Differential gene expression revealed by transcriptomic analyses of male germ cells

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1. Introduction

Male germ cell development in mammals is complex and involves expression of multiple genes and transcript isoforms. The flow of genetic information to primary transcripts, their splicing, and their ultimate translation into protein presents many points of regulation that can affect differentiation and function of spermatogenic cells. The goal of transcriptomic analysis is to understand the global system of transcripts present in germ cells and how this changes over time, with differentiation and after perturbation.

The number of protein-coding genes in the human genome is estimated to be 20,488 or 23,244 (Griffiths-Jones 2007; Pennisi 2007) [(22,592 genes according to the Ensembl database (<http://www.ensembl.org/>)], which accounts for only about 1% of the DNA in the human genome. However, of these genes, more than 90% have multiple isoforms (Pan *et al.* 2008), and thus the resulting number of transcripts can be estimated at approximately 100,000. The complete set of all RNA molecules – or transcriptome – of a given cell type, which includes mRNA, rRNA, tRNA and non-coding RNA, varies among cell types or stages during a process of differentiation, such as spermatogenesis. Studying the transcriptome can provide a systems-level of insight into the processes of a biological system.

Gametes, the sperm of males and ova of females, are the cells that carry genetic information to the next generation. Cells in the testicular germ cell lineage include mitotic spermatogonia, meiotic spermatocytes and haploid spermatids, which are precursors to mature spermatozoa (Figure 1). Key features of the developmental program of male germ cell development include the highly conserved process of meiosis, complex spermiogenic differentiation of cellular features, and precise and stage-specific regulation of many germ cell-specific genes and variants (Eddy 2002).

However, despite much study, many of the genes essential for spermatogenesis remain undiscovered. Here, we review studies that seek to resolve this problem, showing how our emerging understanding of the unique transcriptome of male germ cells is enlarging our view of the repertoire of genes that play a role in spermatogenesis.

2. Methods of transcriptome analysis

Technological developments have greatly impacted on how we have analyzed the transcriptome over the past decade. The methods used can be roughly characterized as unbiased approaches based on a prior knowledge of coding sequences, such as microarrays and serial analysis of gene expression (SAGE), or more recent unbiased RNA sequencing approaches that rely on sequencing the RNA transcriptome, RNA-seq. All of the methods are fraught with problems of sample size, throughput, coverage, statistical analysis, and cost-effectiveness.

DNA microarrays, until recently, were commonly used to estimate expression levels of multiple genes, or, more effectively, to estimate differential expression among different experimental and/or biological conditions. In this method, synthetic DNAs made from mRNA (complementary DNAs or cDNAs) are hybridized to a large collection of microscopic spots, each of a single, specific DNA sequence (collectively known as probes), derived from bioinformatic assemblies of known coding regions. In contrast, the SAGE method applies a cloning technology making use of a whole mRNA library from a given sample. Cleaved cDNAs are bound to streptavidin beads, for modification and cloning for sequencing (Velculescu *et al.* 1995). Because SAGE is based

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on sequencing mRNA output, it is less biased than microarrays and has the potential of new gene discovery, while microarrays are more cost-effective and technically accessible for investigators. Although these methods enable quantitative acquisition of most transcripts expressed in samples, they cover only about one third of the genes in the mouse genome. Nonetheless, these methods have led to finding more than 10,000 genes differentially expressed throughout the spermatogenic process (Laiho *et al.* 2013; Shima *et al.* 2004).

A step forward, from which we can expect much more information about the male germ cell transcriptome, is RNA-seq. This is founded on the so-called next-generation sequencing (NGS), also known as “deep sequencing”. Currently, NGS can produce billions of short reads (50-250 nt) from a sample in days; therefore it is relatively easy to access and generate three-four orders of magnitude more

information than the more conventional and biased approaches, since, theoretically the whole transcriptome present in a sample can be sequenced. Although expensive, the cost is rapidly decreasing and RNA-seq is becoming a method of choice that is cost-effective on a per-nucleotide basis. Among the advantages other than providing a comprehensive transcriptome, analysis of RNA-seq data can inform about alternatively spliced transcripts, post-transcriptional modifications, and mutations, all of which can be subjected to quantitative analysis. Because of all these advantages, use of RNA-seq has been escalating in recent years, with consequent growth of our knowledge about transcriptional and post-transcriptional regulation during spermatogenesis.

3. Differential gene expression during spermatogenesis

Transcriptome profiling has been especially

Figure 1 Gene expression regulation and the transcriptome during male germ-cell differentiation. Spermatogenesis is initiated after birth. It is comprised of a mitotic phase, a meiotic phase, and after the two meiotic divisions, spermiogenic differentiation. The prolonged meiosis I prophase is classified into several substages based on chromatin configuration; preleptotene (PL), leptotene (L), zygotene (Z), pachytene (P) and metaphase (M). During spermiogenesis chromatin is compacted as round spermatids (R) are differentiated into the elongating spermatids (E) and then mature spermatozoa (SZ), during which time histones in chromatin are replaced with protamines. Transcription is active throughout spermatogenic process, especially during pachytene substage, but ceases as spermatids elongate. Transcription of the sex chromosomes is completely or greatly suppressed during the pachytene stage. The transcriptome of germ cells also contains ncRNAs, including miRNAs, piRNAs or lncRNAs, which are differentially expressed during spermatogenesis. RNA translation is actively regulated, especially during later stages of spermatogenesis.

informative for revealing changes in gene expression during spermatogenesis. Even before the advent of global transcript profiling, it was known that spermatogenesis, including the meiotic substages, is mediated by expression of many testis-specific genes (Eddy 2002). Strategies other than transcriptome analysis have been used to identify those germ-cell-specific genes required for production of healthy gametes. For example, unbiased mutagenesis and phenotype screening identified some novel genes essential for infertility (Handel *et al.* 2006; O'Bryan & de Kretser 2006). These findings have been greatly augmented by transcriptome analyses (Chalmel *et al.* 2007; Schultz *et al.* 2003).

In spite of advances provided by transcriptome profiling, the study of gene expression during spermatogenic differentiation is challenging because of developmental changes and complexity of the germ cell population due to continuously ongoing spermatogenesis after the juvenile onset of spermatogenesis. Additionally, transcription is gradually suppressed during spermiogenesis, when chromatin histones are replaced by transition proteins and protamines (Rathke *et al.* 2014) (Figure 1). Several approaches have been used to reduce the complexity of cell populations subjected to transcriptome analysis. Spermatogenesis is initiated about 8 days after birth and a part of spermatogonia enter meiotic phase (Bellve *et al.* 1977), and these meiotic cells progress semi-synchronously. Experimental analysis of this first round of spermatogenesis, called the 'first wave', is advantageous for following the leading edge of spermatogenic development. Transcriptome analyses of cells during the first wave mice have provided informative gene expression profiles (Shima *et al.* 2004). A major improvement on the practice of following developmental leading edge was recently provided by manipulating retinoic acid (RA) availability in testes of neonatal mice (Evans *et al.* 2014). RA is required for the initiation of meiosis (Feng *et al.* 2014), and careful developmental manipulation of RA availability leads to synchronous spermatogenesis. In another approach, Fallahi *et al.* (2010) used physical sorting of specific stages of spermatogenic cells by a FACS-based methodology. Clustering of the resulting gene expression profile yielded two major categories: "before" and "after" early pachytene (Figure 1). The "before-pachytene" genes were predominantly expressed from spermatogonial stages to leptotene/zygotene spermatocytes; and the "after-pachytene"

genes were expressed predominantly at or after the mid-pachytene stage. Several transcriptome studies showed a large amount of transcripts were synthesized and stored during pachytene stage (Fallahi *et al.* 2010; Shima *et al.* 2004).

Recently transcriptome analyses have been used to demonstrate differential gene expression in the spermatogonial population. Techniques for synchronization of the onset of spermatogenesis were particularly valuable for obtaining relatively pure populations and enabling this study (Evans *et al.* 2014). These analyses, coupled with high-resolution live imaging have revealed that the heterogeneous 'undifferentiated' spermatogonia and the 'differentiating' spermatogonia show unique gene expression patterns (Yoshida 2012) suggesting that developmental dynamics of spermatogonia are more complex than previously thought.

How are the complex and dynamic patterns of spermatogenic gene expression so precisely controlled? Transcriptome analyses coupled with genetic tests of function are beginning to provide some answers to this. One point of interests has been the transition of differentiating spermatogonia to meiosis. For example, the *Stra8* gene, regulated by extrinsic RA, has been shown to play a key role in initiating meiosis (Anderson *et al.* 2008), and transcriptome analyses have revealed that this controls the expression of many meiotic genes (Koubova *et al.* 2014). But other genes are also responsible for the dramatic changes in gene expression revealed by transcriptome profiling. For example, the testis-specific transcription factor MYBL1 (also known as A-MYB) plays a key role as a transcription factor for male meiosis, and regulates gene expression required for various processes, especially cell cycle progression (Bolcun-Filas *et al.* 2011).

Transcriptome studies showing global gene expression, and differential expression patterns of these genes during spermatogenesis provide a valuable resource for identifying novel genes essential for regulation of spermatogenesis. Several approaches have been developed to identify cell type-specific or substage-specific transcriptomic signatures. However, these have proven to be challenging and problematic because the mixture of multiple differentiating germ cell types has been difficult to resolve.

4. A special case of differential gene expression: the sex chromosomes

The X and Y chromosomes in germ cells show unique transcriptional features throughout spermatogenesis. Although the XY chromosomes are transcriptionally active until zygotene (Ichijima *et al.* 2012; Turner 2007), they are suddenly silenced during pachytene stage (Turner 2007) (Figure 1). This process is mediated by the formation of heterochromatin during the pachytene substage, so that the sex chromosomes form a unique chromatin structure called the sex body or the XY body (Handel 2004). This process of silencing sex chromosome genes in male germ cells is referred to as meiotic sex chromosome inactivation (MSCI) (Turner 2007), and has long been a subject of intense investigation. Considerably before the advent of transcriptome profiling, it was known that RNA precursors (³H-uridine) failed to be incorporated into sex chromosomes of pachytene spermatocytes (McKee & Handel 1993), and that RNA polymerase did not localize to the XY body (Figure 2). More comprehensive microarray analyses of enriched pachytene spermatocytes also showed that rapid silencing of expression of X-chromosome genes with developmental progress into the pachytene stage (Fallahi *et al.* 2010; Sin & Namekawa 2013), and this sex chromosome silencing is persisted throughout spermiogenesis, referred to as postmeiotic sex chromatin (PMSC) (Turner 2007) (Figure 1). Unlike oocytes, in which 25-30% of X-linked genes are expressed during the pachytene stage (Carrel & Willard 2005), the expression of sex chromosome genes had been thought to be completely suppressed in males. However, recent transcriptome analyses have revealed that a number of X-linked miRNAs, 22-24 nt non-coding RNAs,

are expressed during pachytene stage in spite of silencing of coding gene sequences (Song *et al.* 2009). Since miRNAs are known to play important roles in spermatogenesis (de Mateo & Sassone-Corsi 2014; Yadav & Kotaja 2014), these miRNAs that escape from MSCI might be essential for normal spermatogenesis. Indeed, although the biological function of MSCI is not fully understood, expression of X-linked genes during pachytene stage leads to meiotic arrest (Royo *et al.* 2010). Further transcriptomic analyses, coupled with genetic tests of function are required to resolve the significance of MSCI.

5. Non-coding RNAs in germ cells

In recent years, large-scale transcriptome studies (Jin *et al.* 2008; Yasuda & Hayashizaki 2008), as well as SAGE analyses (Chan *et al.* 2006), showed that the transcriptome sequences are far more complex than simply a subset of coding sequences. The transcriptome includes not only the previously known non-coding RNAs (ncRNAs) (Washietl *et al.* 2005), such as transfer RNA (tRNA), ribosomal RNA (rRNA), but also a significant number of other kinds of small ncRNAs, which are emerging as important for spermatogenic processes (Yadav & Kotaja 2014). Several different ncRNAs are expressed during male germ cell development (Table 1). These include the microRNAs (miRNA), which are about 21-24 nt in length and generated by the RNaseIII-containing enzyme Dicer (Carmell & Hannon 2004). The majority of miRNAs are highly conserved among species, and it has been postulated the miRNAs function as post-transcriptional suppressors by either translational repression or mRNA destabilization through binding to target mRNAs (Kim 2005). Due to

Figure 2 Formation of XY body in pachytene spermatocyte. RNA polymerase is expressed in autosomes but not in X and Y chromosomes (A). Expression of phosphorylated form of histone H2AX (P-H2AX) is restricted to X and Y chromosomes, forming a unique heterochromatin structure called “XY body” (B).

transcriptome analyses, there is increasing evidence for key roles of miRNAs in spermatogenesis. Diverse miRNAs are found in specific cell types of the mouse testis (McIver *et al.* 2012), and many of them are differentially expressed during the first juvenile round of spermatogenesis (Ro *et al.* 2007) (Figure 1). The precise function of miRNAs in germ cells is not clear; however, given that loss or deleted function of miRNAs in the testis leads to reduced fertility in mice, they appear to be essential for male fertility (Kotaja 2014).

Another class of small ncRNAs in germ cells are the Piwi-interacting RNAs (piRNA); these are slightly longer than miRNAs (about 24-30 nt in length) (Ghildiyal & Zamore 2009). The piRNAs are expressed exclusively in the gonads, and associated with silencing of harmful repetitive transposable elements (TEs) (Aravin *et al.* 2001), thus contributing to genome stability during male germline development. A number of studies showed that defects in TE silencing led to aberrant meiosis, suggesting that stable silencing of TEs is required for normal progression of meiosis. Mammalian piRNAs are classified according to the stage of meiosis in which they are expressed: the pre-pachytene and pachytene piRNAs (Figure 1). The pre-pachytene piRNAs are enriched in repeat sequences derived from transposons (Aravin *et al.* 2007). The pre-pachytene

piRNAs bind to Piwi proteins, including MIWI2 and MILI, and mutant mice depleted with these proteins showed loss of DNA methylation marks on transposons (Aravin *et al.* 2008). These findings suggest that pre-pachytene piRNAs are associated with de novo methylation. The pachytene piRNAs predominantly arise from unannotated intergenic sequences (Aravin *et al.* 2006). A recent high-throughput sequencing study provided evidence that the transcription factor MYBL1 is essential for the production of pachytene piRNAs and their precursors (Li *et al.* 2013). Mice deficient for Piwi proteins (Aravin *et al.* 2008) and MYBL1 (Bolcun-Filas *et al.* 2011) fail to produce mature spermatozoa; however, the precise mechanisms of function for the pachytene piRNAs are not known.

Recent RNA-seq analyses document long non-coding RNAs (lncRNA) (>200 nt in length) in the mammalian male germ-cell transcriptome (Carninci *et al.* 2005). Although many of lncRNAs, such as *Xist* (Duret *et al.* 2006), are poorly conserved among mammalian species, they appear to be involved in transcriptional or translational regulation (Feng *et al.* 2006). Interestingly, lncRNAs are differentially expressed throughout the spermatogenic process (Bao *et al.* 2013) (Figure 1). The unique lncRNAs appear to be more abundant than mRNAs in male germ cells; however, their

Table 1. Summary of ncRNA species in spermatogenesis

Species	Length	Origin	Biogenesis	Functions in spermatogenesis
miRNA	21-24 nt	Intergenic regions, miRNA genes	Cleavage from primary transcripts through Drosha, Dicer and Argonote	Post-transcriptional regulation
siRNA	20-24 nt	Intergenic regions, pseudogenes, endo-siRNA loci	Cleavage from dsRNA by Dicer	RNA interference pathway
piRNA	24-30 nt	Transposons, or centromeric/telomeric regions of chromosomes (piRNA loci)	Ping-pong amplification through PIWI proteins	de novo methylation, genome integrity
lncRNA	>200 nt	Intergenic and intragenic regions	Alternative splicing	Transcriptional and translational regulation

functions are poorly understood and require further study.

6. Translational regulation

Transcriptome profiling has revealed great complexity of RNA species in male germ cells, raising the question of how translation into functional proteins is regulated. Translation of mRNA is facilitated by differential polyadenylation and various RNA binding factors (Hecht 1998). For example, both 5' and 3' untranslated regions (UTR) of mRNAs contain translation control elements (Braun 1998). Cellular RNAs are associated with ribonucleoprotein (RNPs) complexes; these protein-RNA complexes and other processes such as miRNA-mediated mechanisms (Huntzinger & Izaurralde 2011) mediate translational regulation by processing, modifying, translating and degrading the RNAs (Figure 1). With the advent of NGS, polysome or ribosome profiling techniques, which provide data on the ribosome-associated transcriptome, have attracted more attention in recent years for their potential to estimate accurate translation efficiency. Overall, global translational analysis such as RNA immunoprecipitation, miRNA profiling or proteome analysis, coupled with use of mutant mice will contribute to understanding mechanisms of mRNA processing and translational regulation mechanisms in germ cells.

7. Future directions

Transcriptome analysis has proven to be a powerful tool to investigate global gene expression, and to assess more focused and nuanced quantitative patterns of gene expression as well. Because the use of NGS is becoming more cost-effective and the number of analysis tools is growing, transcriptome research will become more and more widely used. Given that spermatogenesis is one of the most complex genetic and cell biological processes, and that many testis-expressing genes are differentially expressed, transcriptomic analyses are beginning to elucidate mechanisms that regulate spermatogenesis. There is significant "extra" value of the large-scale transcriptome analysis; just one example of how transcriptomes turned out to be far more complex than expected was discovery and annotation of ncRNAs, which were once thought to be "junk" RNAs. We can expect that high-throughput gene expression analysis will contribute significantly to understanding gametogenesis. Already, transcriptome studies

are increasing our knowledge of splicing variants during spermatogenesis (Laiho *et al.* 2013). However, to enhance the value of these advances, more accurate high-throughput profiling of whole transcriptomes along with new bioinformatics methods are greatly needed to elucidate cell type-specific features during spermatogenesis. An exciting new advance is our ability to use deep sequencing technology on a single cell. Transcriptomes of single cells will provide detail on how individual cells behave in a biological system, and holds much potential for elucidating cell interactions and variability throughout spermatogenesis. From these promising beginnings, it is clear that further improvements in the basic technology, data analysis and interpretation of transcriptome studies will broaden their application to basic research and clinical treatment.

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