Chromatoid body and small RNAs in male germ cells

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Abstract

The chromatoid body (CB) is a germ granule in the cytoplasm of postmeiotic haploid round spermatids that is loaded with RNA and RNA-binding proteins. Following the discovery of small non-coding RNA-mediated gene regulation and the identification of PIWI-interacting RNAs (piRNAs) that have crucial roles in germ line development, the function of the CB has slowly begun to be revealed. Male germ cells utilise small RNAs to control the complex and specialised process of sperm production. Several microRNAs have been identified during spermatogenesis. In addition, a high number of piRNAs are present both in embryonic and postnatal male germ cells, with their expression being impressively induced in late meiotic cells and haploid round spermatids. At postmeiotic stage of germ cell differentiation, the CB accumulates piRNAs and proteins of piRNA machinery, as well as several other proteins involved in distinct RNA regulation pathways. All existing evidence suggests a role for the CB in mRNA regulation and small RNA-mediated gene control, but the mechanisms remain uncharacterised. In this review, we summarise the current knowledge of the CB and its association with small RNA pathways.

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Introduction

Male germ cell differentiation, or spermatogenesis, is a complex developmental programme that produces highly specialised spermatozoaa (Hess & Renato de Franca 2008). Spermatogonial stem cells provide a source of undifferentiated cells and enable cyclic production of sperm throughout the whole period of sexual maturity. Spermatogenesis begins with mitotic proliferation of spermatogonia. Germ cells then enter the meiotic pathway and become spermatocytes that undergo homologous chromosome pairing, synaptonemal complex formation, meiotic recombination and two subsequent meiotic divisions, which reduce the chromosome number by half. Finally, haploid germ cells begin a dramatic differentiation phase called spermiogenesis, which is divided into 16 steps in the mouse (Hess & Renato de Franca 2008). Haploid differentiation includes acrosome and flagellum formation, nuclear reshaping and chromatin compaction by replacing histones with protamines (Fig. 1). Similar to the germ line of many different organisms, mammalian male germ cells are characterised by distinct RNA- and protein-rich cytoplasmic domains that are clearly observed by electron microscopy and occasionally discernible by light microscopy (Eddy 1970). These non-membranous structures are called germinal granules, or germ line granules, or germ granules, or nuage (meaning ‘cloud’, in French) because of their amorphous cloud-like appearance. In this review, we collectively call them germ granules.

The appearance and transformation of germ granules in mammalian male germ cells has been thoroughly described by light and electron microscopy during 1970s and 1980s by several authors. Different forms of germ granules have been identified (Russell & Frank 1978, Soderstrom 1978, Chuma et al. 2009). The most prominent of them are the intermitochondrial cement (IMC) and the chromatoid body (CB; Fig. 1). IMC is found in spermatogonia and mid-to-late pachytene spermatocytes, and it appears as a substance that is observed among mitochondrial clusters completely filling the gap between mitochondria. It is particularly abundant in late pachytene spermatocytes at stages VII–XI of the seminiferous epithelial cycle. In late pachytene spermatocytes, another kind of nuage appears concurrently with the IMC. It is associated with nuclear envelope and intermingled with small vesicles, but not associated with mitochondria, and it is believed to provide precursor material for the CB. By late diplotene stage, prominent germ granule structures are disintegrated, but in secondary spermatocytes, the CB-like material aggregates again into large (0.5 μm) dense bodies. After meiosis in step 1 round spermatids, these dense bodies aggregate and form one single big (∼1 μm) granule per cell, which is commonly called the CB (Fig. 2). Mitochondria are dispersed during and after...
meiotic divisions, and IMC is not anymore detectable in haploid cells.

The CB is easily visible by phase contrast microscopy during all the stages of round spermatid differentiation (steps 1–8; Fig. 2). Due to its big size and distinct characteristics, the CB was first described already by von Brunn (1876) in early rat spermatids where it was referred as ‘Protoplasmaanhäufungen’. Benda (1891) observed similar cytoplasmic granules in the spermatocytes of guinea pig and called them ‘chromatoide Nebenko¨rper’. Regaud (1901) described the rat spermatogenesis in detail, called these cytoplasmic granules ‘corps chromatoides’ (chromatoid bodies). The name CB derives from the fact that this cytoplasmic granule is strongly stained by basic dyes similarly to chromosomes and nucleoli.

In step 7 round spermatids, the CB starts decreasing in size and its degradation proceeds in the course of spermatid elongation (Onohara et al. 2010). During steps 7–8 the CB also starts moving towards the basis of the flagellum (Fawcett et al. 1970). In elongating spermatids, the CB dissociates into two different structures: a ring around the basis of the flagellum and a dense sphere (Shang et al. 2010). This functional transformation of the CB requires testis-specific serine/threonine kinases TSSK1 and TSSK2 that specifically localise in the late form of the CB (Shang et al. 2010). The ring migrates to the caudal end of the developing middle piece of the flagellum, moving in front of the mitochondria that are engaged in mitochondrial sheath morphogenesis. The dense sphere is instead discarded with most of the cytoplasm in the residual body (Shang et al. 2010). In step 16 elongating spermatids, the characteristic structure of the CB is no longer visible.

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The germ granules have been described in different organisms. They are called germinal granules in *Xenopus laevis*, polar granules or *nuage* in *Drosophila melanogaster* and P granules in *Caenorhabditis elegans*. In these organisms, the germ granules constitute ribonucleoprotein particles containing maternal mRNA that is required for germ cell specification (Leatherman & Jongens 2003). Germ granules direct the timing of maternal mRNA translation to promote germ cell development in the
early embryo and establish the germ line for the next generation. Contrastingly, in mammals, prospective germ cells are determined among the population of pluripotent epiblast cells, depending on intercellular inductions from surrounding somatic cells (McLaren 2003, Hayashi et al. 2007). Mammalian germ granules are discernable at later stages of germ cell differentiation and therefore do not share the same function than the germ granules in lower organisms. Nevertheless they share homologues components between different species indicating similar mechanisms of action.

Given the timing of its appearance, the dynamic localisation, as well as other distinct features and the known protein and RNA composition, the CB is likely to have a specialised role in RNA regulation during postmeiotic stages of male germ cell differentiation.

In the following part of the review, we summarise the existing data of the CB composition in relation to its suggested roles in mRNA control and in small RNA-mediated gene silencing.

**The CB and mRNA regulation in spermatogenesis**

Male germ cell differentiation is characterised by temporally and spatially controlled gene expression patterns, and broad spectrum of regulatory mechanisms is to govern epigenetic, transcriptional and posttranscriptional events during spermatogenesis (Kimmins et al. 2004, Kimmins & Sassone-Corsi 2005). The late steps of spermatogenesis include a massive reorganisation of chromatin structure when histones are replaced by sperm-specific protamines to enable tight
compaction of the sperm nucleus (Fig. 1). During this time the translational control of mRNAs is prominent. The protamine-bound genes are largely silenced, thus the mRNAs for many spermatozoon proteins are synthesised already in meiotic cells or early haploid spermatids, and translationally inhibited and stored until needed in elongating spermatids. It has been demonstrated that every meiotic and postmeiotic mRNA is at least partially translationally regulated (Kleine 2003). This is reflected by high number of RNA-binding proteins in spermatogenic cells, many of them being testis-specific (Paronetto & Sette 2010). Another special feature of gene expression in round spermatids is the very high level of transcriptional activity that supports the dramatic induction of haploid gene expression. For example, TATA-binding protein, a component of the basic transcription machinery, accumulates at much higher levels in early haploid germ cells than in any somatic cell type, and TFIIB and RNA polymerase II are also overexpressed in the testis (Tanaka & Baba 2005). Therefore, there are high requirements for posttranscriptional mRNA control to secure the correct timing of protein expression and the quality of the extensive mRNA synthesis in male germ cells.

The CB has been suggested to be involved in male germ cell-specific mRNA regulation (Parvinen 2005, Kotaja & Sassone-Corsi 2007). Already in 1970s, tritiated uridine was demonstrated to accumulate in the CB as analysed by high-resolution autoradiography (Soderstrom & Parvinen 1976). It was also shown that haploid cell transcriptional activity is required for the proper morphology of the CB (Soderstrom 1977, Parvinen et al. 1978). In situ hybridisation experiments have confirmed the accumulation of poly(A)-containing RNA molecules in the CB (Kotaja et al. 2006a, Nguyen Chi et al. 2009). To support the role of the CB in mRNA regulation, a very high number of poly(A)-binding proteins (PABP) are found in this structure (Meikar et al. 2010), and many important CB components, such as MIWI, GRTH/DDX25 and HuR have been demonstrated to bind a specific set of protein-coding mRNAs (Deng & Lin 2002, Tsai-Morris et al. 2004, Nguyen Chi et al. 2009). Furthermore, the timing of the CB appearance correlates well with the high requirement of mRNA control and translational regulation in these cells (Fig. 1). These findings form the basis of the hypothesis that the CB has a role in storing and regulating the haploid mRNA transcripts whose translation is repressed until the proteins are required in elongating spermatids.

The role of microRNAs in the regulation of spermatogenesis

Small regulatory RNAs are non-coding RNAs that control gene expression at transcriptional or posttranscriptional levels and their action usually results in gene silencing. Several classes of small RNAs have been characterised, many of which are present in plant cells or in lower organisms (Ghildiyal & Zamore 2009; Table 1). The best characterised small RNAs in mammals are microRNAs (miRNAs) and PIWI-interacting RNAs (piRNAs), which are both important regulators of male germ cell differentiation, and will be discussed in more detail.

miRNAs have been identified as a large and diverse group of gene regulators that have important regulatory functions in numerous biological processes, such as developmental timing, cell death, cell proliferation, hematopoeisis and the patterning of the nervous system, and they are also connected to several cancers. miRNAs are small, about 22–23 nucleotides (nt) in length, endogenous RNAs that target gene expression by pairing with mRNAs of protein-encoding genes to direct their repression (Krol et al. 2010). Each miRNA may target the miRNAs of hundreds of distinct genes and thus it has been estimated that most of the protein-coding genes are controlled by these small RNAs (Friedman et al. 2009). miRNAs are transcribed by RNA polymerase II as hairpin-loop-folded primary miRNAs (pri-miRNAs; Lee et al. 2004). These precursors are cleaved by RNase III-related enzymes, first in the nucleus by DROSHA and then after the export of precursor-miRNAs (pre-miRNAs) to the cytoplasm by DICER (Krol et al. 2010; Fig. 3). Mature miRNAs then recognise their target mRNAs by sequence-specific pairing in the RNA-induced silencing complex together with Argonaute proteins (AGO). Initially the main mechanism of mammalian miRNA function was thought to be the repression of protein output by posttranscriptional translational regulation with little or no influence on the mRNA levels. However, a recent report demonstrates that the destabilisation of target mRNAs is the predominant function for mammalian miRNAs (Guo et al. 2010).

miRNA-dependent gene control is widely used in different tissues and cellular processes, and many miRNAs are expressed in a highly tissue-specific manner. miRNA pathways are also active in testis. The mRNA transcripts for all the AGO proteins (AGO1–AGO4) as well as DROSHA and DICER have been demonstrated to be present in germ cells and Sertoli cells (Gonzalez-Gonzalez et al. 2008). Detailed description of the expression and localisation of miRNA pathway components at the protein level during spermatogenesis is still missing. miRNAs and some miRNA pathway proteins have been demonstrated to accumulate in the CBs in haploid round spermatids, suggesting that the CB has a role in miRNA-dependent gene regulation (Kotaja et al. 2006a). Since these pathways localise also outside the CB, the functional importance of these findings is still unclear. Several miRNAs are expressed either specifically or predominantly in testis, indicating a crucial role of miRNAs in the control of the precisely timed and highly organised

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<table>
<thead>
<tr>
<th>miRNAs</th>
<th>Plant (Arabidopsis thaliana)</th>
<th>Pre-pachytene (mammals)</th>
<th>Pachytene (mammals)</th>
<th>Drosophila</th>
<th>Zebrafish</th>
</tr>
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<tbody>
<tr>
<td>Average length (nt)</td>
<td>21–23</td>
<td>20–24</td>
<td>26–28</td>
<td>28–33</td>
<td>22–30</td>
</tr>
<tr>
<td>Genomic origin/target</td>
<td>Protein-coding genes</td>
<td>Protein-coding genes</td>
<td>Mostly repetitive elements</td>
<td>Large clusters across the genome, non-repetitive, non-annotated</td>
<td>Pericentromeric or telomeric heterochromatin, mostly repetitive elements</td>
</tr>
<tr>
<td>3’ Met</td>
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<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>AGO proteins involved</td>
<td>AGO1–4</td>
<td>AGO1</td>
<td>MIWI2, MILI</td>
<td>MIWI, MILI</td>
<td>PIWI, AUB, AGO3</td>
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<tr>
<td>Expression</td>
<td>Ubiquitous</td>
<td>Ubiquitous</td>
<td>Male germ line: proSg, Sg, early Spc</td>
<td>Male germ line: late Spc, RS</td>
<td>Female and male germ line, somatic cells</td>
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<tr>
<td>Precursors</td>
<td>Partially homologous hairpins</td>
<td>Partially homologous hairpins</td>
<td>ssRNA?</td>
<td>Long ssRNA transcripts?</td>
<td>ssRNA?</td>
</tr>
<tr>
<td>Biogenesis</td>
<td>DROSHA and DICER-dependent dsRNA cleavage</td>
<td>dsRNA cleavage by DCL1</td>
<td>Ping-pong mechanism\textsuperscript{a}</td>
<td>Putative primary processing pathway</td>
<td>Ping-pong mechanism\textsuperscript{a}</td>
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<tr>
<td>Functions</td>
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<td>mRNA regulation</td>
<td>Transposon mRNA silencing, transcriptional silencing of transposon genes</td>
<td>Unknown</td>
<td>Transposon mRNA silencing</td>
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</tbody>
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Animal and plant miRNAs are similar in the respect of their wide expression, size and similar silencing machinery but differ in the level of complementarity to their targets and the 3’-terminal 2’ O-methylation. All piRNAs share the 3’-terminal methyl group and they bind to the PIWI proteins. Mammalian pre-pachytene piRNAs resemble Drosophila and zebrafish piRNAs in their targets and biogenesis mechanism. The pachytene piRNAs stand out as a separate subclass of piRNAs, having a different function and biogenesis mechanism, which are so far not understood. ProSg, prospermatogonia; Sg, spermatogonia; Spc, spermatocytes; RS, round spermatid.

\textsuperscript{a}For initiation, the ping-pong mechanism requires primary processing pathway.
process of spermatogenesis (Ro et al. 2007, Chiang et al. 2010). Meiotic spermatocytes and haploid spermatids seem to express the highest number of miRNAs during male germ cell differentiation (Ro et al. 2007). Interestingly, many of the testis-expressed miRNA genes are located on the X chromosome where they are found in X-linked miRNA gene clusters (Song et al. 2009). X chromosomal genes are usually transcriptionally silenced in mid-to-late pachytene spermatocytes by a process called meiotic sex chromosome inactivation (MSCI). MSCI takes place in a specialised nuclear territory known as the XY body, or sex body, by heterochromatin formation and transcriptional repression of sex chromatin. However, many X-linked miRNAs escape the inactivation and are thus likely to have important roles during this stage of differentiation (Song et al. 2009).

A large number of miRNA-regulated genes have been identified in somatic cells but not extensively characterised in germ cells. Some examples of miRNA-mediated regulation of gene expression in spermatogenesis exist, such as the control of heat shock factor 2, a transcription factor that is required for normal spermatogenesis in mouse, by miR-18 (Bjork et al. 2010) and the involvement of miR-17-92 cluster in the regulation of E2F-1 transcription factor during normal spermatogenesis and in carcinoma in situ (Novotny et al. 2007). The impact of DICER-dependent small RNA pathways in male fertility has begun to be revealed by studies on different genetically modified mouse lines. Sertoli cell-specific deletion of Dicer in mouse demonstrated that DICER is crucial for the normal function of these somatic nursing cells of the seminiferous epithelium (Papaioannou et al. 2009, 2011). The ablation of DICER...
in Sertoli cells resulted in spermatogenic malfunction and infertility. Mutant Sertoli cells showed defective maturation and were not able to support germ cell differentiation (Papaioannou et al. 2009). Proteomic analysis revealed major alterations in protein expression in the mutant testis (Papaioannou et al. 2011). The importance of intrinsic miRNA pathways for male germ cells has been studied by a mouse line with specific deletion of Dicer in primordial germ cells (PGCs) during embryonic development (Hayashi et al. 2008, Maatouk et al. 2008). These papers demonstrated that the presence of functional DICER in male germ line is essential for normal fertility. Mutant mice showed defects in PGC proliferation and problems in spermatogenesis (Maatouk et al. 2008). However, due to the early deletion of Dicer in PGCs, it is not yet clear whether the spermatogenic problems arise because of defective functions of precursor cells or because DICER is needed in postnatal differentiation. This is why the role of DICER in adult spermatogenesis should be assessed in different mouse lines that undergo Dicer deletion in postnatal germ cells.

In addition to the posttranscriptional control, DICER-dependent RNA silencing may also function at transcriptional level (Moazed 2009). The connection between RNA silencing and the formation and maintenance of heterochromatin is well established in Schizosaccharomyces pombe and plants. Evidence of small RNA-mediated transcriptional gene silencing in animals is also emerging even though the mechanistic aspects still remain unclear (Moazed 2009). Interestingly, mammalian DICER has been implicated in silencing of centromeric repeat sequences in mouse embryonic stem cells (Kanellopoulou et al. 2005, Murchison et al. 2005). However, whether this is mediated by epigenetic chromatin modifications or by other mechanisms is uncertain. The localisation of mouse DICER on pericentromeric heterochromatin during mammalian meiosis indicates that DICER-dependent RNA silencing may also have a role in heterochromatin silencing in male germ line (Khalil & Driscoll 2010). Due to the increasing complexity of small RNA pathways and their functional diversity, it is clear that a lot of further studies are required to understand the exact roles of miRNAs and other DICER-dependent RNA pathways in male germ cell biology.

Discovery and properties of piRNAs

piRNAs were isolated from adult mouse testis independently by several groups in 2006 (Aravin et al. 2006, Girard et al. 2006, Grivna et al. 2006, Watanabe et al. 2006). piRNAs comprise the biggest and most complex class of small non-coding RNAs, and they are defined by their association with PIWI subclade members of AGO (Table 1). Argonautes, which are well-known key proteins in small RNA-mediated silencing complexes, are divided into AGO and PIWI subclades (Faehnle & Joshua-Tor 2007). AGOs are involved in RNA interference and in miRNA-mediated gene silencing and are ubiquitously expressed, while PIWIs are directly associated with piRNAs. In mammals, PIWI proteins and piRNAs seem to be specific for male germ line, but in Drosophila, piRNA machinery is present also in gonadal somatic cells in addition to male and female germ line (Thomson & Lin 2009). Each one of the three mouse PIWI proteins MIWI/PIWIL1, MIWI/PIWIL2 and MIWI2/PIWIL4 binds a specific subset of piRNAs and they have different expression patterns (Thomson & Lin 2009; Table 1 and Fig. 1). MIWI expression starts in PGCs and lasts throughout the spermatogenesis until the round spermatid stage at around 20 days post partum (dpp). MIWI2 can be detected in a narrow time-window from 15 days post coitum (dpc) to three dpp corresponding to the time when cell cycle arrests and de novo methylation takes place. MIWI is expressed later during spermatogenesis, from the pachytene stage in meiosis through the haploid round spermatid phase. Each PIWI protein is essential for spermatogenesis in mouse. In Mili- and Miwi2-knockout mice, spermatogenesis stops with meiotic arrest (Kuramochi-Miyagawa et al. 2004, Carmell et al. 2007). Germ cells in Miwi-null testis undergo meiosis but haploid round spermatids fail to differentiate (Deng & Lin 2002).

Compared with miRNAs that are around 22 nt long, piRNAs are longer, falling mostly in the range of 26–32 nucleotides (Table 1). The biosynthesis of piRNAs is different from that of miRNAs and siRNAs (Aravin et al. 2007a, Thomson & Lin 2009). piRNAs do not derive from self-folding long transcripts and their production is DROSHA and DICER independent. Based on the genomic organisation, piRNAs are believed to derive from long single-stranded transcripts. Two possible pathways are suggested for piRNA biogenesis: the primary processing pathway and the ping-pong cycle, which will be discussed below (Fig. 3). Like other small regulatory RNAs, piRNAs harbour a 5' monophosphate and 3' hydroxyl group, and in general, they share the preference for 5'U. piRNAs in different organisms have also been shown to be 2'-O-methylated at their 3' terminus (Horwich et al. 2007, Kirino & Mourelatos 2007, Ohara et al. 2007, Saito et al. 2007). In mouse, piRNAs are 3'end methylated by mHen1, a methyltransferase that is specifically expressed in testis (Kirino & Mourelatos 2007b). piRNAs have been identified widely within the animal kingdom in the human, mouse, rat, fish, fly, worm and even in the sea anemone (Nematostella vectensis) and in one of the simplest animals, a sponge (Amphimedon queenslandica) but not in plants or fungi (Crimson et al. 2008). Interestingly, PIWI proteins and piRNA-like small RNAs bearing the 3'-terminal 2'-O-methyl group have also been described phylogenetically as far as in Tetramerina thermophila, a ciliated protozoan (Kurth & Mochizuki 2009). All this refers to the ancient origin of PIWI-piRNA machinery.
There are two distinct subsets of mammalian piRNAs that share molecular characteristics but differ in their sequence characteristics, genomic origin, PIWI binding partners, expression time and suggested biosynthesis and functions (Table 1 and Fig. 3). Pre-pachytene piRNAs bind to MILI and MIWI2 and participate in silencing of transposable elements both at epigenetic and posttranscriptional level in foetal and neonatal germ cells (Aravin et al. 2007b, 2008, Carmell et al. 2007, Kuramochi-Miyagawa et al. 2008). The piRNA populations in foetal and neonatal testis are not completely overlapping, and the composition of pre-pachytene piRNAs changes throughout the development (Aravin et al. 2008). However, due to the consistencies in their properties and reported functions, they are discussed as one group in this review. A second subset of piRNAs, the pachytene piRNAs, are associated with MILI and MIWI and are expressed later in spermatogenesis in exceptionally large amounts, but their function has remained elusive (Aravin et al. 2006, 2007b, Girard et al. 2006, Grivna et al. 2006). It is important to note that a great deal of discoveries about piRNA functions and biogenesis has been made in fly and zebrafish, which possess only one class of piRNAs that is the equivalent to mammalian pre-pachytene piRNAs corresponding only to a fraction of the whole mammalian piRNA population.

Pre-pachytene piRNAs

Mammalian pre-pachytene piRNAs resemble Drosophila piRNAs – the repeat-associated small interfering RNAs that originate from repeat sequences related to transposable elements and heterochromatic regions. The link between the pre-pachytene piRNAs and the silencing of transposable element expression has been demonstrated convincingly (Aravin et al. 2007b, 2008, Carmell et al. 2007, Kuramochi-Miyagawa et al. 2008). In MilI- and Miwi2-knockout mice, intracisternal A particle and long interspersed nuclear element 1 transposable elements are aberrantly expressed in both foetal and neonatal testis (Aravin et al. 2007b, Carmell et al. 2007, Kuramochi-Miyagawa et al. 2008). In mammals, the repression of transposon genes requires CpG DNA methylation (Bourc’his & Bestor 2004). In foetal non-proliferating spermatogonia, there is a dramatic resetting of DNA methylation during which novel gametic epigenetic patterns are generated by de novo DNA methylation. At the time of this epigenetic remodelling, most of the early piRNAs derive from areas in the genome that represent individual transposons, and act via MIWI2–MILI pathway to silence these mobile elements posttranscriptionally by the so-called ping-pong amplification cycle (see below) (Aravin et al. 2008, Kuramochi-Miyagawa et al. 2008). Both Miwi2 and MilI mutant mice show defects in the methylation of transposon genes, and their testicular phenotypes resemble remarkably that of the mutant mouse deficient in DNMT3L, a member of the DNA methyltransferase family that is responsible for de novo methylation of transposable elements (Bourc’his & Bestor 2004, Aravin et al. 2007b, 2008, Kuramochi-Miyagawa et al. 2008). Therefore, it has been suggested that in addition to posttranscriptional control of transposon silencing, piRNAs lie upstream of known DNA methylation mediators and serve as sequence-specific guides that direct the de novo DNA methylation machinery to transposable elements (Aravin et al. 2008). MIWI2 seems to be responsible for the nuclear functions of the pathway since it mainly localises in the nucleus of prospermatogonia in contrast to MILI, which is a cytoplasmic protein.

Transposable elements are shown to be transiently derepressed also during postnatal spermatogenesis at the onset of meiosis (Branciforte & Martin 1994, Soper et al. 2008). MILI is the only PIWI protein that is expressed at this stage of germ cell differentiation. A big proportion of MILI-binding piRNAs isolated from ten dpp testis match with active transposons, indicating that these early meiotic piRNAs share similar functions with foetal piRNAs in transposon silencing (Aravin et al. 2007b). One protein suggested to be involved in the control of the meiotic transposon expression, probably via the PIWI–piRNA pathway, is Maelstrom (MAEL), and Mael-knockout testis show elevated and temporally aberrant transposon expression during meiotic processes (Soper et al. 2008).

More detailed analysis of the MILI- and MIWI2-bound pre-pachytene piRNA sequences suggests that they participate in the so-called ping-pong amplification cycle that utilises the piRNA machinery to silence mRNAs of active transposons (Aravin et al. 2008; Fig. 3). The ping-pong cycle starts when a pool of primary piRNAs is generated by the primary processing pathway by as yet uncharacterised mechanisms. The produced primary piRNAs bind to MILI and direct the MILI-mediated cleavage of the precursor RNA at the position of ten nucleotides from the 5’ end of the original piRNA. This first cutting site becomes the 5’ end of the secondary piRNAs. The second cleavage at their 3’ end is required, but this reaction still needs characterisation. The newly cut secondary piRNAs bind the MIWI2 complexes and enter into the MILI–MIWI2 feed-forward loop, which generates more piRNAs and enhances the silencing. Primary piRNAs share a preference for 5’uridine (1U), thus the respective secondary piRNAs have a preference for adenine in their tenth position (10A). These are used as footmarks of the possible ping-pong cycle when defining pre-pachytene piRNAs from the whole piRNA population (Aravin et al. 2008). It has to be kept in mind that even though the pre-pachytene piRNA pathway has elegantly been demonstrated to act on transposon silencing, sequence analyses have revealed that not all the pre-pachytene piRNAs match with transposon sequences, and the functions of this remaining piRNA population are still unclear.
Pachytene piRNAs

Vast majority of mammalian piRNAs are pachytene piRNAs, which are produced later in spermatogenesis in spermatocytes and round spermatids. Compared with the pre-pachytene piRNAs, they are different in many aspects (Table 1). In the mouse, the expression of pachytene piRNAs starts around day 14 pp as revealed by the emergence of the characteristic ~30-nucleotide piRNA band in total testis RNA extract (Aravin et al. 2006, Girard et al. 2006, Grivna et al. 2006). This is the time when pachytene spermatocytes appear, giving name to the late piRNAs. The pachytene piRNA expression peaks in postmeiotic round spermatids and disappears during spermiogenesis. The prominent piRNA band is not present in the total RNA extract of epididymis (Girard et al. 2006, Grivna et al. 2006). It is estimated that there are about 1 million piRNA molecules per mouse spermatocyte or round spermatid (Aravin et al. 2006). While being expressed in large quantities, the individual copy number of each piRNA is probably low, because many of the piRNAs revealed by deep sequencing were found only as single hits. The total number of distinct mammalian piRNAs has been estimated to be in hundreds of thousands (Aravin et al. 2007b).

Similarly to pre-pachytene piRNAs, the pachytene piRNAs share the preference to 5′U, but they do not have clear signs of the ping-pong cycle footprint. These two piRNA subsets are derived from different genomic locations. Pachytene piRNAs originate mostly from non-annotated regions of the genome and map into large clusters from tens to hundreds of kilobases along the genome with most of the clusters being derived from one of the two genomic strands (Aravin et al. 2007a, 2007b). There is very little conservation of individual piRNA sequences between different mammals, but surprisingly there is a significant conservation of the genomic locations of mammalian piRNA clusters (Betel et al. 2007). This might refer that the pachytene piRNAs act as a population, representing areas of their specific locations on chromosomes. Pachytene piRNAs are devoid of sequences relative to active transposons. Furthermore, in Miwi mutant mice, no activation of transposons has been detected (Aravin et al. 2007a). Altogether this indicates a different biosynthesis mechanism and functions of pachytene piRNAs.

Taking into account the onset of MIWI and pachytene piRNA expression in late pachytene spermatocytes, it is tempting to speculate that they are involved in the processes controlling the late steps of meiosis. piRNAs are expressed at very high level still in postmeiotic round spermatids, and furthermore, in Miwi-null testes that are devoid of pachytene piRNAs, a lot of round spermatids are generated demonstrating that meiosis can be completed without MIWI and MIWI-binding pachytene piRNAs (Deng & Lin 2002, Grivna et al. 2006). The main problem in the seminiferous epithelium of the Miwi mutant mice seems to be the haploid differentiation, since spermatogenesis in Miwi mutant mice is blocked at the round spermatid stage with a failure to start the elongation of spermatids (Deng & Lin 2002). The exact functions of MIWI and pachytene piRNAs, whether targeting late meiosis or differentiation of haploid cells or both, remain to be characterised. There are many basic questions on pachytene piRNAs that need to be answered before we can draw conclusions about their functions: how are they produced? How dynamic is the expression of pachytene piRNAs – is the population changing during the development from pachytene spermatocytes to round spermatids, and during the course of round spermatid differentiation? Do they have nuclear or cytoplasmic functions? Do all late piRNAs bind to MIWI and MILI and what are the other players involved?

The CB as a scene for the piRNA pathway

We have developed a protocol to isolate CBs from mouse testis and we have shown that CBs accumulate high concentrations of piRNAs (Meikar et al. 2010). Mass spectrometric analysis revealed six main CB components that are MVH/DDX4, MIWI/PIWIL1, Tudor domain-containing protein 6 (TDRD6), TDRD7, gonadotropin regulated testicular RNA helicase (GRTH/DDX25) and PABPC3 (PABP, cytoplasmic 3). These proteins comprise over 90% of the total CB content by mass. Four of these proteins, MIWI, DDX4, TDRD6 and TDRD7 have been implicated in piRNA-mediated RNA regulation (Arkov & Ramos 2010, Kuramoto-Miyagawa et al. 2010). In addition, MILI and TDRD1 have been localised to the CB by immunostaining experiments (Chuma et al. 2006, Vagin et al. 2009). The CB contains also Maelstrom, a protein that is functionally linked to the piRNA pathway (Soper et al. 2008). Spermatogenesis in Mili−/−, Ddx4−/− and Mael-knockout mice is blocked at meiotic prophase, demonstrating that they have important meiotic functions already before the appearance of the CB (Tanaka et al. 2000, Kuramoto-Miyagawa et al. 2004, Soper et al. 2008). The nature of the phenotype makes it impossible to analyse the status of the CB in these mice. Instead, round spermatids with clearly CB-deficient morphology are detected in mice lacking MIWI, GRTH/DDX25, TDRD6 or TDRD1. This reveals a connection between the function of these proteins and their localisation in the CB (Tsai-Morris et al. 2004, Chuma et al. 2006, Kotaja et al. 2006b, Vasiljeva et al. 2009). On the basis of all these findings, it is logical to assume that the CB functions include piRNA-dependent actions.

It appears that the organisation of RNA control pathways in germ granules is required in a wide range of different organisms. Despite the fact that the CB in mouse and the germ granules in lower organisms (fly, nematodes and frogs) apparently support different
biological functions, they still share many protein components. These include the DEAD-box RNA helicase Vasa (DDX4 or MVH in mouse), Tudor domain-containing proteins as well as PIWI proteins (Arkov & Ramos 2010). In Drosophila, the connection of small RNA-mediated gene silencing and germ granules (nuage) in the ovarian germ line has been nicely described. Nuage has several well-characterised components, such as Vasa, Aubergine, Spindle-E, Maelstrom and Kripper and many of them have also been indicated in RNA silencing (Khurana & Theurkauf 2010). Mutation of RNA silencing proteins disrupts nuage, and vice versa, the mutation of nuage components disrupts small RNA-mediated repression of selfish genetic elements (Lim & Kai 2007, Khurana & Theurkauf 2010). A member of the heterochromatin protein 1 subfamily of chromo box proteins, Rhino (Rhi), is required for the production of piRNAs from dual-strand heterochromatic clusters and for piRNA-mediated transposon silencing in fly (Klattenhoff et al. 2009). Interestingly, mutation of Rhi disrupts localisation of Vasa and PIWI proteins in the perinuclear germ granule that is normally enriched in piRNA pathway components. It was also demonstrated that if piRNA homology sequences are artificially introduced to introns of protein-coding genes, they escape silencing by the piRNA pathway. This suggests that the recognition of transcripts with piRNAs takes place after splicing (Klattenhoff et al. 2009). These observations led to the speculation that nuage functions as a perinuclear surveillance machine that scans RNAs after splicing and nuclear export and destroys transcripts with piRNA complementarity.

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PIWI proteins specifically interact with TDRD proteins through symmetrically dimethylated arginines in the
processing bodies (P-bodies, PBs or GW/P-bodies) that in mammalian cultured cells are known to be involved in the posttranscriptional control of mRNAs through mRNA degradation, transport, stabilisation and translational repression (Kulkarni et al. 2010).

Since foetal prospermatogonia in mouse (Aravin et al. 2009, Shoji et al. 2009), but also germ line cells in C. elegans (Gallo et al. 2008) and Drosophila (Lim et al. 2009) seem to contain processing body-like granules in addition to the ‘traditional’ germ granules, it is possible that the interplay between different cytoplasmic granules is of general importance for many RNA regulation pathways. Interestingly, pachytene spermatids and round spermatids contain a distinct granule in the cytoplasm that is easily distinguishable from the CB under electron microscopy. This satellite body (SB), or chromatoid satellite, is often found to be closely associated with the CB with frequently observed connections between these two types of granules by bridges of dense material (Onohara et al. 2010; Fig. 4). A Tudor domain-containing protein RNF17 is not localised to the CB but specifically occupies the granules in spermatocytes and elongating spermatids that resemble the SB (Pan et al. 2005). Mouse Maelstrom protein has been reported to localise to both the CB and a second nuage in round spermatids that possibly corresponds to the SB (Soper et al. 2008). On the contrary, most of the CB components do not localise to the SB. It remains to be revealed what is the molecular composition of the SB and whether there is functional interplay between the CB and the SB in postmeiotic spermatids.

The close association of IMC-type germ granules in prospermatogonia and spermatocytes with mitochondria is prominent, but the functional importance of this connection has been unclear. Mitochondrial ribosome-dependent translation has been demonstrated to be necessary for Drosophila germ line formation (Amikura et al. 2005), and mitochondrial ribosomes are also involved in the translation of nuclear encoded mammalian sperm proteins (Gur & Breibart 2006). Taking into account the likely function of germ granules in RNA regulation, it will be interesting to find out if the interplay with mitochondria has a role in the germ granule-mediated control of gene expression. Interestingly, two recent papers demonstrated that mitochondrial membrane protein MitoPLD is required for piRNA pathways and the correct localisation of mitochondria and piRNA pathway components in distinct cytoplasmic germ granules in mouse male germ cells (Huang et al. 2011, Watanabe et al. 2011). It was suggested that mitochondrial-surface lipid phosphatic acid generated by MitoPLD recruits or activates germ granule components that are critical for piRNA production (Huang et al. 2011). The association of germ granules with mitochondrial clusters is lost in haploid spermatids, indicating that the CB does not have similar functional connection with mitochondria.

The CB as a dynamic regulator of cellular processes

The CB is not a static structure, but its shape, location and composition are dynamically changing (Fig. 5). We have analysed the full proteome of the CB and identified more than 100 proteins in addition to the six main ones discussed above (Meikar et al. 2010; data not shown). Many of these proteins are believed to localise in the CB transiently instead of being stable components. Some reports of transient CB proteins exist, for example the RNA-binding protein HuR accumulates with its target mRNAs in the CB only in early round spermatids (Nguyen Chi et al. 2009). Future studies will give more insights to the changes in the CB composition during differentiation. The area of the nuclear envelope that is associated with the perinuclear CB is rich in nuclear pore

The CB is the piRNA machinery including MIWI, MILI, DDX4, TDRD1,6,7 and MAEL, and it is likely that the CB has a role in piRNA-mediated RNA regulation. The CB could be also involved in sharing material between neighbouring cells through intercytoplasmic bridges, and it has been hypothesised that the CB is involved in sharing material between haploid cells (3). There is also a link between the CB and mRNA translation (4), since many of the RNA-binding proteins in the CB have been demonstrated to associate with translational machinery. The known CB components include several RNA-binding proteins, RNA helicases and other protein involved in different RNA regulation pathways. According to mass spectrometric analysis, the six most abundant proteins in the CB are DDXY, MIWI, GRTH/DDX25, TDRD6, TDRD7 and PABPs. mRNAs, miRNAs and piRNAs are also concentrated in the CB. The most prominent functional pathway localised in the CB is the piRNA machinery including MIWI, MIIL, DDX4, TDRD1, TDRD6, TDRD7 and MAEL, and it is likely that the CB has a role in piRNA-mediated RNA regulation. The CB could be also involved in scanning the transcripts exported from the nucleus to direct them to the correct pathways and destinations. KIF17 is a microtubule-associated kinesin motor protein that is potentially involved in the regulation of CB movement and/or dynamics of the CB composition.

Figure 5 Summary of the CB and its interactions with other cellular compartments. The CB in the cytoplasm of round spermatids moves both along the nuclear envelope and perpendicularly to the nuclear envelope and makes frequent contacts with the nucleus. Nuclear pore complexes are concentrated on the areas where the CB is found, and the CB appears to receive material directly from the nucleus (1). The CB is associated with vesicles and membrane-bound organelles, and it stays in close contact with the Golgi complex (2). The CB and small dense granules dissociated from the CB can move between neighbouring cells through intercytoplasmic bridges and it has been hypothesised that the CB is involved in sharing material between haploid cells (3). There is also a link between the CB and mRNA translation (4), since many of the RNA-binding proteins in the CB have been demonstrated to associate with translational machinery. The known CB components include several RNA-binding proteins, RNA helicases and other protein involved in different RNA regulation pathways. According to mass spectrometric analysis, the six most abundant proteins in the CB are DDXY, MIWI, GRTH/DDX25, TDRD6, TDRD7 and PABPs. mRNAs, miRNAs and piRNAs are also concentrated in the CB. The most prominent functional pathway localised in the CB is the piRNA machinery including MIWI, MIIL, DDX4, TDRD1, TDRD6, TDRD7 and MAEL, and it is likely that the CB has a role in piRNA-mediated RNA regulation. The CB could be also involved in scanning the transcripts exported from the nucleus to direct them to the correct pathways and destinations. KIF17 is a microtubule-associated kinesin motor protein that is potentially involved in the regulation of CB movement and/or dynamics of the CB composition.
complexes, and material continuities between the nuclear pores and the CB have been demonstrated (Parvinen 2005). Therefore, it appears that the CB can actively collect RNA and proteins directly from the nucleus. On the other hand, the CB is also associated with cytoplasmic multivesicular bodies and several small vesicles resembling lysosomes and containing lyosomal markers (Ventela et al. 2003, Haraguchi et al. 2005, Yokota 2008). This indicates that the CB can be involved in the lysosomal degradation pathway of material originating from the nucleus, probably via autophagocytosis of CB material and subsequent processing or transport to other destinations. The CB has also been shown to have frequent transient contacts with the Golgi complex (Parvinen 2005).

One intriguing but less studied feature of the CB is its active movements inside the cytoplasm (Parvinen 2005). Immediately after the second meiotic division in rat, the Golgi complex and the CB are dispersed in the cytoplasm of young round spermatids. During step 1 of spermatid differentiation, when the small dense precursor bodies aggregate into the typical CB structure, the CB moves along the nuclear envelope on a wide area surrounding the Golgi complex. These kinds of movements are also observed during step 2. In step 3 round spermatids, the CB shows a different pattern of movement, this time perpendicular to the nuclear envelope. During step 4 the movements of the CB slow down and practically cease at step 5. The CB movement is dependent on intact microtubule network (Ventela et al. 2003). Interestingly, a microtubule-associated motor protein KIF17b has been demonstrated to interact with MIWI in the CB, thus providing a possible mechanistic explanation for the CB dynamics (Kotaja et al. 2006b).

A characteristic feature of spermatogenesis is that the dividing meiotic cells do not complete cell division and stable cytoplasmic bridges are formed that interconnect newly generated haploid cells. The function of cytoplasmic bridges is to facilitate the sharing of cytoplasmic constituents and to allow haploid germ cell differentiation to be directed by the products of both parental chromosomes making the syncytium functionally diploid. The CB and smaller granules dissociating from the CB are able to move from one spermatid to the other passing through the cytoplasm bridge connecting the two cells (Ventela et al. 2003). Therefore, the CB is not only moving dynamically inside a cell, but it could also provide an intercellular trafficking system to share material between connected round spermatids.

Future perspectives

The CB is packed with RNA, including mRNAs, piRNAs and miRNAs, and most of the characterised CB proteins are RNA-binding proteins or other proteins involved in RNA processing pathways. There is no doubt about the involvement of the CB in RNA regulation. However, the exact mechanisms and processes acting in the CB are yet to be characterised. Proteomics of the CB has revealed that, in addition to piRNA pathway, a great number of other proteins involved in distinct RNA regulation pathways are found in the CB (data not shown). Therefore, the CB could actually be involved in organising the RNA regulation in much more diverse way than it was first anticipated. Because of its biochemical composition, non-random movements, close association with the nucleus and its possible involvement in sharing material between neighbouring spermatids, our working hypothesis is that the CB provides a dynamic platform that scans RNA directly from the nucleus interconnecting specific pathways and coordinating RNA regulation processes in an efficient and accurate manner (Fig. 5). The presence of germ granules with conserved protein components in earlier stages of mammalian spermatogenesis as well as in the germ line of non-mammalian organisms suggest that these granules share similar mechanisms for their actions. There has been evident progress in the functional characterisation of germ granules during the last few years. Further elegant studies and provocative approaches are required to reveal the peculiarities of germ granule-associated processes and their functional importance in the control of germ cell differentiation and fertility.

Declaration of interest

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

Funding

This work was supported by Academy of Finland, Emil Aaltonen Foundation and Turku Graduate School of Biomedical Sciences.

Acknowledgements

We thank Prof. emeritus Martti Parvinen for all his insights and critical reading of the manuscript.

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Received 28 February 2011
First decision 12 April 2011
Accepted 6 June 2011