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INVITED REVIEW

Sperm Biology

Transcriptome research on spermatogenic molecular drive in mammals

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It is known that spermatogenic disorders are associated with genetic deficiency, although the primary mechanism is still unclear. It is difficult to demonstrate the molecular events occurring in testis, which contains germ cells at different developmental stages. However, transcriptomic methods can help us reveal the molecular drive of male gamete generation. Many transcriptomic studies have been performed on rodents by utilizing the timing of the first wave of spermatogenesis, which is not a suitable strategy for research in fertile men. With the development of separation methods for male germ cells, transcriptome research on the molecular drive of spermatogenesis in fertile men has seen great progress, and the results could be ultimately applied to improve the diagnosis and treatment for male infertility.

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INTRODUCTION

Gametogenesis is a unique and complex biological process, which requires undifferentiated diploid germ-line cells possessing the ability to self-renew undergo meiosis, halve their genetic information, and produce haploid gametes exponentially. The process of spermatogenesis is more intricate. To produce the mature male gamete called spermatozoon, male germ cells must experience two divisions in series, and this story does not end yet when haploid cells appear.[1] Drastic changes take place to the spermatid, which is the initial haploid cell. Special structures such as the acrosome,[2] mitochondrial sheath,[3] and tail will take shape,[4] the nucleus will be condensed,[5] and the cytoplasm and most organelles will be discarded during spermiogenesis. The remolding process makes male gametes more robust and adaptive for transmitting the hereditary material to the oocyte.

Besides the incredible morphological changes during spermatogenesis, the distinguishing characteristics found during the development of male germ cells are different from those of female germ cells. For example, even in the adult testis, undifferentiated germ cells can proliferate as well as differentiate.[6–8] The balance between proliferation and differentiation of the undifferentiated male germ cells, termed spermatogonial stem cells, is critical for the continuous production of male gametes.[1,8,9]

It has been reported that, 10%–15% coulples are infertile, and 50% of the cases are caused by male factors.[10,11] Many of these cases exhibit a disorder in spermatogenesis. With the help of a rodent model, the associations of hundreds of gene defects with abnormal spermatogenesis have been elucidated.[12,13] However, the pathogenesis in many infertility cases is still unknown. Hence, it is necessary to determine the general molecular drive of normal spermatogenesis. Unfortunately, little is known about what happens at the molecular level during spermatogenesis. High-throughput technologies such as RNA-sequencing and microarray could help achieve it.

RESEARCH ON RODENTS

As the most widely used model animals in mammalian reproductive research, rodents have been the focus of research on the changes in RNA profiles during spermatogenesis.[14]

To monitor and describe the changes during spermatogenesis, it is necessary to determine the time points at which these changes occur. The adult mammalian testis is comprised of hundreds of seminiferous tubules at different spermatogenic phases,[1,15] each of which contains germ cells at different stages of differentiation. Therefore, for transcriptomic studies, researchers had to isolate different types of cells from the tissues, which was difficult and time-consuming. Fortunately, the timing of the first wave of spermatogenesis in rodents has been determined.[16–18] It is feasible to sacrifice rodents at different ages and to collect seminiferous tubules at specific spermatogenic phases, with their unique patterns of germ cell composition.[14] After the testicular tissue of rodents at different ages is collected, RNA can be extracted, and sequencing or microarray hybridization can be performed with or without cDNA subtraction. By comparing the RNA profiles of the testis at different stages, researchers could determine the dynamics of the transcriptional profile during spermatogenesis, demonstrate the molecular mechanism of male germ cell development, and elucidate the genes critical for spermatogenesis (**Figure 1**).

Schultz *et al*.[19] collected testes from C57BL/6 mice at different days postpartum individually and performed microarray processing to monitor the changes in the transcriptome profiles from birth to adulthood. By comparing the differences in the transcriptome profiles, it was easy to characterize the expression of specific genes initiated at

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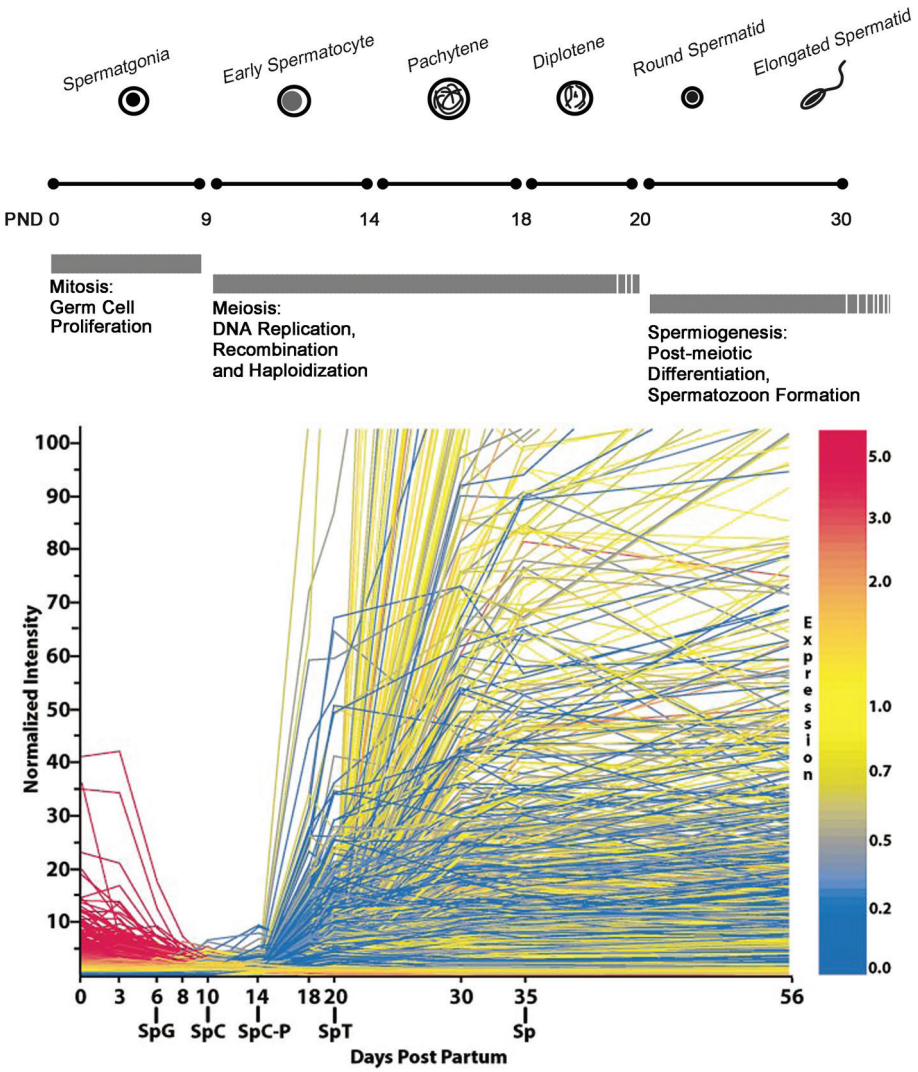

Figure 1: The timing of the first wave of mouse spermatogenesis. Typical germ cells appear successively in the first wave of mouse spermatogenesis according to the observations of Bellve *et al*.[18] are shown in the first line. The duration of each stage is approximately indicated by the bars with dots. The numbers shown below these bars indicate the timing of the appearance of specific cell types. The process of spermatogenesis can be divided into three stages: mitosis, meiosis, and spermiogenesis. The time points are arranged on an approximate scale. The plot shown below is a representative result from spermatogenic timing to monitor the transcriptome profile of spermatogenesis.[34] PND: postnatal day.

different ages. For example, at day 11 postpartum, when meiosis is initiated, the abundance of a group of genes increased markedly. These genes were considered to be related to meiosis or later spermiogenesis. A total of 1652 genes were identified as being expressed solely in meiotic or postmeiotic germ cells, with 351 genes appearing to be expressed only in the male germline. Targeted disruption of 19 of these genes in mice has been reported to lead to an infertility phenotype,[20–33] indicating that these genes could be potentially used as contraceptive targets.

Similarly, in 2004, the localization of the transcripts within the testis was described by using a method based on *in silico* subtraction techniques for two categories of transcripts: enriched and specific.[34] This method helped the authors describe the changes in RNA profiles of specific cell types in the testis during spermatogenesis more accurately.

Other work described an overview of genetic events during spermatogenesis by using a novel specifically targeted gonadal gene set.[35] Two subtracted cDNA libraries, derived from the testes, were collected from mice at different days postpartum, enriched for testis-specific and germ cell-specific genes, and constructed and analyzed by microarray. The expression patterns of the identified genes were classified into 24 clusters. In addition, four selected germ cell-deficient models were used to correlate the expression of groups of genes with the appearance of defined germ cell types, suggesting their cellular expression patterns within the testis. A total of 1748 previously uncharacterized genes showed significant changes during spermatogenesis, indicating their potential roles during spermatogenesis.

Previously, researchers had elucidated the importance of testis-specific gene expression while the exact transcripts and comprehensive gene expression patterns remained unknown. In 2013, large-scale sequencing techniques were introduced into transcriptome research on spermatogenesis.[36] The SOliD 4 next-generation sequencing platform was employed to investigate the gene expression patterns at five different time points during the first wave of murine

spermatogenesis. This work highlighted the up-regulation of spermatogenesis-related biological processes and associated cellular components. In all, 2494 differentially expressed genes were identified, with 160 000 gene isoforms, of which 29% were novel transcripts. Furthermore, a total of 947 up-regulated long noncoding RNAs during the first wave of spermatogenesis were identified. This study provided a very valuable basis for investigating gene isoforms and regulation factors contributing to male fertility.

Transcriptome research has also been used to study the effect of cytokine, hormonal, and environmental factors on germ cells. Aguilar *et al*.[37] have studied the effect of chronic cyclophosphamide treatment on male rat germ cells. A velocity sedimentation cell separator apparatus (STA-PUT) was used to isolate different types of germ cells by their sedimentation coefficient as described by Bellve *et al*.[38] According to their morphology, cell types were identified in different fractions and purities, and fractions with high-purity (>85%) were pooled. Germ cells were separated into pachytene spermatocytes, round spermatids and elongated spermatids. RNA was extracted from these cells and hybridized to rat stress and toxicology atlas arrays. The alterations in stress response genes' expression in different germ cells caused by chronic cyclophosphamide treatment were recorded. Genes were classified according to their response to the treatment in every cell type, and 12 different profiles of expression were obtained. The authors found that the treatment reduced the number of genes detected in all germ cell types. The altered genes included those involved in DNA repair, posttranslational modifications, and anti-oxidant defense of male germ cells. These alterations might have adverse consequences on male fertility and progeny outcome.

Similar work has been done to explore the regulatory effect of androgens on gene expression in the neonatal mouse testis, and the expression of over 100 genes was found to be up- or down-regulated, most of which were expressed by testicular somatic cells, which play a role in forming the spermatogenic micro-environment.[39] These data provided insights into what might occur in normal androgen-supported spermatogenesis in the adult testis.

Other works included a study with the STA-PUT method and immunomagnetic separation to enrich type A spermatogonia to study the changes in the RNA profile to glial cell line-derived neurotrophic factor (GDNF) *in vitro*.[40] GDNF is a known cytokine that promotes the proliferation of cells. It plays a key role in regulating the proliferation and self-renewal of spermatogonial cells. In this work, it was described that the isolated cells proliferated in culture and underwent the first step of spermatogenesis after GDNF stimulation. A total of 19 genes related to cell proliferation, 24 related to cell differentiation, and 7 related to stem cell fate were revealed to be up-regulated, thus identifying signaling pathways that might play a crucial role in maintaining germline stem cell proliferation and renewal.

RESEARCH ON HUMAN BEINGS

Although many informative and intriguing features of spermatogenesis and its dysfunction have been identified in rodent models,[12,41] little is known about the process in human beings. The findings on rodents cannot translate to human beings because of the differences between human and rodent in spermatogenesis. As an ancient and critical reproductive process for all mammals, the basic molecular drive of gametogenesis is essentially conserved, while many assignable differences during the course of evolution have been derived. It has been reported that there are about 15 000 protein-coding genes expressed in the human, versus about 10 000 in the mouse.[42] The most convincing example of the different in gene expression in humans and rodents during spermatogenesis may be an expression in human male germ cells of the *DAZ* gene, which does not exist in the rodent genome.[43] Derived from its autosomal homolog *DAZL*, *DAZ* is the major candidate of the Y chromosome microdeletion region *AZFc*,[44] which usually relates to a disorder of spermatogenesis.[45–48] Therefore, it is obvious that the study of human beings is indispensable.

To study the transcriptional profile of the human germ cell, semen containing mature spermatozoa is the most accessible resource.

Historically, spermatozoa were thought to be a desert for transcription because the highly condensed sperm nucleus was believed to be transcriptionally silent, and most RNA was discarded during spermiogenesis along with the cytoplasm.[49,50] However, in the late 1980s, reports showed that RNA exists in the sperm nuclei of rats and humans.[51,52] Thirteen years later, by using a suite of microarrays containing 27 016 uniquely expressed sequence tags (ESTs), human spermatozoa RNA profiles were determined.[53] About 3000 ESTs were hybridized, demonstrating that the ejaculated spermatozoa contained a variety of RNAs, although the total amount may be low. Why did these RNAs remain in spermatozoa? Some researchers thought that these transcripts were simply residues from the process of spermatogenesis, which reflected the events that occurred during spermatogenesis, while others believed they would play an essential role in postfertilization events. A serial analysis of gene expression (SAGE) library of fertile human ejaculated sperm cDNA was established in 2006, and mRNA transcripts from these spermatozoa were characterized and quantified.[54] More than 2000 unique tags were identified, and 25 functional gene groups were revealed. Besides these, 54 novel tags were identified, which were thought to be candidates of new genes. In subsequent years, the existence of at least 5 novel human testis-specific expressed genes was confirmed via rapid amplification of cDNA ends (RACE) on the basis of these tags (data not published).

From the RNA profile of fertile human spermatozoa, we can speculate on some events that take place during spermatogenesis. By comparing sperm RNA profiles between fertile and infertile men, we could speculate on the importance of these events. However, the time point at which the differences develop is still unknown. With regard to patients with nonobstructive azoospermia (NOA), who have no spermatozoa in their semen, can we explain what went wrong during the generation of their gametes? To answer these questions, it is necessary to distinguish the different transcript profiles from germ cells at various differentiation stages.

Obviously, the methods employed in rodent research are inapplicable to research on the RNA profile of human gametogenesis since the samples are difficult to collect ethically. Therefore, researchers have turned to infertile patients with spermatogenic dysfunction.

Spermatogenic dysfunction can be divided into different histopathological types, from Sertoli cell only (SCO) to hypo-spermatogenesis,[55] and each can serve as a natural model for research on spermatogenesis. Some patients with NOA can use a spermatozoon obtained from the testis via surgery for intracytoplasmic sperm injection (ICSI),[56] and even round spermatids have been reported to fertilize oocytes via round spermatid injection (ROSI).[57] Many NOA patients would choose testicular sperm extraction (TESE) or testicular sperm aspiration (TESA) as the last hope to conceive their own baby.[58] A portion of the tissues obtained from these surgeries were sent for pathological analysis, and a Johnsen's score from 1 to 10 would be given, according their most advanced spermatogenic level present, and which represent SCO, spermatogonia arrest, spermatocyte arrest,

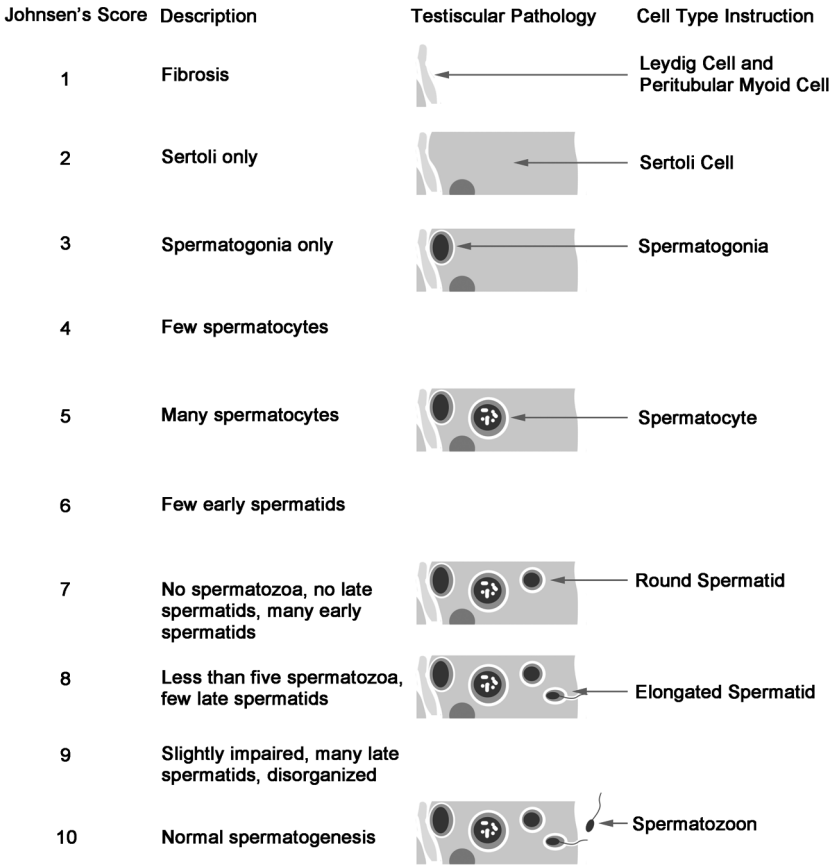

Figure 2: Diagram of Johnsen's score and corresponding testicular appearance. The appearance of spermatogenic dysfunction is classified into 10 levels based on the Johnsen's score. The grading standard is listed in the second panel. The third panel shows the corresponding schematic of the cellular composition of testicular samples used in the work of Chalmel *et al*.[61]

spermatid maturation arrest, hypo-spermatogenesis, and normal spermatogenesis[59,60] (**Figure 2**). These tissues could be used for research purpose with the signed informed consent of the patients. By collecting testicular tissue from patients with different spermatogenic dysfunction, researchers can distinguish genes specifically expressed in germ cells of various differentiation stages by comparing their RNA profiles.

Chalmel *et al*. screened the transcript profiles of 47 biopsies from prepubescent children diagnosed with undescended testis, infertile adult patients whose spermatogenesis was arrested at consecutive stages, and fertile control individuals via GeneChip analysis. The results were integrated with data from enriched normal germ cells, nontesticular expression data, phenotype information, predicted regulatory DNA-binding motif, and interactome data.[61] A total of 3580 genes were recorded and, of them, 933 genes were considered "specific expression in testis," 754 genes were "preferential expression in testis," 676 genes were "intermediate expression in testis" and 1552 genes were "ubiquitous expression." By combining a set of testicular samples lacking a specific cell population and by ranking them by age and an increasingly severe spermatogenic phenotype, the researchers assembled a global transcript profile of postnatal human male gonads before and after puberty. According to their expression patterns and functional annotations, these transcripts were clustered into 13 groups, and we could draw a dynamic curve of the molecular drive at key points of spermatogenesis (**Figure 3**). Furthermore, motif-enrichment profiles in germ cell patterns and their relation to corresponding transcript factors were also analyzed in this work, providing an overview of the key transcription factors involved in the generation of male gametes, which can also be regarded as the switches of germ cell regeneration and differentiation.

This excellent work contributed many valuable findings. However, it is uncertain whether the data derived from patients with deficient spermatogenic function can represent the process occurring in healthy people, and prior research was cited to clarify.[62] In that study, cDNAs derived from enriched germ cells provided by fertile donors were used to hybridize with GeneChip, and the data were compared to those of rats and mice in order to determine the conservation of the transcriptome from humans and rodents in male gametogenesis. On the basis of the diverse size and nucleo-cytoplasmic ratio of germ cells at various differential stages, a modified density gradient centrifugation method was used to enrich pachytene spermatocytes and spermatids (**Figure 4**). An elutriation rotor was used to separate germ cells. This method was first introduced in 1981 to purify rat germ cells,[63] and in 1996, it was used to separate human germ cells in the study of the expression of human leukocyte antigen (HLA) class I genes in meiotic and postmeiotic stages.[64] Germ cells of different morphology could be identified by microscopy in different fractions flowing from the rotor. Enriched pachytene spermatocytes and spermatids, with a purity of 86% and 92%, respectively, could be acquired by collecting specific fractions. With the help of such a method, the authors reported a cross-species whole-genome expression profiling analysis of the male germ-line in mammals. Conserved and differentially expressed meiotic genes in the mouse, rat, and human were identified. Though many

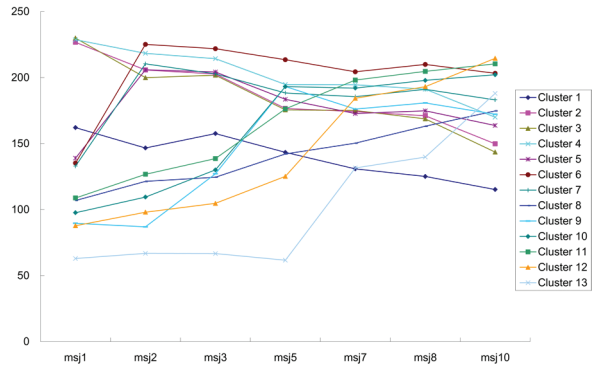

Figure 3: The dynamic curve of molecular drive during spermatogenesis. The curve was drawn according to the information provided by Chalmel *et al.*[61] Different spermatogenic levels (represented in the form of Johnsen's score) are arranged according to the appearance of specific germ cells. Genes with different expression patterns fall into 13 clusters. Enriched gene ontology annotations of some clusters are listed; the number in parentheses indicates the number of corresponding genes. Cluster 1: cell-cell adhesion (13), cell-cell adherens junction (7), nervous system (38) and heart (16) development, cell differentiation (42), signaling (61), plasma membrane (68). Cluster 2: collagen (5). Clusters 3 and 4: cell differentiation (61), cell adhesion (31), regulation of cell proliferation (37), extra-cellular region (93), cell surface (23), extra-cellular matrix organization (17), lipid metabolic processes (42), steroid metabolism (19), cellular hormone processes (9), anatomical structure development (89), platelet alpha granule (9), platelet degranulation (10), collagen (7). Clusters 5 and 6: anatomical structure development (62), nervous system (34), reproductive structure (10), and male gonad (7) development, cell adhesion (23), cell differentiation (43), migration (16). Cluster 9: regulation of gene expression (46), gene silencing (7), DNA methylation (5), cell cycle (32), mitosis (14), cell division (13), nucleus (88), chromosome (15), reproduction (23), spermatogenesis (13), *P* granule (5). Cluster 10: cell cycle (108), nucleus (201), cell-cycle checkpoint (30), DNA repair (23), replication (19), histone exchange (6), cell division (54), mitosis (50), cytoskeleton (72), microtubule (25), spindle (26), meiosis (26), chromosome (46), synaptonemal complex (6), chromosome organization (45), reproduction (43), spermatogenesis (27), germ cell development (13). Cluster 11: cytoskeleton (56), microtubule (20), cilium (26), flagellum (8), axoneme (9), dynein complex (6), spermatogenesis (29), spermatid differentiation (8), cilium morphogenesis (7), cell division (21), cell cycle (50), mitosis (20). Cluster 12: reproduction (42), spermatogenesis (28), flagellum (7), acrosome vesicle (8), dynein complex (6). Cluster 13: reproduction (52), sperm motility (8), fertilization (11), spermatogenesis (35), flagellum (7), acrosome vesicle (7).

differentially expressed genes existed, it was revealed that there were hundreds of conserved genes expressed in all three species during the critical meiotic and postmeiotic stages of sexual reproduction. Some of these genes are listed in **Table 1**.

RESEARCH ON NONCODING RNA PROFILES

For a long time, the research on transcripts had been focused on the expression of mRNAs that could be translated into proteins because proteins were considered the executors of life processes. However, previous work revealed that noncoding RNAs also play important roles in the regulation of biological functions.[65] These RNAs include microRNAs (miRNAs), PiWi-interacting RNAs (piRNAs), and long noncoding RNAs (lncRNAs). Many papers have reported the essential roles of miRNAs and piRNAs during the process of spermatogenesis. miRNAs can silence the translation of their targets, thus playing a posttranscriptional regulatory role. Since germ cells are periodically transcriptionally silenced during spermatogenesis, posttranscriptional regulation is essential for germ cells.[66] Disruption of miRNA synthetic pathways can reduce the spermatogonial population in mice, even leading to an SCO phenotype.[67,68] The expression of many miRNAs has

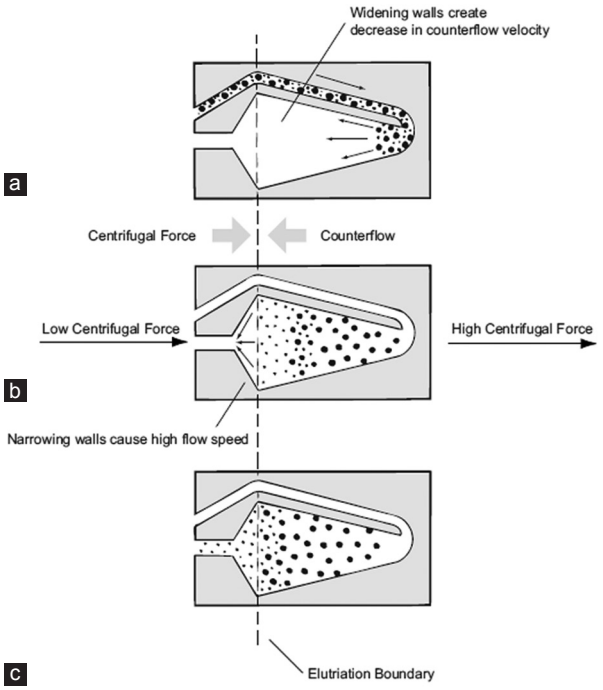

Figure 4: The elutriation process. This figure is derived from the user's manual of the JE-5.0 Elutriation System (Beckman Coulter, Inc., Canada) to illustrate how an elutriation rotor separates different cells according their sedimentation. (a) Cells suspended in a medium are pumped into the chamber; (b) Centrifugal sedimentation of cells is balanced by flow velocity; (c) Flow increased smaller, slowly sedimenting cells elutriate out of the chamber.

been shown to change significantly during male germ cell development, which is coincident with the highly active posttranscriptional control of gene expression during this process.[66,69,70] Large-scale gene transcription occurs before the cells enter quiescence during the process of meiosis and postmeiotically before nuclear silencing. Two-thirds of the transcripts generated at these stages are stored for later translation. These findings suggest a potential role of miRNA in posttranscriptional regulation during spermatogenesis. However little is known about the changes in the germ cell miRNA expression profile during spermatogenesis.

Microarrays have been widely used in miRNA expression profile studies. Yan *et al.*[70] employed an miRNA microarray covering 892 miRNAs to evaluate the expression of miRNAs in the mouse testis. It was found that the expression of 19 miRNA was significantly different between immature and mature individuals, including 14 up-regulated and 5 down-regulated miRNAs in immature mice. The putative target genes and the crucial roles of these miRNAs during spermatogenesis were predicted and identified (**Table 2**). Similar work was performed in 2010 – an miRNA microarray containing 1260 miRNA probes was used to profile miRNA expression between sexually immature and mature pig testes.[71] More differentially expressed miRNAs were identified and associated with spermatogenesis. Yan *et al.*[72] screened miRNA expression in the testis of primates via microarray. Samples included the adult human testis and mature and immature rhesus monkey testes. In all, 62 miRNAs were found to be differentially expressed in mature and immature rhesus monkey testes, while a total of 76 miRNAs were found to be differentially expressed in adult human and mature rhesus monkey testes. This work suggested a putative function of miRNA in spermatogenesis, along with a species-specific expression of miRNA in the mammalian testis.

Table 1: Genes that are conserved, differentially-expressed and specific to the testis[62]

Expression stage	*Conserved genes*
Somatic	*Gata4*, *Abcal*
Mitotic	*Osr2*, *Pcdh18*, *Dmrt1*
Meiotic	*4632434I11Rik*, *Gprk2l*, *Spdy1*, *3300001k11Rik*, *D930005D10Rik*, *4933417K04Ril*, *Ccna1*, *Acrbp*, *Adam18*, *4921513E08Rik*, *Cage1*, *Als2cr11*, *4930550C14Rik*, *4930451G09Rik*, *Nmnat3*, *Aurkc*, *2700059L22Rik*, *Zpbp*, *Gsto2*, *Rnf190*, *Adam2*, *9630025C22*, *Ldhal6b*, *Ppp3r2*, *Tnp1*, *Dmrtb1*, *Ypel1*, *4930518F03Rik*, *Nasp*, *Tcfl5*, *Lrrc27*, *Ribc2*, *Pla2g6*, *Tsga10*, *A530057A03Rik*, *Als2cr15*, *Hexim2*, *4930524B15Rik*, *Igf2bp3*, *4932413O14Rik*, *Kbtbd8*
Postmeiotic	*Tulp2*, *Fscn3*, *4933417A18Rik*, *Ttll9*, *4933401K09Rik*, *Odf1*, *Odf3*, *Ubqln3*, *Capza3*, *1700095F04Rik*, *Tssk2*, *4931407G18Rik*, *Mell1*, *Sufu*, *Socs7*, *4921517J23Rik*, *Rffl*, *2510048L02Rik*, *4930432J16Rik*, *Slco6b1*, *4931417G12Rik*, *Spaca3*, *Akap3*, *Ankrd5*, *Klhl10*, *Tnp2*, *Akap3*, *Cst8*, *1700027D21Rik*, *Zfp294*, *1700120E14Rik*, *C230086A09Rik*, *1700026H06Rik*

Table 2: Target genes for miRNA identified in germ cells[70]

miRNA name	*Chromosome location*	*Fold change*	*Putative targets*
mmu-miR-411	12	77.3211	*Usp42*
mmu-miR-335	6	11.7969	*Ccnt2*, *CcnD2*, *Rsbn1*
mmu-miR-434-5p	12	10.957	N/A
mmu-miR-337	12	8.4267	*Ybx1*, *Ap1g1 Taf5*, *Taf12*, *Creb1*, *Ccnl1*
mmu-miR-379	12	8.266	*Eif4g2*, *Edn1*, *Rnf6*
mmu-miR-127	12	7.2991	*Brd2*
mmu-miR-376a	12	6.8128	N/A
mmu-miR-214	1	5.2029	*Hspd1*, *Tex27*, *Adcyap1r1*, *Hbp1*, *Ap1g1*, *Ssr1*
mmu-miR-181c	8	4.9265	*Sox6*, *Sox5*, *Rsbn1*, *Adm*, *Tnpo1*, *Dazap2*, *Kpnb1*
mmu-miR-361	X	4.1878	*Kpnb1*, *Zfp148*, *Bmpr2*, *Calm2*
mmu-let-7e	17	4.1362	*Nr6a1*, *Taf5*, *Fasl*, *Eif4g2*, *Suv39h2*, *Dzip1*, *Dd×19b*
mmu-miR-181b	1	3.6619	*Tbp11*, *Rsbn1*, *Adm*, *Dazap2*, *Pik3r3*, *Rnf6*
mmu-miR-34a	4	0.4448	*CcnD2*, *Bcl2*, *Gmfb*
mmu-miR-29b	6	0.1871	*Creb5*, *Bak1*, *Usp42*, *Mlf1*, *Hbp1*, *Snx24*
mmu-miR-449	13	0.067	*Sox11*, *CcnE2*, *Bcl2*, *Gmfb*
mmu-miR-34c	11	0.0126	*SPAG4*, *Ccnl1*, *Zfp148*, *Gmfb*

Work has also been done in comparing the expression of miRNA in the testis of patients with NOA to those of fertile controls, in attempts to determine the relationship between miRNA and spermatogenic dysfunction.[73] Again, a microarray was employed to screen the differentially expressed miRNAs. A total of 154 down-regulated and 19 up-regulated miRNAs was observed. Several of these miRNAs were identified as having oncogenic potential.

Since NOA can be divided into different histopathologic patterns, such work could be considered crude. Therefore, Abu-Halima *et al*. screened miRNA expression profiles in human testicular tissue from infertile men with different histopathological patterns.[74] The testicular biopsies derived from patients with azoospermia were classified into four groups – SCO, mixed atrophy, germ cell arrest at the spermatocyte stage, and normal spermatogenesis. Hundreds of differentially expressed miRNAs were identified via microarray, of which five miRNAs were validated and shown by bioinformatics to be involved in apoptosis, cell proliferation, and differentiation. The putative targets of these five miRNAs are known to be involved in spermatogenesis.

Another group of noncoding RNAs, piRNA, is also important in spermatogenesis.[75–77] These small RNAs are abundantly expressed in animal gonads. By suppressing the activity of retro-transposons, piRNAs ensure the stability of the transfer of genetic material.[78]

By using a gravity sedimentation procedure, Gan *et al*.[79] isolated type A spermatogonia, pachytene spermatocytes, and round spermatids from mice of different ages, according to the timing at the first wave of spermatogenesis. Then, deep sequencing was employed to determine the expression profile of small RNAs in these cells. The authors focused on the expression of potential piRNAs. It was found that the transcripts of a large number of genes involved in spermatogenesis were precursors of piRNAs and were intricately regulated by alternative splicing and antisense transcripts.

Other work profiled small RNAs in normal human testes via next-generation sequencing technology.[80] A total of 775 miRNAs and 20 121 piRNAs were detected. Again, the important roles played by these small RNAs were indicated by the nature of their putative targets.

Besides the small RNAs noted above, lncRNAs are also considered important for regulating biological processes.[81–84] Although the mechanism is still unclear, their potential roles in spermatogenesis have attracted researchers. Bao *et al*. collected mouse testes at 6 times points, corresponding to the specific timing of murine spermatogenesis.[85] The transcriptomes of these organs were profiled by microarray, and thousands of lncRNAs were shown to be differentially expressed at the six critical time points during spermatogenesis. The results indicated that lncRNAs might function to regulate gene expression during male germ-line development. Other similar work compared the lncRNA profile obtained via microarray between neonatal and adult mice, and 3025 differentially-expressed lncRNAs were identified.[86] Many of these RNAs were related to key transcription factors and other genes involved in spermatogenesis, again emphasizing the importance of lncRNAs during spermatogenesis.

ONLINE DATABASE

There is a huge amount of data obtained from transcriptome research, and the perspectives of researchers may vary. Therefore, it is necessary to establish a database to share the data obtained from different works.

The National Center for Biotechnology Information's (NCBI) Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) is a public genomics data repository supporting MIAME-compliant data submissions, which may be the most widely used database for array- and sequence-based data. Any researcher can download data from GEO and upload their own data. Some works mentioned in this article cited the data from former research provided by GEO to draw comparisons to their own experimental data, thus supporting their findings. For example, Chalmel *et al*.[62] reported 45 nontesticular data sets, each covering one tissue sample from GEO, to determine the extent to which transcripts are specifically present in male gonads before and after puberty. They also reported that 57 mouse MG430 2.0 CEL files were downloaded from the NCBI GEO public repository, corresponding to five testicular cell types (spermatogonia A and B, pachytene spermatocytes, round spermatids, total testis), two female gonadal tissues, including the oocyte and ovary, and 17 normal somatic tissues, including the aorta, brain, CD4+ naïve cells, day 7 embryo, and eye, etc., GEO shares a wide range of transcriptome data derived from related studies. Sharing the research data can save many resources for transcriptome research and make full use of existing research results, thus helping researchers achieve more by referring to prior research.

GEO is a comprehensive database for transcriptome research, but it also has some limitations. Since germ cells are so different from other cell types, a database focused on germ cells is needed.

In 2003, GermOnline (http://www.germonline.org), a cross-species community annotation database on germ-line development and gametogenesis, was developed.[87] GermOnline includes an interactive platform for cross-species gene annotation by research scientists. It allows researchers to contribute directly and update knowledge about genes of interest covering mitosis, meiosis, and germ cell development, in co-operation with a team of database developers and scientific curators. This system permits up-to-date information to be readily available and facilitates species comparisons in a wide variety of areas that are now laborious to execute. Predictably, with the help of GermOnline, the study of germ-line transcriptomes will be more efficient.

RESEARCH STRATEGY

As mentioned above, the key to transcriptome research on the molecular drive of spermatogenesis is to determine the specific gene expression profiles of germ cells at the various differential stages, although the separation of different germ cells is rather difficult.

cDNA subtraction was a good idea in the research of germ cells; there was no need to isolate target germ cells from the testis when this method was applied. If we know the timing of the first spermatogenetic wave, we could know the exact cell composition of the corresponding developmental stages. By comparing the transcriptional profiles of whole testicular tissue at different developmental stages, we could extract the expression profile of the specific type of cells. Such a method has been widely used in the work on rodents[19,34,35,88] but is not suitable for human beings owing to ethical concerns. Although men with a particular spermatogenesis arrest could provide a natural model for such research,[61] the limitations are also obvious. First, the data obtained from patients with deficient spermatogenesis cannot represent normal human spermatogenesis completely; second, the subtraction method can only provide "differential expression" data, but fails to offer "common expression" data. The data obtained via the subtraction method reflect the changes in the abundance of genes caused by the absence of specific cell types, not the changes caused by the differentiation of the germ cells. Therefore, it is essential to separate every type of germ cell for transcriptome research.

Since there are obvious morphological differences that distinguish various cell types in the testis, altered sedimentation conditions can be used to isolate cells of diverse types from the total testicular cell population. As stated, density gradient centrifugation and STA-PUT have been widely used in this field.[40,63,64,89–94] Each method can produce high-purity specific cell populations if operated properly. However, these methods are limited because of the requirement for specific devices, such as an elutriation rotor or STA-PUT, which are not popular in laboratories. The cellular yield is also inversely proportional to cell purity. Large amounts of tissue are required if we want to obtain a sufficient yield of high-purity samples. However, the samples collected from surgery are usually very small and nearly impossible to separate with these methods, possibly explaining why there are few reports on the research on the transcriptome during human spermatogenesis. A comparison of the reported methods and their applications is shown in **Table 3**.

Another feasible method would be fluorescence-activated cell sorting (FACS). Flow cytometry has been widely used in ploidy analysis. Germ cells in the testis consist of haploid cells (spermatids and spermatozoa), diploid cells (including spermatogonial stem cells, spermatogonia, and secondary spermatocytes), and tetraploid (double diploid) cells, which are primary spermatocytes. These cells possess different numbers of chromosomes that can be stained with DNA dyes. When stained with a fluorescent DNA dye, different fluorescence intensities reflect the difference in DNA content, and cells with different ploidy can be distinguished easily by flow cytometry (**Figure 5**). It has been reported that germ cells derived from the testis can be separated into spermatogonia, preleptotene, leptotene-zygotene, early-pachytene, middle-pachytene, late-pachytene, diplotene, and round spermatids via FACS.[95] Before sorting, testicular-derived cells would be cultured for at least 24 h to remove Sertoli cells and other somatic cells that attach to the culture dishes. The cell suspension, which mainly consists of germ cells, is collected, and the cells stained with Hoechst 33342, a dye that can stain the DNA in living cells. These cells would then be sorted by a fluorescence-activated cell sorter into haploid cells, diploid cells, and tetraploid cells.

As the diploid cells mainly comprise spermatogonial cells and secondary spermatocytes, further processing should be carried out to enrich the spermatogonial cells for research purposes.

Magnet-activated cell sorting (MACS) is a generally accepted method to separate spermatogonial cells. Surface antigens such as G protein-coupled receptor 125 (GPR125) and GDNF family receptor alpha 1 (GFRA1) are used as specific markers to isolate spermatogonial stem cells via MACS.[96,97] Since there are not magnetic beads labeled directly with antibodies for these antigens, the cells need to be incubated with a corresponding primary antibody overnight, before incubation with the magnetic beads labeled with a secondary antibody. This procedure may influence cell viability. Fortunately, there are commercially available anti-CD90 antibody-labeled magnetic beads, and CD90 is one of the markers of undifferentiated spermatogonial cells.[98] Though CD90 is also expressed by hematopoietic cells and mesenchymal stem cells,[97] considering the low abundance of these cells in the testis, it is reasonable to use CD90 to enrich undifferentiated spermatogonial cells from FACS-sorted diploid germ cells.

The combination of FACS and MACS could provide highly enriched undifferentiated spermatogonial cells, primary spermatocytes, and spermatids/spermatozoa for transcriptome research purposes.

Besides the methods used to isolate different germ cells, the methods used to profile the transcriptome are also important for transcriptome research on spermatogenesis. There are two kinds of methods used to generate the expression profile of genes: microarrays (GeneChips) and RNA-Seq.

Microarrays, or GeneChip, are a technology that uses immobilized probes to detect large numbers of transcripts. The probes are designed according to known or predicted transcript sequences. When the probes hybridize with the corresponding transcripts, fluorescent signals can be detected, and the intensity of these signals can be correlated to the abundance of these transcripts. Such high-throughput technology is very efficient, and the cost is relatively low. However, it cannot detect novel transcripts whose sequences are unknown, and it can hardly recognize mutants of the transcripts. On the other hand, RNA-Seq, a technology based on sequencing, can compensate for these shortages.

In the RNA-Seq procedure, a cDNA library is constructed, and cDNAs are broken into small fragments connected in series. The library is sequenced and the recorded sequences assembled according to the information provided by the genome database. The abundance of the original transcripts is reflected by "reads." RNA-Seq is more sensitive than microarrays. About 18% of low abundance transcripts will be lost during microarray analysis, which can be detected using RNA-Seq.[99] With the progress of sequencing technology, RNA-Seq has become increasingly sensitive, and even trace transcripts can be detected.

Table 3: A comparison of methods and their applications

Principle	*Species*	*Methods to distinguish transcriptomic profile*	*Main discovery*	*Pros*	*Cons*	*References*
Subtraction	Mouse	Using testes derived tissue from mice 3 days from days 1–29 and at day 60 postpartum. Transcriptome profiled by Affymetrix Murine Genome U74v2 A, B, and C arrays	A series of meiotic/postmeiotic specific genes	No need to isolate germ cells from the various differential phases and is easy to perform	The actual abundance of these genes in specific cell types remains unknown. Not suitable for research on humans	Schultz *et al*. 2003[19]
	Mouse	Using testes derived from mice at days 0, 3, 6, 8, 10, 14, 18, 20, 30, 35, and 56 postpartum. Transcriptome profiled by Affymetrix Murine Genome U74v2 A, B, and C arrays	Types A and B spermatogonia demonstrated a similar expression profile to that of Sertoli cells, many of which are down-regulated when germ cells enter meiosis. The spermatocytes and spermatids had enriched genes, including genes involved in forming sperm structure, DNA reorganization and condensation, unique metabolism, and sperm-egg binding and motility			Shima *et al*. 2004[34]
	Mouse	Using testes derived from mice at days 1, 5, 10, 15, 23, 35, and 56 postpartum and 4 mutant mouse models of male infertility. Transcriptome profiled by a customized specifically-targeted gonadal gene array	The majority of clones showed their largest changes in expression between days 10 and 23, which are associated with the presence of primary spermatocytes or round spermatids			Ellis *et al*. 2004[35]
	Mouse	Using testes derived from mice at days 7, 14, 17, 21, and 28 postpartum. Transcriptome profiled by SOliD 4 next-generation sequencing	Cell cycle processes and chromosome processes were upregulated during the transition from spermatogonia to early meiotic cells. Acrosome reaction and sperm-egg fusion-related terms were enriched during postmeiotic spermiogenesis			Laiho *et al*. 2013[36]
	Human	Using testes derived from patients with different types of spermatogenic dysfunction, the transcriptome was profiled by U133 Plus 2.0 GeneChips	Provides an initial glimpse into the complex regulatory network controlling germ-line development	No need to isolate germ cells of different differential phases, easy to perform	The actual abundance of these genes in specific type of cells remains unknown. Results cannot represent normal people	Chalmel *et al*. 2012[61]
Density gradient sedimentation	Mouse, rat, and human	Isolating different germ cell from an elutriation rotor. Transcriptome profiled by Affymetrix human U133 Plus 2.0, mouse MG 430 2.0 and rat RG 230 2.0 GeneChips, respectively	Hundreds of conserved genes were obtained in all three species during the critical meiotic and postmeiotic stages of sexual reproduction	The actual abundance of these genes in specific cell types was clearly demonstrated	Low yield of isolated cells, litter individual repeat	Chalmel *et al*. 2007[62]

Because of this sensitivity, results from both of these methods need to be validated to check for false positive results. As it is reported that data obtained from RNA-Seq are more consistent with the results of later qPCR verification than those of microarrays,[99] the application of RNA-Seq in transcriptome research should increase. Especially in the research on human germ cell development, RNA-Seq should be more feasible owing to the limitation of human samples.

PROSPECT

Transcriptome research on spermatogenesis could provide an overall view of the molecular events accompanying male gamete production. There is a view that the proteome is more important because proteins carry out biological functions. However, many genes are found to be noncoding genes, which have no corresponding protein products. These genes still play an important role in biological processes, including spermatogenesis, by regulating the transcription and translation of other genes and even epigenetic modifications.[65] These genes include lncRNAs,[100] miRNAs,[101,102] and piRNAs,[103,104] which are neglected by proteomic research. Transcriptome research can provide more complete information about the molecular drive of spermatogenesis.

Transcriptome research on spermatogenesis will likely generate massive amounts of data, which will be analyzed using various methods. We could compare the difference in the RNA profiles of germ cells at various differentiated stages, which would allow us to determine the molecules involved in specific processes during spermatogenesis. We could screen the RNA profiles of the germ cells from infertile patients with spermatogenic dysfunction and determine the molecular deficiency causing germ cell differentiation arrest or hypo-spermatogenesis. Finally, we could reveal the key genes that

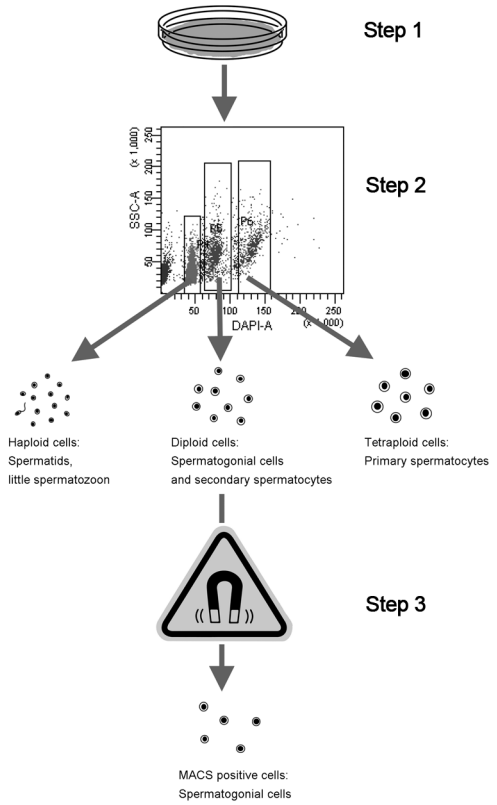

Figure 5: A diagram of germ cell separation by the use of FACS and MACS. Testicular cells can be sorted into spermatogonial cells, primary spermatocytes, and spermatids via FACS and MACS. Step 1: testicular samples are treated with a two-step enzymatic digestion. The derived cells are cultured on gelatin-coated dishes to remove Sertoli cells and other somatic cells, which attach to the dish. Step 2: the nonbinding cells are collected and stained with Hoechst 33342 nuclear fluorescent dye. The stained cells are sorted via FACS according their DNA content. Step 3: since both spermatogonial cells and secondary spermatocytes are diploid cells, MACS is employed to enrich the spermatogonial cells by using an antibody against spermatogonial cell-specific surface antigens. These sorted cells represented three typical germ cell populations appearing during spermatogenesis and are capable of being used for transcriptome research purposes. FACS: fluorescence-activated cell sorting; MACS: magnet-activated cell sorting.

regulate the process of spermatogenesis; without the drive from their products, this process would be impaired. It would be possible to customize a biochip to detect these genes as a marker of male infertility for clinical purposes, and then we could determine the causes of infertility and provide targeted treatments.

AUTHOR CONTRIBUTIONS SECTION

ZJZ wrote this review manuscript. SY and ZL helped to revise the paper.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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REFERENCES

1 Clermont Y. Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiol Rev* 1972; 52: 198–236.
2 Moreno RD, Ramalho-Santos J, Sutovsky P, Chan EK, Schatten G. Vesicular traffic and golgi apparatus dynamics during mammalian spermatogenesis: implications for acrosome architecture. *Biol Reprod* 2000; 63: 89–98.
3 Cataldo L, Baig K, Oko R, Mastrangelo MA, Kleene KC. Developmental expression, intracellular localization, and selenium content of the cysteine-rich protein associated with the mitochondrial capsules of mouse sperm. *Mol Reprod Dev* 1996; 45: 320–31.
4 Oko R. Occurrence and formation of cytoskeletal proteins in mammalian spermatozoa. *Andrologia* 1998; 30: 193–206.
5 Meistrich ML, Mohapatra B, Shirley CR, Zhao M. Roles of transition nuclear proteins in spermiogenesis. *Chromosoma* 2003; 111: 483–8.
6 de Rooij DG. Stem cells in the testis. *Int J Exp Pathol* 1998; 79: 67–80.
7 de Rooij DG, Russell LD. All you wanted to know about spermatogonia but were afraid to ask. *J Androl* 2000; 21: 776–98.
8 Huckins C. The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. *Anat Rec* 1971; 169: 533–57.
9 Caires K, Broady J, McLean D. Maintaining the male germline: regulation of spermatogonial stem cells. *J Endocrinol* 2010; 205: 133–45.
10 Comhaire F. Towards more objectivity in the management of male infertility. The need for a standardized approach. *Int J Androl* 1987; 7: 1–53.
11 De Kretser D, Baker H. Infertility in men: recent advances and continuing controversies. *J Clin Endocrinol Metab* 1999; 84: 3443–50.
12 Jamsai D, O'Bryan MK. Mouse models in male fertility research. *Asian J Androl* 2011; 13: 139–51.
13 Massart A, Lissens W, Tournaye H, Stouffs K. Genetic causes of spermatogenic failure. *Asian J Androl* 2012; 14: 40–8.
14 Wrobel G, Primig M. Mammalian male germ cells are fertile ground for expression profiling of sexual reproduction. *Reproduction* 2005; 129: 1–7.
15 Russell LD, Ettlin RA, Hikim APS, Clegg ED. Histological and Histopathological Evaluation of the Testis. International journal of andrology 1993; 16: 83.
16 Oakberg EF. Duration of spermatogenesis in the mouse. *Nature* 1957; 180: 1137–8.
17 Oakberg EF. Duration of spermatogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium. *Am J Anat* 1956; 99: 507–16.
18 Bellve AR, Cavicchia JC, Millette CF, O'Brien DA, Bhatnagar YM, *et al*. Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization. *J Cell Biol* 1977; 74: 68–85.
19 Schultz N, Hamra FK, Garbers DL. A multitude of genes expressed solely in meiotic or postmeiotic spermatogenic cells offers a myriad of contraceptive targets. *Proc Natl Acad Sci U S A* 2003; 100: 12201–6.
20 Miki K, Willis WD, Brown PR, Goulding EH, Fulcher KD, *et al*. Targeted disruption of the Akap4 gene causes defects in sperm flagellum and motility. *Dev Biol* 2002; 248: 331–42.
21 Adham IM, Nayernia K, Burkhardt-Göttges E, Topaloglu Ö, Dixkens C, *et al*. Teratozoospermia in mice lacking the transition protein 2 (Tnp2). *Mol Hum Reprod* 2001; 7: 513–20.
22 Ren D, Navarro B, Perez G, Jackson AC, Hsu S, *et al*. A sperm ion channel required for sperm motility and male fertility. *Nature* 2001; 413: 603–9.
23 Shamsadin R, Adham IM, Nayernia K, Heinlein UA, Oberwinkler H, *et al*. Male mice deficient for germ-cell cyritestin are infertile. *Biol Reprod* 1999; 61: 1445–51.
24 Narisawa S, Hecht NB, Goldberg E, Boatright KM, Reed JC, *et al*. Testis-specific cytochrome c-null mice produce functional sperm but undergo early testicular atrophy. *Mol Cell Biol* 2002; 22: 5554–62.
25 Cho C, Bunch DO, Faure JE, Goulding EH, Eddy EM, *et al*. Fertilization defects in sperm from mice lacking fertilin β. *Science* 1998; 281: 1857–9.
26 Nayernia K, Adham IM, Burkhardt-Göttges E, Neesen J, Rieche M, *et al*. Asthenozoospermia in mice with targeted deletion of the sperm mitochondrion-associated cysteine-rich protein (Smcp) gene. *Mol Cell Biol* 2002; 22: 3046–52.
27 Kashiwabara SI, Noguchi J, Zhuang T, Ohmura K, Honda A, *et al*. Regulation of spermatogenesis by testis-specific, cytoplasmic poly (A) polymerase TPAP. *Science* 2002; 298: 1999–2002.
28 Cho C, Willis WD, Goulding EH, Jung-Ha H, Choi YC, *et al*. Haploinsufficiency of protamine-1 or-2 causes infertility in mice. *Nat Genet* 2001; 28: 82–6.
29 Uhrin P, Dewerchin M, Hilpert M, Chrenek P, Schöfer C, *et al*. Disruption of the protein C inhibitor gene results in impaired spermatogenesis and male infertility. *J Clin Invest* 2000; 106: 1531.
30 Baba D, Kashiwabara SI, Honda A, Yamagata K, Wu Q, *et al*. Mouse sperm lacking cell surface hyaluronidase PH-20 can pass through the layer of cumulus cells and fertilize the egg. *J Biol Chem* 2002; 277: 30310–4.
31 Pearse RV, Drolet DW, Kalla KA, Hooshmand F, Bermingham JR, *et al*. Reduced fertility in mice deficient for the POU protein sperm-1. *Proc Natl Acad Sci* 1997; 94: 7555–60.
32 Mannan AU, Nayernia K, Mueller C, Burfeind P, Adham IM, *et al*. Male mice lacking the Theg (testicular haploid expressed gene) protein undergo normal spermatogenesis and are fertile. *Biol Reprod* 2003; 69: 788–96.

33 Yu YE, Zhang Y, Unni E, Shirley CR, Deng JM, *et al*. Abnormal spermatogenesis and reduced fertility in transition nuclear protein 1-deficient mice. *Proc Natl Acad Sci* 2000; 97: 4683–8.
34 Shima JE, McLean DJ, McCarrey JR, Griswold MD. The murine testicular transcriptome: characterizing gene expression in the testis during the progression of spermatogenesis. *Biol Reprod* 2004; 71: 319–30.
35 Ellis PJ, Furlong RA, Wilson A, Morris S, Carter D, *et al*. Modulation of the mouse testis transcriptome during postnatal development and in selected models of male infertility. *Mol Hum Reprod* 2004; 10: 271–81.
36 Laiho A, Kotaja N, Gyenesei A, Sironen A. Transcriptome profiling of the murine testis during the first wave of spermatogenesis. *PLoS One* 2013; 8: e61558.
37 Aguilar-Mahecha A, Hales BF, Robaire B. Chronic cyclophosphamide treatment alters the expression of stress response genes in rat male germ cells. Biol Reprod 2002; 66: 1024–32.
38 Bellve AR, Millette CF, Bhatnagar YM, O'Brien DA. Dissociation of the mouse testis and characterization of isolated spermatogenic cells. *J Histochem Cytochem* 1977; 25: 480–94.
39 Zhou Q, Shima JE, Nie R, Friel PJ, Griswold MD. Androgen-regulated transcripts in the neonatal mouse testis as determined through microarray analysis. *Biol Reprod* 2005; 72: 1010–9.
40 Hofmann MC, Braydich-Stolle L, Dym M. Isolation of male germ-line stem cells; influence of GDNF. *Dev Biol* 2005; 279: 114–24.
41 Jamsai D, O'Bryan MK. Mouse models as tools in fertility research and male-based contraceptive development. *Handb Exp Pharmacol* 2010; 198: 179–94.
42 Su AI, Wiltshire T, Batalov S, Lapp H, Ching KA, *et al*. A gene atlas of the mouse and human protein-encoding transcriptomes. *Proc Natl Acad Sci U S A* 2004; 101: 6062–7.
43 Reynolds N, Cooke HJ. Role of the DAZ genes in male fertility. *Reprod Biomed Online* 2005; 10: 72–80.
44 Yu YH, Lin YW, Yu JF, Schempp W, Yen PH. Evolution of the DAZ gene and the AZFc region on primate Y chromosomes. *BMC Evol Biol* 2008; 8: 96.
45 Fernandes S, Huellen K, Goncalves J, Dukal H, Zeisler J, *et al*. High frequency of DAZ1/DAZ2 gene deletions in patients with severe oligozoospermia. *Mol Hum Reprod* 2002; 8: 286–98.
46 Fernandes S, Paracchini S, Meyer L, Floridia G, Tyler-Smith C, *et al*. A Large AZFc Deletion Removes DAZ3/DAZ4and Nearby Genes from Men in Y Haplogroup N. *Am J Hum Genet* 2004; 74: 180–7.
47 Foresta C, Moro E, Ferlin A. Y chromosome microdeletions and alterations of spermatogenesis. *Endocr Rev* 2001; 22: 226–39.
48 Rives N. Y chromosome microdeletions and alterations of spermatogenesis, patient approach and genetic counseling. *Ann Endocrinol* 2014; 75: 112–4.
49 Cooper TG. Cytoplasmic droplets: the good, the bad or just confusing? *Hum Reprod* 2005; 20: 9–11.
50 Dadoune JP. The nuclear status of human sperm cells. *Micron* 1995; 26: 323–45.
51 Rejon E, Bajon C, Blaize A, Robert D. RNA in the nucleus of a motile plant spermatozoid: characterization by enzyme-gold cytochemistry and *in situ* hybridization. *Mol Reprod Dev* 1988; 1: 49–56.
52 Pessot CA, Brito M, Figueroa J, Concha, II, Yanez A, *et al*. Presence of RNA in the sperm nucleus. *Biochem Biophys Res Commun* 1989; 158: 272–8.
53 Ostermeier GC, Dix DJ, Miller D, Khatri P, Krawetz SA. Spermatozoal RNA profiles of normal fertile men. *Lancet* 2002; 360: 772–7.
54 Zhao Y, Li Q, Yao C, Wang Z, Zhou Y, *et al*. Characterization and quantification of mRNA transcripts in ejaculated spermatozoa of fertile men by serial analysis of gene expression. *Hum Reprod* 2006; 21: 1583–90.
55 Ali A, Derar R, Al-Sobayil F, Mehana S, Al-Hawas A. Impotentia generandi in male dromedary camels: clinical findings, semen characteristics, and testicular histopathology. *Theriogenology* 2014; 82: 890–6.
56 Esteves SC, Miyaoka R, Agarwal A. Sperm retrieval techniques for assisted reproduction. *Int Braz J* 2011; 37: 570–83.
57 Tesarik J, Mendoza C, Testart J. Viable embryos from injection of round spermatids into oocytes. *N Engl J Med* 1995; 333: 525.
58 Esteves SC, Miyaoka R, Orosz JE, Agarwal A. An update on sperm retrieval techniques for azoospermic males. *Clinics (Sao Paulo)* 2013; 68 Suppl 1: 99–110.
59 Johnsen S, Agger P. Quantitative evaluation of testicular biopsies before and after operation for varicocele. *Fertil Steril* 1978; 29: 58–63.
60 Agger P, Johnsen S. Quantitative evaluation of testicular biopsies in varicocele. *Fertil Steril* 1978; 29: 52–7.
61 Chalmel F, Lardenois A, Evrard B, Mathieu R, Feig C, *et al*. Global human tissue profiling and protein network analysis reveals distinct levels of transcriptional germline-specificity and identifies target genes for male infertility. *Hum Reprod* 2012; 27: 3233–48.
62 Chalmel F, Rolland AD, Niederhauser-Wiederkehr C, Chung SS, Demougin P, *et al*. The conserved transcriptome in human and rodent male gametogenesis. *Proc Natl Acad Sci U S A* 2007; 104: 8346–51.
63 Meistrich ML, Longtin J, Brock WA, Grimes SR Jr, Mace ML. Purification of rat spermatogenic cells and preliminary biochemical analysis of these cells. *Biol Reprod* 1981; 25: 1065–77.
64 Guillaudeux T, Gomez E, Onno M, Drenou B, Segretain D, *et al*. Expression of HLA class I genes in meiotic and post-meiotic human spermatogenic cells. *Biol Reprod* 1996; 55: 99–110.
65 Mukherjee A, Koli S, Reddy KV. Regulatory non-coding transcripts in spermatogenesis: shedding light on 'dark matter'. *Andrology* 2014; 2: 360–9.
66 Papaioannou MD, Nef S. microRNAs in the testis: building up male fertility. *J Androl* 2010; 31: 26–33.
67 Hayashi K, Chuva de Sousa Lopes SM, Kaneda M, Tang F, Hajkova P, *et al*. MicroRNA biogenesis is required for mouse primordial germ cell development and spermatogenesis. *PLoS One* 2008; 3: e1738.
68 Maatouk DM, Loveland KL, McManus MT, Moore K, Harfe BD. Dicer1 is required for differentiation of the mouse male germline. *Biol Reprod* 2008; 79: 696–703.
69 Ro S, Park C, Sanders KM, McCarrey JR, Yan W. Cloning and expression profiling of testis-expressed microRNAs. *Dev Biol* 2007; 311: 592–602.
70 Yan N, Lu Y, Sun H, Tao D, Zhang S, *et al*. A microarray for microRNA profiling in mouse testis tissues. *Reproduction* 2007; 134: 73–9.
71 Luo L, Ye L, Liu G, Shao G, Zheng R, *et al*. Microarray-based approach identifies differentially expressed microRNAs in porcine sexually immature and mature testes. *PLoS One* 2010; 5: e11744.
72 Yan N, Lu Y, Sun H, Qiu W, Tao D, *et al*. Microarray profiling of microRNAs expressed in testis tissues of developing primates. *J Assist Reprod Genet* 2009; 26: 179–86.
73 Lian J, Zhang X, Tian H, Liang N, Wang Y, *et al*. Altered microRNA expression in patients with non-obstructive azoospermia. *Reprod Biol Endocrinol* 2009; 7: 13.
74 Abu-Halima M, Backes C, Leidinger P, Keller A, Lubbad AM, *et al*. MicroRNA expression profiles in human testicular tissues of infertile men with different histopathologic patterns. *Fertil Steril* 2014; 101: 78–86e2.
75 Aravin AA, Sachidanandam R, Girard A, Fejes-Toth K, Hannon GJ. Developmentally regulated piRNA clusters implicate MILI in transposon control. *Science* 2007; 316: 744–7.
76 Grivna ST, Pyhtila B, Lin H. MIWI associates with translational machinery and PIWI-interacting RNAs (piRNAs) in regulating spermatogenesis. *Proc Natl Acad Sci U S A* 2006; 103: 13415–20.
77 Lau NC, Seto AG, Kim J, Kuramochi-Miyagawa S, Nakano T, *et al*. Characterization of the piRNA complex from rat testes. *Science* 2006; 313: 363–7.
78 Aravin AA, Sachidanandam R, Bourc'his D, Schaefer C, Pezic D, *et al*. A piRNA pathway primed by individual transposons is linked to *de novo* DNA methylation in mice. *Mol Cell* 2008; 31: 785–99.
79 Gan H, Lin X, Zhang Z, Zhang W, Liao S, *et al*. piRNA profiling during specific stages of mouse spermatogenesis. *RNA* 2011; 17: 1191–203.
80 Yang Q, Hua J, Wang L, Xu B, Zhang H, *et al*. MicroRNA and piRNA profiles in normal human testis detected by next generation sequencing. *PLoS One* 2013; 8: e66809.
81 Mattick JS. Long noncoding RNAs in cell and developmental biology. *Sem Cell Dev Biol* 2011; 22: 327.
82 Mattick JS. The central role of RNA in human development and cognition. *FEBS Lett* 2011; 585: 1600–16.
83 Wapinski O, Chang HY. Long noncoding RNAs and human disease. *Trends Cell Biol* 2011; 21: 354–61.
84 Bhan A, Mandal SS. Long noncoding RNAs: emerging stars in gene regulation, epigenetics and human disease. *Chem Med Chem* 2014; 9: 1932–56.
85 Bao J, Wu J, Schuster AS, Hennig GW, Yan W. Expression profiling reveals developmentally regulated lncRNA repertoire in the mouse male germline. *Biol Reprod* 2013; 89: 107.
86 Sun J, Lin Y, Wu J. Long non-coding RNA expression profiling of mouse testis during postnatal development. *PLoS One* 2013; 8: e75750.
87 Primig M, Wiederkehr C, Basavaraj R, de Menthiere CS, Hermida L, *et al*. GermOnline, a new cross-species community annotation database on germ-line development and gametogenesis. *Nat Genet* 2003; 35: 291–2.
88 Small CL, Shima JE, Uzumcu M, Skinner MK, Griswold MD. Profiling gene expression during the differentiation and development of the murine embryonic gonad. *Biol Reprod* 2005; 72: 492–501.
89 Grabske RJ, Lake S, Gledhill BL, Meistrich ML. Centrifugal elutriation: separation of spermatogenic cells on the basis of sedimentation velocity. *J Cell Physiol* 1975; 86: 177–89.
90 Meistrich M, Bruce W, Clermont Y. Cellular composition of fractions of mouse testis cells following velocity sedimentation separation. *Exp Cell Res* 1973; 79: 213–27.
91 Bellvé AR. Purification, culture, and fractionation of spermatogenic cells. *Methods Enzymol* 1993; 225: 84–113.
92 Romrell L, Bellvé AR, Fawcett DW. Separation of mouse spermatogenic cells by sedimentation velocity: a morphological characterization. *Dev Biol* 1976; 49: 119–31.
93 Meistrich ML. Separation of mouse spermatogenic cells by velocity sedimentation. *J Cell Physiol* 1972; 80: 299–312.
94 Meistrich M, Trostle PK. Separation of mouse testis cells by equilibrium density centrifugation in renografin gradients. *Exp Cell Res* 1975; 92: 231–44.
95 Getun IV, Torres B, Bois PR. Flow cytometry purification of mouse meiotic cells. *J Vis Exp* 2011; Available from: http://www.jove.com/video/2602/flow-cytometry-purification-of-mouse-meiotic-cells.

96 He Z, Kokkinaki M, Jiang J, Zeng W, Dobrinski I, *et al*. Isolation of human male germ-line stem cells using enzymatic digestion and magnetic-activated cell sorting. In: Germline Development. New York, United States, Humana Press, Springer; 2012. p. 45–57.
97 Izadyar F, Wong J, Maki C, Pacchiarotti J, Ramos T, *et al*. Identification and characterization of repopulating spermatogonial stem cells from the adult human testis. *Hum Reprod* 2011; 26: 1296–306.
98 Dym M, Kokkinaki M, He Z. Spermatogonial stem cells: mouse and human comparisons. *Birth Defects Res C Embryo Today* 2009; 87: 27–34.
99 Wang C, Gong B, Bushel PR, Thierry-Mieg J, Thierry-Mieg D, *et al*. The concordance between RNA-seq and microarray data depends on chemical treatment and transcript abundance. *Nat Biotechnol* 2014; 32: 926–32.
100 Shen C, Zhong N. Long Non-coding RNAs: the epigenetic regulators involved in the pathogenesis of reproductive disorder. *Am J Reprod Immunol* 2015, 73: 95–108.
101 Khazaie Y, Nasr-Esfahani MH. MicroRNA and Male Infertility: a potential for diagnosis. *Int J Fertil Steril* 2014; 8: 113–8.
102 Ran M, Chen B, Yin J, Yang A, Jiang M. Advances in miRNA research related to testis development and spermatogenesis. *Yi Chuan* 2014; 36: 646–54.
103 Kang HJ, Moon MJ, Lee HY, Han SW. Testosterone alters testis function through regulation of piRNA expression in rats. *Mol Biol Rep* 2014; 41: 6729–35.
104 Bortvin A. PIWI-interacting RNAs (piRNAs) – A mouse testis perspective. *Biochemistry* 2013; 78: 592–602.