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microRNAs in the Testis: Building Up Male Fertility

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Running title: Role of microRNAs in spermatogenesis

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ABSTRACT

Spermatogenesis is a strictly regulated process, both at the transcriptional and the post-transcriptional level, which allows continuous gamete production throughout adulthood. A novel mechanism of post-transcriptional control mediated by microRNAs (miRNAs) has lately emerged as an important regulator of spermatogenesis. miRNAs are endogenous, small, non-coding RNAs produced through a multi-step enzymatic process, which involves the action of Dicer, an RNaseIII endonuclease. Here, we first present a short overview of classic post-transcriptional control during spermatogenesis, and then concentrate on recent findings that have unraveled the important role of miRNAs in male reproductive function. Particular focus is given to the *in vivo* role of miRNAs that has been demonstrated through the generation of Sertoli- or germ-cell specific Dicer knockouts, as well as the potential application of these findings in the treatment of human male infertility and the development of male contraceptives. It is anticipated that unraveling miRNA functions in the testis will further our understanding of the regulatory mechanisms of mammalian spermatogenesis.

Keywords: microRNAs, testis, Dicer, Sertoli cells, germ cells, spermatogenesis

Mammalian Spermatogenesis

Spermatogenesis refers to the development of mature haploid spermatozoa from diploid spermatogonial cells. It is typically divided into three phases: the *mitotic*, during which spermatogonial renewal and proliferation occur, the *meiotic*, during which spermatogonia first duplicate their DNA to give rise to primary spermatocytes and then primary spermatocytes undergo two divisions to generate secondary spermatocytes and haploid spermatids respectively, and finally, the phase of *spermiogenesis*, which culminates with the release of spermatozoa (spermiation). During spermiogenesis, spermatids undergo a metamorphosis which does not involve cell division, but a number of morphological changes (acrosome formation, nuclear condensation, development of the flagellum and cytoplasm reorganization) that eventually result in the generation of mature spermatozoa.

The nuclear condensation that occurs during spermiogenesis has long been of great interest not only because it is essential for successful sperm production, but also because it involves fine mechanisms of post-transcriptional control. Before and during meiosis, histones are gradually replaced by testis specific arginine-rich histone variants (H1t, TH2A, TH2B, TH3) (Meistrich, et al., 1978). In fact, histones become hyperacetylated (Hazzouri, et al., 2000, Marcon and Boissonneault, 2004), thereby facilitating nucleosome disassembly and histone displacement (Oliva and Mezquita, 1986). Histone variants are subsequently replaced by the basic transition proteins TP1 and TP2 , which in their turn are replaced by protamines (PRMs), thus transforming the chromatin into a highly compact form (reviewed in (Pradeepa and Rao, 2007)).

Classic post-transcriptional control during spermatogenesis

Spermatid chromatin compaction is incompatible with transcription, therefore any transcript essential for later stages of spermatogenesis must be generated well in advance of its use and is thus under translational control. In the mouse, transcription occurs all throughout spermatogonial proliferation and meiosis, but *ceases* before the completion of spermiogenesis, at the transition from round to elongating spermatids (reviewed in (Braun, 1998)). In fact, transcription occurs massively after meiosis; post-meiotic transcripts accumulate in large amounts, become deadenylated and are stored in the spermatid cytoplasm for 4-5 days until their translation (Braun, 1998, Kleene, 1993). In mammalian haploid GCs, about two thirds of all mRNAs are at least partially stored in translationally inactive free-mRNPs (mRNA ribonucleoprotein particles) (Kleene, 1993, Schmidt, et al., 1999).

Translational control involves protein repressors that bind either to the poly(A)-tail or to other specific sequences in the 3’UTR (3’ untranslated region) of an mRNA, although it now becomes

clear that translational control can also be mediated by 5'UTR sequence elements (Yang, et al., 2003). Two sequence elements in the 3'UTR of an mRNA are involved in translational control: the polyadenylation consensus motif (PCM) [5'aauaaa3'] and the cytoplasmic polyadenylation element (CPE) [5'uuuuuau3'] to which CPEB (CPE-binding protein) binds in order to promote polyadenylation (Hake, et al., 1998).

Perhaps the best –of numerous- characterized example of translational control during spermatogenesis is provided by the expression of *Prm1* and *Prm2* which encode for protamines 1 and 2. Several proteins have been found to bind to their 3'UTR and control their expression, for example the 26kDa TB-RBP (testis/brain RNA-binding protein). TB-RBP interaction with the 3'UTR Y-box elements of *Prm1* and *Prm2* depends on phosphorylation and dimerization (Wu, et al., 1998). In fact, TB-RBP not only controls translation of the *Prm1* and *Prm2* mRNAs, but also facilitates their movement from the nucleus to the cytoplasm where they are temporarily stored (Morales, et al., 1998). Importantly, although fertile, male mice lacking TB-RBP show decreased sperm count numbers and severe abnormalities within the seminiferous epithelium (Chennathukuzhi, et al., 2003). Another Y-box protein binding to the 3'UTR and thus controlling translation of *Prm1* is MSY4: Male mice in which *Msy4* expression is extended beyond its normal window frame are completely infertile (Giorgini, et al., 2002), thus demonstrating that repression of Prm1 translation must be relieved in a timely manner in elongating spermatids so as to successfully complete spermatogenesis.

Translational control has been a field of intense study for many years, and favors a 'classical' dogma according to which binding of a protein on an mRNA is what controls its translation. However, several 3'UTR consensus sequence elements have been identified as mediators of translational repression but for the moment, no specific protein binding to them has been found (Steger, 2001). The Z-box element is an example (Kwon and Hecht, 1991, Kwon and Hecht, 1993): it is a conserved 17nt motif present in the 3'UTR of the *Prm1* and *Prm2* mRNAs that has the ability to mediate translational repression, yet no RNA-binding protein has been found to interact with it, thus leaving the question open as to what actually binds and mediates translational control of certain transcripts. Could it be a molecule other than a protein?

microRNAs: A novel mechanism of post-transcriptional control

Among the different classes of small RNAs, siRNAs (small interfering RNAs), piRNAs (Piwi-interacting RNAs) and microRNAs (miRNAs) have emerged as important regulators of development across eukaryotes (for a review see (Carthew and Sontheimer, 2009, Ghildiyal and Zamore, 2009). Fifteen years ago, a breakthrough discovery, that of the first miRNAs in

C.Elegans (Lee, et al., 1993, Wightman, et al., 1993) was about to change the classical dogmas of post-transcriptional control, but as we have witnessed it in the last year, the way we study reproductive biology as well.

miRNA genomics: genes and biogenesis- miRNAs are single-stranded, non-coding small RNAs of approximately 22 nucleotides that have been found in plants and animals, but also in viruses (Pfeffer, et al., 2004) and more recently, in the unicellular algae *C. reinharddtii* (Molnar, et al., 2007, Zhao, et al., 2007); they function as post-transcriptional regulators of gene expression. miRNAs are endogenous molecules; they are encoded by miRNA genes which are found either within introns –in which case they are transcribed as part of the gene in which they reside in- (Aravin, et al., 2003, Lagos-Quintana, et al., 2003, Lai, et al., 2003, Lim, et al., 2003) or intergenic regions –in which case they originate from independent transcriptional units (Lagos-Quintana, et al., 2001, Lau, et al., 2001, Lee and Ambros, 2001). Until now, 706, 547 and 286 miRNAs have been identified in human, mouse and rat respectively (Sanger Database, v13.0, March 2009, http://microRNA.sanger.ac.uk/sequences/).

In animals, miRNA genes are typically transcribed by polymerase II (reviewed in (Kim, et al., 2009)). Their transcription produces a primary miRNA transcript (pri-miRNA) which folds in a hairpin stem loop structure. The Microprocessor complex which contains Drosha**,** an RNaseIII endonuclease, and DGCR8 (in mammals) or Pasha (in flies) ((Han, et al., 2004, Landthaler, et al., 2004), cleaves the ends of both strands of the pri-miRNA and thus produces the precursor miRNA (pre-miRNA), a stem-loop intermediate with a 5' phosphate and ~2nt 3' overhangs (Lee, et al., 2003). Interestingly, Drosha cleavage occurs during transcription –independently of the miRNA's gene location, intronic or intergenic-, and in the case of a miRNA gene embedded in an intron of a host gene, intron processing and degradation occurs before splicing of the nascent mRNA (Morlando, et al., 2008). Alternatively, a pre-miRNA can be produced through splicing of a pre-mRNA during which an intron that precisely mimics the structural features of a 'canonical' pre-miRNA (the so called mirtron) is liberated (Berezikov, et al., 2007, Okamura, et al., 2007). pre-miRNAs are transported by Exportin into the cytoplasm (Lund, et al., 2004, Yi, et al., 2003), where Dicer (Dcr), another RNaseIII endonuclease, cleaves the hairpin structure at about two helical turns away from the stem loop (Grishok, et al., 2001, Hutvagner, et al., 2001, Ketting, et al., 2001). The resulting miRNA:miRNA* duplex is then unwound by a helicase, and while the miRNA* strand is degraded, the miRNA strand (the mature strand) is loaded on to the RNA-induced silencing complex (RISC), which in addition to other proteins, contains members of the Argonaute (AGO) protein family. The strand that gets preferentially loaded on RISC is almost always the one with the thermodynamically less stable 5'

end ((Khvorova, et al., 2003, Schwarz, et al., 2003). After the assembly of RISC on the mature miRNA strand, the complex is tethered on an mRNA target and, depending on the miRNA-mRNA sequence complementarity, induces either translational repression or mRNA degradation. Of note, of the mammalian Ago proteins (Ago1, Ago2, Ago3, Ago4) that can be part of the RISC complex, only Ago2 has the capacity to endonucleolytically cleave a target mRNA ('Slicer' activity) (Liu, et al., 2004, Meister, et al., 2004, Pillai, et al., 2004), whereas all four are equally potent translational repressors (Su, et al., 2009).

miRNA mechanisms of function- Some studies have proposed that translational repression upon miRNA binding on an mRNA target occurs after at the initiation step (Olsen and Ambros, 1999, Petersen, et al., 2006), while others suggest that suppression occurs at the initiation step (Humphreys, et al., 2005, Mathonnet, et al., 2007, Pillai, et al., 2005, Wakiyama, et al., 2007, Wang, et al., 2006). Of note, one study has proposed that miRNA-mediated translational repression can also occur during the elongation phase of translation, through proteolytic cleavage of the nascent polypeptide (Nottrott, et al., 2006). mRNA degradation is the other possible effect upon miRNA binding. It was shown that upon exogenous miRNA expression in mammalian cells, a large number of transcripts is downregulated and that the 3'UTRs of these transcripts are significantly enriched for miRNA binding sites (Lim, et al., 2005). This study also showed that a far greater than expected number of transcripts are actually regulated by miRNAs. Additional studies later on confirmed the findings of Lim and colleagues; miRNAs in *C. Elegans* were found to induce degradation of their target mRNAs (Bagga, et al., 2005), and in zebrafish, miR-430 was shown to be responsible for the clearance of maternal mRNAs during early embryogenesis (Giraldez, et al., 2006).

In either case –translational repression or mRNA degradation-, miRNAs obviously act as negative post-transcriptional regulators of gene expression. Recently however, an elegant study presented data supporting an unexpected role for miRNAs as positive regulators: Vasudevan and colleagues showed that although miRNAs repress translation of their targets in proliferating cells, they induce translational up-regulation upon cell cycle arrest (Vasudevan, et al., 2007), thus broadening the potential effect of miRNAs on gene expression and raising new, stimulating questions about the way miRNAs are –or should be- studied.

Taking out Dicer: miRNA functions unraveled

Given that Dcr plays a central role in the biogenesis of miRNAs, it quickly became a great pole of attraction for all those interested in understanding the biological functions of miRNAs. The first mouse *Dcr* knockout was generated by Bernstein and colleagues in an effort to

study RNAi mechanisms in mammals (Bernstein, et al., 2003). Loss of DCR led to early embryonic lethality (E7.5), and was characterized by an almost complete absence of embryonic stem cells, thus demonstrating the absolute necessity of Dcr for murine embryonic development. Thereafter, numerous studies unraveled an essential role for Dcr –and thus miRNAs- in diverse developmental processes (for example (Harfe, et al., 2005, Harris, et al., 2006, Kobayashi, et al., 2008, O'Rourke, et al., 2007, Suarez, et al., 2008, Yi, et al., 2006)).

Not surprisingly, several groups turned their attention to the potential involvement of Dcr-dependent mechanisms in the regulation of the reproductive function. The first conditional *Dcr* knockout in the reproductive tract was done in mouse oocytes; two studies showed that although Dcr is dispensable for oocyte growth and development, it is essential for meiosis of the female germline. Deletion of *Dcr* in mouse oocytes results in an arrest of meiosis I, spindle disorganization and chromosome congression defects. In addition, abnormally high levels of transposon transcripts are found in Dcr-depleted oocytes, thus suggesting that Dcr might somehow protect oocytes from abnormal activity of transposable elements (Murchison, et al., 2007, Tang, et al., 2007). The role of DCR in the somatic compartment of the female reproductive tract was also studied; *Dcr* was specifically inactivated in the mesenchyme of developing Müllerian ducts and in ovarian granulosa cells and led to infertility due to diverse reproductive defects such as reduced ovulation rates, smaller oviduct and uterine horns' size, appearance of bilateral oviductal cysts harboring unfertilized, degenerate oocytes, and adenomyosis (Gonzalez and Behringer, 2009, Nagaraja, et al., 2008).

microRNAs in the testis: A new era for male fertility

miRNAs were first cloned from the testis only few years ago, in an effort undertaken by several groups to establish techniques reliable for genome-wide miRNA profiling (Barad, et al., 2004, Liu, et al., 2004). Shortly afterwards, a more detailed analysis of the testicular miRNAome reported that a number of miRNAs are differentially expressed during testicular development (Yu, et al., 2005). The same study also presented evidence for *in vitro* targeting of the *Tp2* mRNA by a testicular miRNA, miR-122a. The first however physical evidence for the potential involvement of the miRNA pathway in the regulation of male GC development came when Kotaja and colleagues reported that DCR and other components of the miRNA pathway, namely Ago2, Ago3, and testis-expressed miRNAs (miR-21, let-7a, miR-122a), localize in the chromatoid body of male GCs, from where they might control post-meiotic GC differentiation (Kotaja, et al., 2006). Another elegant study discussing the potential involvement of miRNAs in post-transcriptional regulation in the testis reported that the miR-17-92 cluster is activated upon

c-Myc expression and eventually leads to the translational repression of E2F1, thereby preventing apoptosis during meiotic recombination (Novotny, et al., 2007). Two studies published shortly afterwards reported the miRNA profile of the murine testis using either microarrays (Yan, et al., 2007) or cloning analysis (Ro, et al., 2007); in the first study, whole testis was used as starting material, whereas in the second, purified spermatogenic cells were used. Later on, another study described the expression patterns of several members of the miRNA pathway in the testis; Dcr, Drosha, Ago1, Ago2, Ago3 and Ago4 are all expressed in pachytene spermatocytes, round and elongated spermatids, as well as Sertoli cells (Gonzalez-Gonzalez, et al., 2008). Shortly afterwards, in an effort to understand the mechanism of meiotic gene silencing, Marcon and colleagues showed that miRNAs localize to the chromosome cores, telomeres and the XY body of spermatocytes, but also in the nucleolus of Sertoli cells (Marcon, et al., 2008). Another interesting study recently reported that about 86% of X-linked miRNAs actually escape meiotic sex chromosome inactivation (MSCI) during spermatogenesis: the study showed that although transcriptional silencing of genes on chromosomes X and Y occurs in mid-to-late pachytene spermatocytes, most of the miRNA genes located on chromosome X are *de novo* transcribed and processed at this stage, thus suggesting that either they are involved themselves in MSCI or that they are essential for post-transcriptional control of autosomal mRNAs during late meiosis and early post-meiosis (Song, et al., 2009).

Two groups described an *in vivo* model that studies the involvement of miRNAs in spermatogenesis. Mice harboring two floxed Dcr alleles were mated with PGC (primordial germ cell)-specific, Cre recombinase-expressing mice (TNAP-Cre), so as to generate animals in which Dcr is absent in germ cells; Loss of *Dcr* in the germ lineage of the testis was found to result in defective PGC proliferation and eventual late adult infertility due to spermatogenic arrest probably caused by defective proliferation and/or differentiation of spermatogonia. Interestingly though, no defects were seen when instead of Dcr, Ago2 was deleted in germ cells, suggesting that Dcr-knockout defects are independent of Ago2 (Hayashi, et al., 2008). Slightly different results were obtained in the study of Maatouk and colleagues, who, using the same Cre expressing transgenic mouse, found that males lacking Dcr in germ cells were subfertile due to both a defect in the transition from round to elongating spermatids and production of sperm with abnormal motility (Maatouk, et al., 2008). The results of both studies should be interpreted with caution since the TNAP-Cre transgenic mouse does not seem to be fully penetrant (only ~50% of germ cells express Cre), nor to be specific uniquely to germ cells. It should also be noted that since the expression of TNAP-Cre begins as early as E10 (Lomeli, et al., 2000), it is difficult to understand how and when exactly spermatogenesis is affected in the adult. The primary effects

appear in the PGC population and inevitably, the defect is carried on to adult germ cells, however, it is not possible to measure the importance of miRNAs in adult germ cells; a postnatal germ-cell-specific deletion of Dcr would be required to assess this.

Given the essential role of Sertoli cells in spermatogenesis, we addressed the possibility of miRNA-mediated post-transcriptional control in this compartment of the testis, and provided *in vivo* evidence for the absolute requirement of Sertoli cell-Dcr for the normal occurrence of spermatogenesis (Papaioannou, et al., 2009). We showed that selective ablation of *Dcr* in Sertoli cells leads to infertility due to complete absence of spermatozoa and progressive testicular degeneration. The first morphological alterations appeared already at postnatal day 5 and correlated with a severe impairment of the prepubertal spermatogenic wave, due to defective Sertoli cell maturation and incapacity to properly support meiosis and spermiogenesis. An initial increase in SC proliferation was followed by massive Sertoli and germ cell apoptosis in prepubertal testes. Importantly, we found several key genes such as *Gdnf, Kitl, Man2a2,* and *Serpina5*, all known to be essential for spermatogenesis, to be significantly down-regulated in neonatal testes lacking Dcr in Sertoli cells. We hypothesized that this downregulation could account –at least partially- for the numerous spermatogenic defects we observe; however, we do not neglect all those genes that were found to be upregulated, given that miRNA effects are lately becoming broader than originally thought.

A novel class of small RNAs in the testis- We should note here that a new, male GC-specific class of small RNAs called piRNAs (PIWI-interacting RNAs) has recently been discovered (Aravin, et al., 2006, Girard, et al., 2006, Grivna, et al., 2006, Watanabe, et al., 2006), although for the moment their biogenesis is thought to be DCR-independent (Houwing, et al., 2007, Vagin, et al., 2006). piRNAs are slightly bigger in size than miRNAs (26-31nts), and two distinct groups have been identified: the first includes pre-pachytene-expressed piRNAs which derive from repeat- and transposon-rich clusters and are associated with MIWI and MIWI2, two members of the AGO protein family (Aravin, et al., 2008, Aravin, et al., 2007, Kuramochi-Miyagawa, et al., 2008), and the second includes an extremely abundant group of pachytene-expressed piRNAs associated with MILI and MIWI (Aravin, et al., 2008, Aravin et al., 2007, Girard et al., 2006). Most piRNAs correspond to intergenic repetitive sequences including retrotransposons, and are thus thought to have a role in the silencing of selfish genetic elements; in favor of this hypothesis is the finding that MIWI and MIWI2 are essential for the repression of male GC transposons through *de novo* DNA methylation (Aravin et al., 2008, Aravin et al., 2007, Kuramochi-Miyagawa et al., 2008, (Carmell, et al., 2007)). The abundant presence of piRNAs in male GCs strongly suggests that they might play key roles in the regulation of spermatogenesis; in favor of

this notion is the fact that loss of both Mili and Miwi leads to infertility. Spermatogenesis in Mili-/- mice is blocked at the early prophase of meiosis I, probably at the zygotene or pachytene stage, and sperm production is therefore almost completely absent (Kuramochi-Miyagawa, et al., 2004), whereas in Miwi-/- males spermatogenesis is blocked at the round spermatid stage, and neither chromatin condensation nor spermatid elongation occur (Deng and Lin, 2002).

Conclusions and Perspectives

The discovery of miRNAs has inevitably had its impact on the field of reproduction; reproductive biology is slowly entering into a new era, which demands a thorough revision of the current theories, so as to complement them with the most recent findings from RNA biology. Novel essential roles played by the Dcr-dependent pathway in the male reproductive function have been revealed. It is now clear that a global loss of miRNAs, whether in the supporting or the germ lineage of the testis, has detrimental effects on male fertility and as such, should be taken into consideration when discussing about treatment of male infertility or –on the opposite-, male contraception. Surely, more detailed analyses at the molecular level are required in order to identify individual miRNAs with key roles during spermatogenesis: the need to generate knockouts for individual miRNAs now seems evident. This will allow us to identify their target genes and thus understand in depth how the process of spermatogenesis is controlled by the miRNA machinery. Certainly, the functional redundancy between miRNAs will not make the task easy, however the first attempts have opened new promising routes and will soon deliver their fruit.

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