOP2 after fertilization during sperm decondensation and pronuclear formation. However, it is not clear whether this activity in the oocyte is due to paternally or maternally derived enzyme (Bizzaro *et al*. 2000, St Pierre *et al*. 2002, Tateno & Kamiguchi 2004). Regardless, inheritance of an intact sperm nuclear matrix, regulated by TOP2, is expected to be essential to the initial stages of development as it likely orders the paternal chromatin structure.

Support for the hypothesis that the sperm nuclear matrix mediates a form of non-genetic information between parent and offspring has also been inferred from the studies of transgenerational genetic instability following germline exposure to toxins or radiation (reviewed in de Boer *et al*. (2010)). Chronic paternal exposure to low doses of cyclophosphamide (CPA) is correlated with an altered sperm nuclear matrix protein profile as well as abnormal chromatin condensation (Codrington *et al*. 2007*a*, 2007*b*). Pairing treated sires with healthy mares increased preimplantation loss as well as developmental defects. These were correlated with precocious DNA decondensation, an increase in DNA damage, perturbed gene expression, and changes in the timing of ZGA (Harrouk *et al*. 2000*a*, 2000*b*, 2000*c*, Grenier *et al*. 2010). These effects cannot be reconciled by the altered composition of the sperm nuclear matrices alone. Chronic exposure of post-meiotic spermiogenic cells to CPA results in varying types of DNA damage (Codrington *et al*. 2004). The lack of DNA repair in post-meiotic cells propagates these errors. The effects of CPA might be exacerbated by changes to higher order chromatin structure including reordered associations between S/MARs and the nuclear matrix, as these interactions are thought to be essential to early development.

Additional evidence for the sperm nuclear matrix influencing male fertility has been provided (Barone *et al*. 2000, Ankem *et al*. 2002). Infertile cryptorchidic patients presented with sperm nuclear matrix instability. Though hampered by a small sample size, this study supports the view that evaluation of sperm nuclear matrix stability could be informative in certain cases of male factor infertility. Similarly, the level of sperm DNA fragmentation may discriminate between damage to chromatin associated with the nuclear matrix, the proposed toroid linker regions, and that of the toroid DNA itself (Ward 2010). The role of DNA damage and its use in predicting male fertility have been reviewed elsewhere (Leduc *et al*. 2008*b*, Lewis *et al*. 2008, Aitken & Koppers 2011, Barratt *et al*. 2010).

Demonstrating transgenerational inheritance of paternal chromatin structure requires delineation of those DNA sequences associated with the nuclear matrix in sperm and the paternal pronucleus. Though a direct comparison is limited to model species, investigation of these interactions in human sperm is underway. Instrumental to this effort has been the increased sequence resolution afforded by newer high throughput technologies. These include the development of unique genomic array system capable of simultaneously and specifically assaying the single copy transgenic human PRM domain in addition to the endogenous locus (Johnson *et al*. 2011). Utilizing these methods, similar studies have been reported in varied somatic cell types (Linnemann & Krawetz 2009*a*, 2009*b*, Linnemann *et al*. 2007, 2009, Drennan *et al*. 2010). Preliminary analysis of the human sperm nuclear matrix from four donors has yielded intriguing results (Fig. 2A and B). Following extraction with 2 M NaCl and 10 mM DTT, in the presence of 10 mM EDTA, unconstrained DNA loops were released from isolated sperm nuclear matrices by EcoRI digestion. Matrix- and loop-associated DNA fractions were separated by centrifugation, labeled, and competitively hybridized to genomic tiling arrays. Analysis was confined to the PRM locus (Fig. 2). In agreement with previous studies, the coding regions of the domain reside within a nuclease-sensitive loop, which is anchored to the nuclear matrix by flanking S/MARs (Choudhary *et al*. 1995, Kramer & Krawetz 1996). This conformation reflects the prior expressive status of the locus which first becomes potentiated in pachytene spermatocytes (Kramer *et al*. 2000). Interestingly, the S/MARs display a degree of variance between the donors (Fig. 2B) and are comparatively distal of those previously observed (Choudhary *et al*. 1995, Kramer & Krawetz 1996). The majority of these regions show negligible sperm histone enrichment in contrast to the promoters and exons of the PRM locus. However, the large sequence block identified as the 3′ MAR in this study does appear to be strongly bound by nucleosomes,

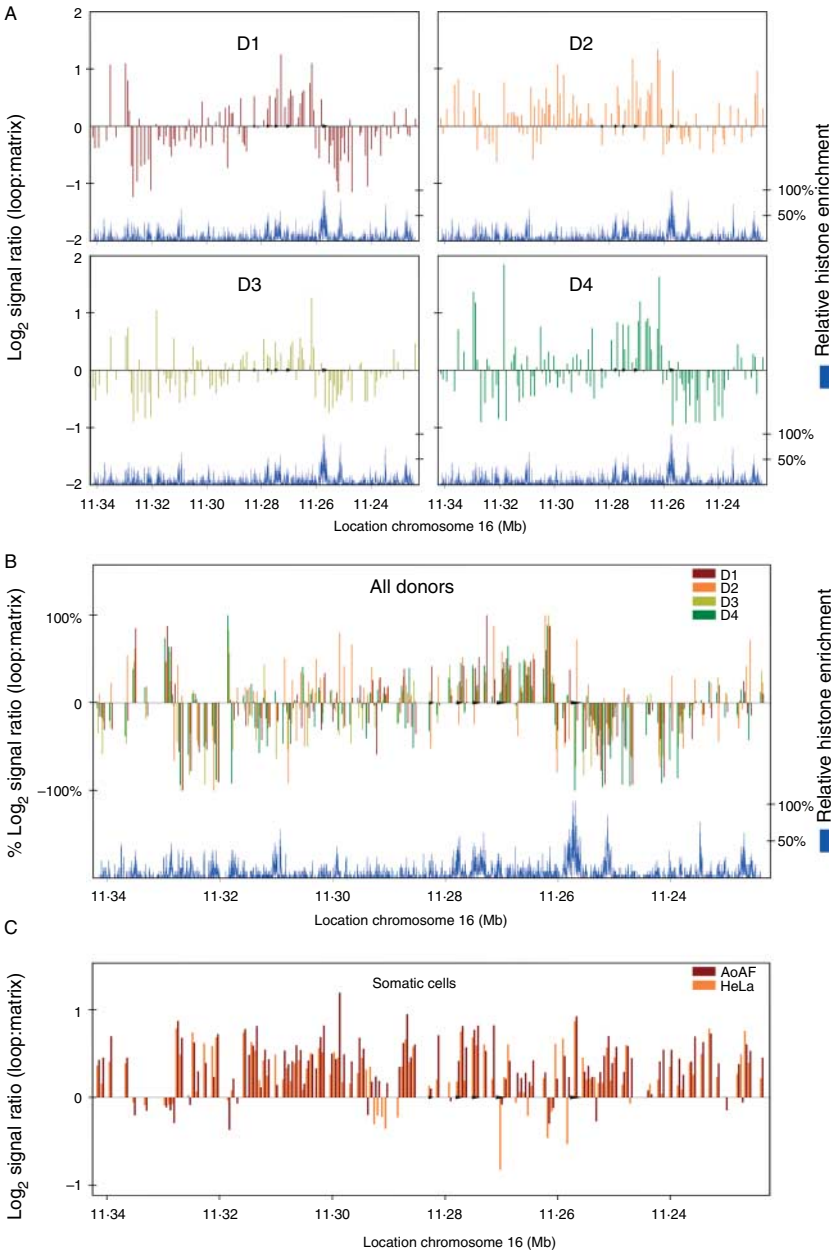

Figure 2 Nuclear matrix association within the protamine locus of sperm and somatic cells. Genomic regions in sperm associated with DNA loops or the nuclear matrix within a ~120 kb region of human chromosome 16 (chr 16: 11 223 803–11 341 499) are displayed as Log_2 values (loop/matrix). This region contains the complete protamine domain as well as the neighboring *SOCS1* gene. Genes are denoted by black arrows: *PRM1*>*PRM2*>*PRM3*>*TNP2*>*SOCS*. The relative histone enrichment across this region is illustrated in blue (GEO Series GSE15690, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE15690). (A) Nuclear matrices were extracted from sperm from four fertile donors. Following EcoRI digestion, matrix- and loop-associated DNA fractions were labeled and competitively hybridized to Nimblegen CGAR0150-WHG8 CGH arrays. Loop- or matrix-association was determined as previously described (Linnemann *et al.* 2007). (B) Composite of percent normalized values from all four fertile donors. (C) Loop- and matrix-associated DNA fractions from HeLa and AoAF cells were identified as previously described (Linnemann & Krawetz 2009*a*, 2009*b*, Linnemann *et al.* 2009).

though this is likely due to the presence of the SOCS1 promoter. This entire region shares a high degree of synteny with sequence downstream of the mouse PRM domain which functions as a MAR in spermatids (Martins & Krawetz 2007*a*). This region also contains a 3′ boundary element that is essential for full expression of the human PRM genes (Martins *et al.* 2004). Mutations in this region have been correlated with decreased PRM expression and infertility in men (Kramer *et al.* 1997). Furthermore, deletion of this element in transgenic mice harboring a copy of the human PRM locus recapitulates this perturbed PRM expression (Martins *et al.* 2004). Irrespective of the above, nuclear matrix association within this region clearly differs from that observed in somatic cells (Fig. 2, Linnemann & Krawetz 2009*a*, 2009*b*, Linnemann *et al.* 2009). Studies of higher order chromatin structure within the orthologous domains of this transgenic model will inform the degree to which this regulation is species specific.

Conclusion

The appreciation that sperm functionally package several layers of developmentally important information has become apparent. In human sperm, the genomic landscape, though dominated by PRMs, is enriched in

histones at both promoters and exons. The presence of nucleosomes in these regions, some of which contain modified histones, is highly suggestive of subsequent epigenetic control in the embryo. Furthermore, nucleosome-associated DNA may also tether individual toroid loops to the nuclear matrix. Following fertilization, these sequences partnered with the sperm nuclear matrix may provide the zygote a platform for the transgenerational inheritance of paternal chromatin structure. These potentially inherited chromatin associations may demarcate replicons utilized in early development. Perhaps some of these events are directed by factors translated from paternally derived mRNAs. This subpopulation of RNAs, like the rest of the transcripts present in sperm, is undoubtedly delivered to the oocyte. But are these transcripts functional?

The nuclear environment of the mammalian sperm continues to yield new discoveries. Many of these will be instrumental in elucidating the mechanisms controlling the early moments following conception. However, this will require the use of non-human models. Irrespectively, male fertility biomarkers may soon emerge as local chromatin structure and/or RNA signatures continue to be developed.

Declaration of interest

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

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