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The Interplay of Coding and Non-Coding Regulations in Mouse Spermatogenesis

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Abstract

Spermatogenesis is an essential biological process to produce functional male gametes from undifferentiated progenitor cells throughout the reproductive age. Success in a series of differentiation of stem cell-like progenitor cells is a complex cascade and involves sophisticated regulations by various types of molecules in the developing germ cell. In this review, we will discuss the role of different groups of germline biomolecules, including some protein-coding genes, microRNAs, piRNAs and long non-coding RNAs (lncRNAs) in leading to a complete cycle of mouse spermatogenesis. We will also share our perspective on future research opportunities on lncRNA regulations in spermatogenesis.

Development of male gametes in mammals is regarded as one of the most efficient biological processes, which is characterized by a continuous production of sperms from puberty till the end of reproductive age, which lasts for more than a half of male lifespan. It would not be possible to achieve successful spermatogenesis without numerous regulatory molecules in germ cells accomplishing their functions. We will discuss in the following parts how four types of regulatory molecules are so important in different stages in spermatogenesis.

Keywords: Spermatogenesis; Regulatory molecules; Spermatogonial stem cells

Pre-Spermatogenesis Stage - Formation of Spermatogonial Stem Cells

It is well accepted that Spermatogonial Stem Cells (SSCs) are the progenitor cells that finally give rise to mature sperms in reproductive age, while SSCs are formed from gonocytes at a period before spermatogenesis takes place. Take mice as a model: since shortly after birth, gonocytes inside the seminiferous tubules migrate from the lumen to the basement membrane, a process known as homing [1]. A successful homing depends on adhesion molecules like β1-integrin [2], Kit [3], and Sox8 [4], which are expressed in both the gonocytes and Sertoli cells for cell-cell attachment and directional migration.

In few days after homing, gonocytes residing on the basement membrane develop to Spermatogonial Stem Cells (SSCs), and the first wave of spermatogenesis starts. Current model for SSC development suggested that single type A spermatogonia (A_{single} or A_s), which appear at 6 days-of-age still retain the stemness: ability to undergo self-renewal to maintain the A_s population and to differentiate. A complete spermatogenesis takes around 35 days for an SSC differentiates to spermatocytes, spermatids, and terminally differentiated mature spermatozoa (sperms) in mice [5]. In order to achieve a continuous production of mature and functional sperms throughout the reproductive age, SSCs have to maintain a balance between self-renewal and differentiation into daughter cells.

Experiment Design to Evaluate Intrinsic and Niche Factors

Generally speaking, both intrinsic factors of progenitor cells and niche factors affect sperm production. It has been observed in many experiments that the number and function of SSCs declined with age [6,7]. However, during normal development, both niches and intrinsic factors keep changing with time, imposing difficulties in accessing the effect of individual factors. Failure of maintaining a true spermatogonial stem cell line by *in vitro* setup also disfavors our investigation on SSC biology.

To study the effect of intrinsic factors under consistent niche, Ryu et al. designed the serial transplantation experiment in mice [7]. Briefly, spermatogonia from donor mice were isolated and injected to seminiferous tubules of recipient mice, whose own endogenous spermatogonia had been depleted by busulfan treatment. The recipient mice were young adults, always around 12-week-old at transplantation, in order to supply a favourable environment in the seminiferous tubules for spermatogonial proliferation and differentiation. Since the transplantation process was repeated every three months, the spermatogonia were kept in a relatively constant and favourable SSC niche. In such setting, the proliferation ability of rodent spermatogonia was found to be quite consistent through the first to the ninth transplantation, equivalent to over 1,000 days of cell age [1,7,8], indicating SSC niche plays an essential role in sperm development. At the same time, a gradual decrease in SSC number was also observed [8], suggesting possible regulation by intrinsic factors during aging.

Proteins that Regulate Spermatogenesis and Mark the Differentiation Stage

Spermatogenesis is a complicated process and each step is strictly regulated. As recently reviewed by Bettegowdas and Wilkinson, a variety of transcriptional factors, such as HSF2 in meiotic stages, and Ovol1 in proliferation of spermatogonia, have been shown to guarding the progression of spermatogenesis by direct interaction with DNA and control the expression of differentiation related genes [9].

Some factors are shown to regulate spermatogenesis by inducing histone modifications. For instance, Jmjd1a is a testis specific histone

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demethylation factor which regulates gene expression during meiotic phases of spermatogenesis by decreasing H3K9me1 and H3K9me2 levels [10]. Knockout of Jmjd1a caused decreased expression of a number of genes essential for spermatid development and thus defects in spermatid elongation [10].

Another histone modifier that regulates spermatid elongation is Pygo2 protein, which is shown to promote global acetylation. With its Plant Homeodomain (PHD) finger domains, Pygo2 induced histone H3 remodeling and affected downstream gene expressions. Reduction in Pygo2 level distorted the chromatin condensation during spermiogenesis and hence leading to infertility [11].

In addition, some proteins are able to bind to mRNA transcripts, either stabilize or destabilize the mRNA for post-transcription regulations. Gonadotrophin-Regulated Testicular RNA Helicase (GRTH) is one of this category of proteins. In mice spermatocytes, GRTH was found to selectively bind to mRNAs of some pro- and anti-apoptotic factors and regulate their half-life, finally leading to a controlled apoptosis [12]. Knockout of GRTH led to an increase in mRNA level of apoptotic genes, such as caspase 3 and caspase 8 and increased the proportion of apoptotic cells.

Besides, there are also a number of protein markers for spermatogenic cell identification [13]. For example, Gfrα1, Pou5f1 (also known as Oct4), and Plzf are common molecular phenotypes for undifferentiated type A spermatogonia including A_{single}, A_{paired} and $A_{aligned}$, while Kit, Sohlh1, and Ngn3 are cell surface markers for differentiating spermatogonia. A recent report also revealed that Nanog and Pou5f1 are two markers specifically for A_{single}, the highly proliferative population of undifferentiated type A spermatogonia [14].

For spermiogenesis particularly, Arp3 has been shown to be essential for actin architecture and facilitate spermatid maturation in rat model [15], while Esp in Sertoli cells regulates the detachment of elongated spermatids [16]. These two proteins also seem to be key in manipulating the actin dynamics and keeping the integrity of Blood-Testis-Barrier (BTB) [17], which is crucial for maintaining the niche for spermatogenesis.

Inhibitory Pathways by MicroRNAs at Different Stages

Apart from protein-coding genes, the non-coding part of transcriptome also plays an active regulatory role in spermatogenesis, where the best-studied member is micro-RNA (miRNA). MiRNAs are actively expressed in adult mouse testes [18]. After transcribed as pre-miRNAs and processed by Drosha or Dicer protein, mature miRNAs of 21-25 nucleotides in size from the RNA-Induced Silencing Complex (RISC) with appropriate Argonaute proteins. This RISC then binds to an mRNA that contains a sequence complementary to the miRNA sequence, repressing the translation or even initiate mRNA degradation [19-21]. Kotaja's team showed miRNA accumulates with Dicer and Argonaute proteins in chromatoid body during meiotic and post-meiotic stages of spermatogenesis, suggesting important regulation by miRNA [22,23]. Hayashi's group supported this idea by demonstrating dicer-knockout primordial germ cell and spermatogonia exhibited poor proliferation [18]. They also showed an early termination of reproducibility and a significant depletion of sperm production in 8-month-old mice with dicer-deficient testes [18], illustrating miRNAs are crucial for spermatogenesis to initiate and to be maintained through reproductive life. Since then, there is a boost in the number of researches on the role of specific miRNAs in spermatogenesis carried out. Some examples of validated miRNAs in each stage of spermatogenesis are summarized in Figure 1.

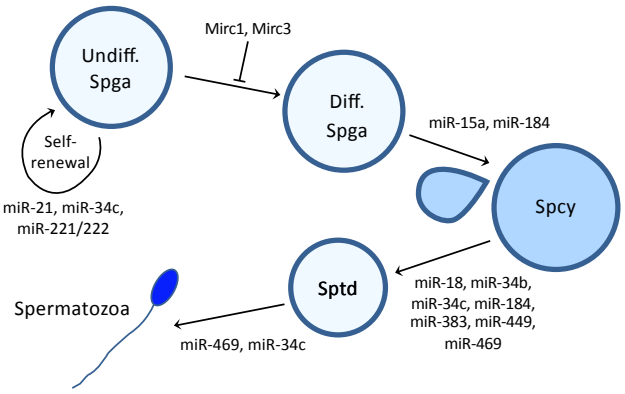

Figure 1: Validated microRNA candidates involved in spermatogenesis

Spermatogenesis is closely regulated by non-coding RNA, including: (a) Self-renewal of undifferentiated spermatogonia (undiff. Spga): miR-21 [25], miR-34c [25]; (b) Differentiation of spermatogonia (Diff. Spga): Mirc1 [27], Mirc3 [27], Mirlet7 [71]; (c) Differentiation to spermatocytes (Spcy): miR-15a [29], miR-184 [72]; (d) Meiotic division of spermatocytes to spermatids (Sptd): miR-18 [24], miR-34b, miR-34c [28], miR-184 [72], miR-383, mir-449, miR-469 [31]; (e) Differentiation of spermatid to form spermatozoa: miR-34c [28], miR-469 [31].

Mir-18 is another miRNA highly expressed in male germ cells. It was found that *mir-18* can regulate heat shock factor 2 (HSF2), a well-known transcription factor regulating gametogenesis, by binding to the 3'UTR of HSF2 mRNA [24]. Knockdown of mir-18 causes up-regulation of HSF2, and alters the relative abundance of the downstream targets of HSF2. This suggested that, just similar to other systems, the non-coding transcriptome indeed participates actively with the established protein-centric regulatory network.

A recent report described a mechanism by which *miR-21* regulates the fate of mouse SSC. *MiR-21* is shown to preferentially expressed in Thy1-enriched SSCs. Inhibition of *miR-21* promotes apoptosis and reduces self-renewal of SSC, suggesting they are vital for normal self-renewal [25]. *MiR-221* and *miR-222* were found to induce Kit expression in mouse undifferentiated spermatogonia and hence promote differentiation of the progenitor cell [26].

Another recent study demonstrated that two miRNA clusters, namely *Mir-17-92 (Mirc1)* and *mir-106b-25 (Mirc3)* may carry important regulatory role in spermatogenesis. In Thy1-enriched spermatogonia, these two miRNA clusters showed significant down-regulation upon retinoic-acid induced differentiation [27]. Further *in vivo* study clearly showed *Mirc1*-knockout mice at reproductive age had smaller testes with decreased sperm formation. Moreover, an up-regulation of *Mirc3* was observed in *Mirc1*-knockout mice, suggesting these two miRNA clusters work closely together in sperm cell regulation [27].

Other experimentally validated regulatory miRNAs related to the spermatogenesis include *miR-34c* which up-regulates some lineage markers [28], *miR-15a* in early spermatogenesis regulation by binding Ccnt2 [29], *miR-23b* in the translation regulation of Pten and Eps15 in Sertoli cells [30], and *miR-469* that binds TP2 and Prm2 mRNA [31]. Furthermore, clinical observation showed many microRNAs are down-regulated in patients with non-obstructive azoospermia (NOA), including *miR-17-92* [32], *miR-371/2/3* cluster [32], *mir425* and *miR191* [33], *miR-34c-5p*, *miR-122*, *miR-146b-5p*, *miR-181a*, *miR-374b*, *miR-509–5p*, and *miR-513a-5p* [34]. The precise molecular regulatory mechanisms of these short ncRNAs are not yet fully understood. This wide range of evidence demonstrated the

non-coding transcripts are playing a significant role in the regulatory network of spermatogenesis.

Piwi Protein and Piwi-Interacting RNA

Another large family of small RNA is the Piwi-interacting RNA (piRNA), most with length between 24-31 nucleotides. piRNAs were detected in testes of mice only after 20 day post partum (dpp) [35], at which the first wave meiosis is about to take place, suggesting piRNA plays important roles in meiotic regulation. Similar to interaction between miRNAs and Argonaute proteins to form RISC, piRNAs form functional complex, named as PIWI-interacting RNA complex (piRC) with PIWI interacting proteins, another clade of Argonaute. This machinery was first discovered to play a critical role in germline development in *Drosophila* model back to late 1990s [36-38], and is highly conserved from worms to mammals through the evolution [35,37]. The later discovery from high-throughput sequencing data found that most piRNAs of distinct clusters were originated from a single long primary transcript [35,39], which will undergo further complicated but not well-defined processes to produce numerous piRNAs. As a result, over 80% of piRNAs are transcribed from only 42 genomic clusters, and up to over 2,000 piRNAs can be transcribed from a genomic length of 35-80 kilo base-piars (kbp) [39-41]. Furthermore, these clusters were dispersed in different chromosomes, ranging from 1 to 14 clusters per autosome were identified.

There are three types of mouse PIWI (Miwi) proteins, namely Mili [42], Miwi [43], and Miwi2 [44] identified with different expression profile in along spermatogenesis (Table 1) [45]. Mili is specifically expressed from early stages up to pachytene spermatocytes, Miwi in spermatocyte and spermatid stages, while Miwi2 is only detected in fetal stage from embryonic day 14 to postnatal day 3 [46]. These proteins were found to be involved in spermatogonial stem cell maintenance, meiosis and spermiogenesis [35].

One possible regulatory mechanisms of Piwi-interacting RNA complex is through interaction with mouse vasa homologue (Mvh) protein. Since a similar phenotype of Mili-null and Mvh-null mice was observed, and the localization of Mvh altered after knocking out of Mili [35], suggesting Mvh may function through interaction with Mili and Miwi [42].

Another mechanism of piRC regulation was by protecting the germline genome from attacks by Transposable Elements (TEs) during meiosis [46-48]. TEs are mobile DNA fragments which are able to replicate and translocate themselves by transposition process, regardless of host genome replication machinery. If this process is uncontrolled, TE will disrupt the integrity of host genome and influence normal gene expressions and protein synthesis. In normal somatic cell, TE activity is limited by epigenetic modifications like DNA methylation and heterochromatin formation [49]. However during germ cell production, these epigenetic protections have been partially removed during meiotic reprograming, leaving the transposition more active.

Bioinformatics analysis showed a substantial anti-sense correlation between piRNA and TEs in *Drosophila* [50]. Once a transposon is actively transcribed, it is susceptible to binding and restriction by RNP complex formed by PIWI protein and piRNA with an anti-sense sequence. The restriction produces a sense piRNA, which will be loaded to Agonaute 3 to form a RISC. This RISC will in turn act on the long precursor piRNA to cleave out the anti-sense piRNA. This model is called the ping-pong cycle [51], by which both sense and anti-sense piRNA will be amplified in a positive feedback loop in response to any activity in TE. It is believed that mammalian piRNAs are also amplified by similar mechanism [35]. Although the ping-pong model in mice is recently challenged by works from Beyret's group [52], which showed the anti-sense piRNAs is the predominant species, the importance of Piwi proteins and piRNAs in controlling TE invasion has not been questioned.

Long Non-Coding RNA: The Next Blackbox to Open

Based on all above discussions, which shows both protein-coding genes and short non-coding RNAs are active in regulating spermatogenesis, it is reasonable to anticipate that some longer non-coding RNA transcripts are also involved in the regulation of spermatogenesis.

Long non-coding RNAs (lncRNAs) are one of the most abundant ncRNA families, although its functional annotation lags far behind other families. While short RNAs still rely on partnership with proteins for regulatory functions, lncRNA is more independent in carrying out its own functions. Reported regulatory mechanisms by lncRNAs are versatile, which include direct binding to proteins and modulating the level of activated form [53], direct binding to antisense mRNA for post-translational regulations [54], recruiting polycomb repressive complex for transcriptionally repressive chromatin modifications [55], acting as competing endogenous RNA to inhibit the function of miRNA [56], being the precursor other small RNA [57], and directly bind to X chromosome for chromosome-wide inactivation [58,59].

Meiotic recombination hot spot locus (*mrhl*) RNA is so far the only published functional lncRNA in mouse spermatogenesis. It is a 2.4-kb mono-exonic lncRNA in mouse chromosome 8, upstream to a pachytene repair site and located within a 7.2 kb fragment of recombination hot spots [60], and thus it is named after this genomic location. Further

Mice Piwi protein			
	Mili	Miwi	Miwi2
Year of gene discovery (first publication)	2004	2002	2007
Stages of expression [46,47]	From gonocyte to pachytene spermatocyte (post-natal pre-pachytene stage)	From pachytene spermatocyte to round spermatid (pachytene stage)	14 dpc to 3dpp (fetal stage)
Outcomes in male germline with gene knockout or mutant [45]	Arrest in early pachytene spermatocyte. Increase in germline apoptosis. Decrease in testes size. More piRNA loaded to Miwi2.	Arrest at round spermatid. Decrease in testes size. Changes in Mvh localization.	Arrest in leptotene spermatocyte. Increase in germline apoptosis. Germ cell depletion.
Molecular mechanism	Able to form piRC with piRNA. The piRC will target DNA or RNA with sequence complementary to the piRNA and induce inhibition and degradation.		
	Bind and regulate Mvh, an factor essential for early spermatocyte stage [42]	Bind and regulate Mvh. TE control. Amplify responsive piRNAs by 'ping-pong' cycles.	TE control. [44] Amplify responsive piRNAs by 'ping-pong' cycles.

Table 1: Comparison on three groups of Piwi proteins in mice.

study showed *mrhl* RNA regulates spermatogenesis potentially by two pathways. First, acting as a precursor of microRNA, *mrhl* RNA could be restricted by Drosha to form an 80-nucleotide RNA intermediate [61]. Both RNAs were localized in nucleoli of GC1 spermatogonia cell line, suggesting possible interactions with chromatin [61]. Second, *mrhl* RNA inhibited the Wnt signaling pathway through binding to p68 [62]. However, the functional effects on spermatogenesis of this lncRNA remain elusive, and we believe there are many other lncRNA regulators for spermatogenesis.

To understand the developmental programs in male germ cell development, our group previously applied mouse spermatogenesis and gonadogenesis as model systems to study the regulation of cellular differentiation and proliferation in mammalian development. We applied SAGE to examine the transcriptomes of male germ cells [63] and gonads [64], where all data are accessible through GermSAGE [65] and GonadSAGE [66] databases.

In the spermatogenesis study, the transcriptomes of germ cells, namely spermatogonia, pachytene spermatocytes, and round spermatids, were compared at different differentiation stages. SAGE captures all polyadenylated transcripts in the transcriptome, and therefore it offers a comprehensive and unbiased method for novel discovery not found in microarray platforms. Concordant to a similar study in rat spermatogenesis [67], the germ cell transcriptomes of different stages were quite unique, with approximately 30% SAGE tags were specific at each stage [63,65,68]. These stage-specific tags fell in both coding and non-coding regions. Bioinformatic analyses further revealed the dynamic transcriptional regulation of various transcription factors and promoter elements, and the involvement of stage-specific gene networks [68].

The resolution of GermSAGE data was recently increased by incorporating expression data from whole-genome tiling microarrays [69]. Like other high-resolution genomic data, this 'big data' created a great opportunity and challenge to current genome biology research [70]. Importantly, over 45% of transcripts were not annotated in both platforms, and their exact functions are not clear. We hypothesized *Age-lncRNA* species were contained in the un-annotated population and have recently identified two Spga-specific lncRNA candidates, known as *Spga-lncRNA* family. Both demonstrated significant differentiation inhibition *in vitro* in model, suggesting it may be important in maintaining stem cell state in SSCs. We also observed both lncRNA candidates contain retinoic acid binding sites on the promoter region and proved to be functional by comparing the luciferase activities of different deletion constructs (data not shown). Both lncRNAs showed to repress both RNA and protein expression of Stra8, a key gene for initiation of differentiation of SSCs. The validation experiment is currently ongoing.

Conclusions and Perspectives

Spermatogenesis is one of the most important biological processes concerning continuation of a species. Proteins and non-coding RNAs are regarded as the key regulators for this complex and highly regulated process. Although the regulations by proteins, miRNAs and piRNAs have been better studied, more novel pathways are still coming out.

Based on our current understanding in non-coding RNA biology, we expect the current findings of lncRNA regulations in spermatogenesis are vastly incomplete. Here, we hypothesize that lncRNAs are actively involved in age-dependent regulation in SSC aging. If this hypothesis is correct, the current concepts of molecular regulation in SSC development need to be re-assessed.

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