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Review

Gene expression in spermiogenesis

H. Tanaka[a] and T. Baba[b,*]

[a] Department of Science for Laboratory Animal Experimentation, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita City, Osaka 565–0871 (Japan), e-mail: tanaka@biken.osaka-u.ac.jp
[b] Graduate School of Life and Environmental Sciences and Institute of Applied Biochemistry, University of Tsukuba, Tsukuba Science City, Ibaraki 305-8572 (Japan), e-mail: acroman@sakura.cc.tsukuba.ac.jp

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Abstract. Germ cells convey parental genes to the next generation, and only germ cells perform meiosis, which is a mechanism that preserves the parental genes. The fusion of the products of germ cell meiosis, the haploid sperm and egg, creates the next generation. Sperm are the haploid germ cells that contribute genes to the egg. In preparation for this, the haploid round spermatids produced by meiosis undergo drastic morphological changes to become sperm. During this process of spermiogenesis, the nuclear form of the haploid germ cell takes shape, the mitochondria are rearranged in a specific manner, the flagellum develops and the acrosome forms. Spermatogenesis is supported by precise and orderly regulation of gene expression during the changes in chromatin structure, when protamine replaces histone. In this report, we summarize the molecular mechanisms involved in spermiogenesis.

Key words. Spermatogenesis; gene expression; transcription; translation; testis; chromatin.

Introduction

In most multicellular organisms, fertilized eggs differentiate into somatic cells and germ cells. These cells are separated during the early developmental stages and have quite different fates [1]. Germ cells maintain species continuity, whereas somatic cells ensure the construction and activity of the individual. In other words, somatic cells help the germ cells ensure survival and continuity of the species. Thus, germ cells play a fundamental role in multi-cellular organisms. One germ-cell-specific feature is meiotic division, which represents a critical mechanism for the maintenance of species with large genomes [2]. Homologous chromosomal DNA undergoes mosaic recombination during meiosis, such that each chromosome of a haploid germ cell is arbitrarily rearranged from two homologous parental chromosomes. Even if two or more mutations arise in different genes on one chromosome during cell proliferation, one pair of homologous chromosomes that does not include mutations can result from meiotic recombination, thus avoiding the fixation of accumulated mutations on chromosomal DNA during cell proliferation (fig. 1). However, the success of this strategy depends on the fact that only meiotic cells give rise to the next generation. Other major features of germ cells, i.e., maintenance of a telomere [3] and maternal/paternal chromosome imprinting [4], reflect this requirement. In apparent contradiction to this requirement, there have been recent reports of cloned animals [5] and the single pre-implantation of an egg [6]. However, a cloned animal may not produce subsequent generations through cloning [7]. What role does the male germ cell play in the maintenance of species? Studies of male germ cell differentiation, especially the mechanisms following meiosis, are important in understanding the evolutionary strategies of multicellular organisms.

* Corresponding author.

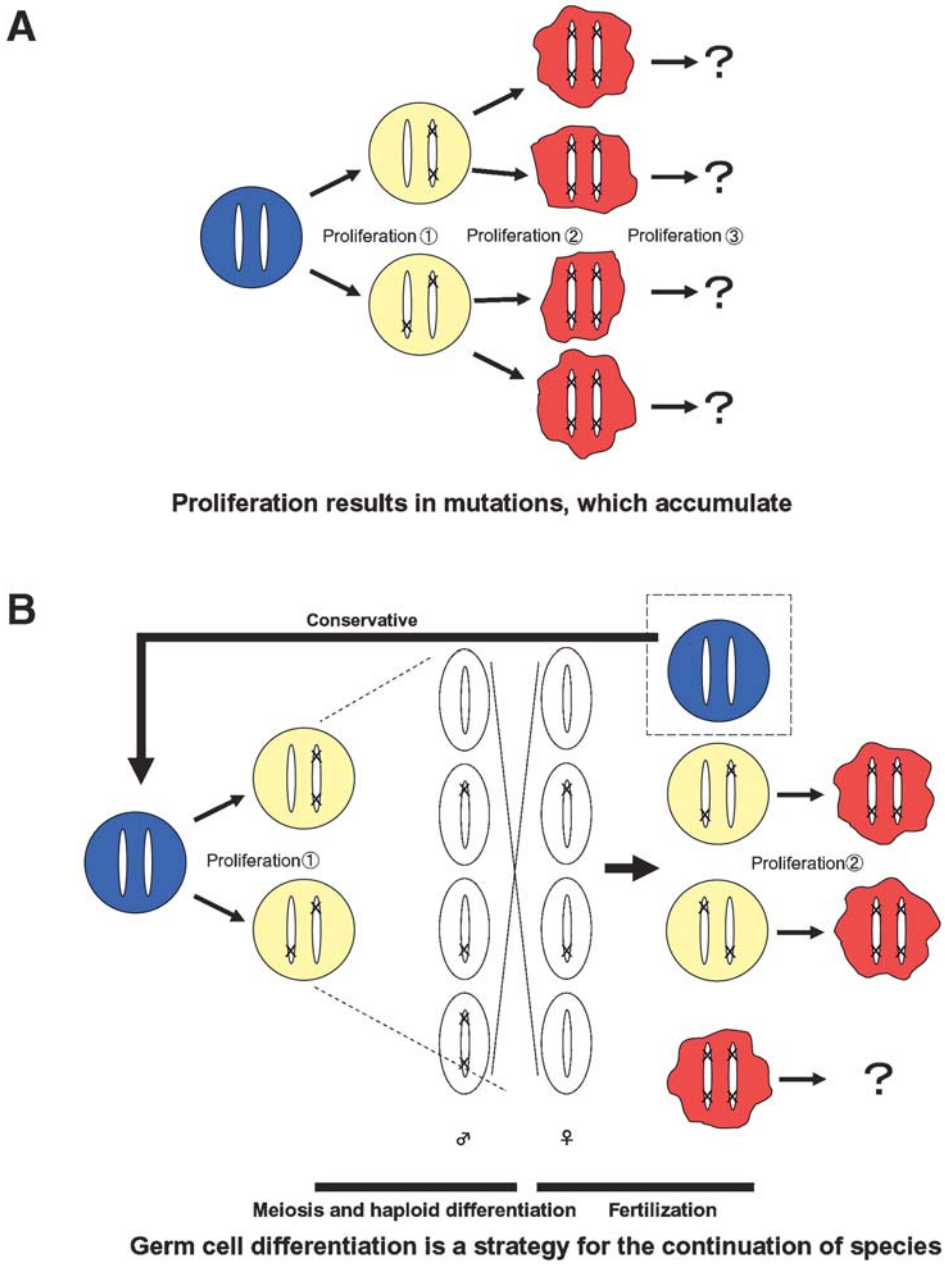

Figure 1. Schematic presentation of cell division. Distribution of chromosomes during mitosis (*A*) and meiosis (*B*). Even if two or more mutations arise in different genes on one chromosome during cell proliferation, meiotic recombination can produce one pair of homologous chromosomes that does not include the mutations, thus avoiding the fixation of mutations in chromosomal DNA during cell proliferation. X chromosomes are repaired in females by this mechanism, while most of the Y chromosome is not repaired. Blue represents a chromosome that contains the original set of genes. A mutation that occurred during cell proliferation is represented in yellow. The accumulation of mutations during mitotic cells divisions can result in a descendant cell that differs from the ancestral cell (red).

Spermatogenesis in testis

In mouse, primordial germ cells (PGCs) are first observed at days 7–8 of pregnancy, in the extra-embryonic mesoderm [8]. PGCs migrate through the hindgut and dorsal mesentery, undergoing repeated multiplicative divisions, and establish the gonadal primordium on days 10–11 of pregnancy. The gonad also contains non-migrating germ cells called gonocytes. Seminiferous cords formed by Sertoli cell precursors appear in the gonadal primordium at days 12–13 of pregnancy. At this stage, it becomes clear whether the gonad will become a testis or ovary. In the testis, the PGCs/gonocytes proliferate until days 13–14 of pregnancy. At this stage, the PGCs/gonocytes arrest their cell cycles, and proliferation does not recommence until a few days after birth. In the

ovary, PGCs/gonocytes enter meiosis and arrest in the prophase of meiosis I.

During the period of cell cycle arrest, PGCs/gonocytes in the testis differentiate to prospermatogonia. Co-culture experiments with PGCs and genital ridges have shown that the commitment of PGCs to male germ cells is not cell-autonomous but is an induced response [9, 10]. The systematic differentiation processes in testis are supported by Sertoli and Leydig cells, which function in response to testosterone. After birth, the prospermatogonia emerge from cell cycle arrest, and the first wave of spermatogenesis begins. Spermatogenesis will occur in seminiferous tubules throughout adulthood, producing huge amounts of sperm [11]. The process of spermatogenesis can be divided into three phases. The first, premeiotic phase is characterized by an increase in cell number as a result of mitotic divisions of diploid spermatogonia. Second, a meiotic prophase leads subsequently to the formation of haploid round spermatids. Third, the post-meiotic phase involves the morphogenetic events that are necessary for sperm formation. As the first sperm cell is completed in the testis ('the first wave'), the spermatogonia, spermatocytes, and spermatids are systematically arranged in the seminiferous tubules: spermatogonia in tubule walls spermatids at the center of tubules and spermatocytes between them. Stem cell proliferation and differentiation, meiosis, generation of haploid germ cells and morphogenesis of the developing sperm require approximately 1 month in mice and 2 months in humans. To function correctly, mature sperm must be able to travel independently for relatively long distances. During haploid germ cell differentiation (or spermiogenesis), the round spermatids undergo marked morphological changes. The nucleus takes on a more compact shape, the mitochondria are rearranged, the flagellum develops and the acrosome is generated [11]. The spermiogenetic period takes about 2–3 weeks in mice [12] and 5–6 weeks in humans [13, 14]. At this stage, the haploid germ cells do not divide, but morphogenesis occurs, indicating that a regulatory mechanism arrests the cell cycle.

Chromatin formation

Dramatic remodeling of chromatin takes place during mammalian spermiogenesis. Nuclear elongation and chromatin condensation occur concomitant with modifications in the nuclear basic proteins associated with DNA. A number of biochemical events accompany the displacement of histones and the appearance of a set of basic nuclear proteins, such as tH2A, tH2B, H1t, spermatid-specific H2B (ssH2B), haploid germ cell-specific nuclear protein 1 (Hanp1), testis-specific HMG (tsHMG), histone H1-like protein in spermatids 1 (Hils 1), transition proteins (TPs) and protamines [15–23] (fig. 2). Histone synthesis ceases during spermiogenesis, and histones are replaced by a set of TPs, which are subsequently replaced by protamines [19]. The completed process results in a greater than six-fold condensation of mitotic chromosomes, producing a tightly packaged chromatin structure.

TPs (TP1, TP2) are major nuclear basic proteins that are expressed during spermatogenesis. Recently, mouse null mutants for TP1 or TP2 were found to be subfertile [24, 25], while mice in which both TPs were lost were infertile [26]. However, the sperm nuclei of TP null mutant mice

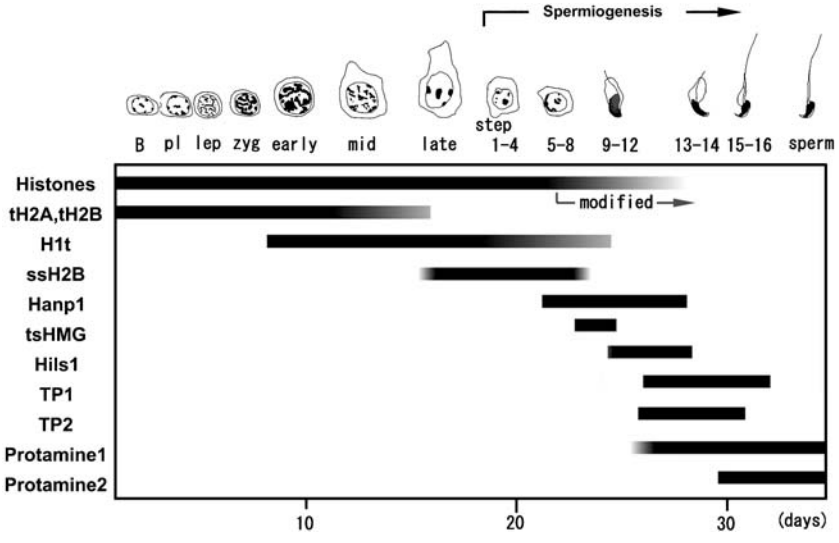

Figure 2. Schematic representation of nucleoprotein expression in germ cells during mouse spermatogenesis. Bars indicate nucleoproteins that are thought to be involved in chromatin structuring. The onset of expression of the testis-specific histone variants tH2A, tH2B, H1t and ssH2B is also shown. tsHMG, testis-specific high-mobility-group box protein; TP1 and 2, transition proteins 1 and 2.

can achieve successful fertilization via round spermatid nuclear injection (ROSNI). Most mammals have one protamine form that replaces the TPs, but a few species, including humans and mice, have two protamine forms. Gene disruption experiments involving protamine 1 or 2 in mice have shown that both protamines are essential for fertility, and that haploinsufficiency is caused by a mutation in one protamine allele [27]. Moreover, when protamine 2 is disrupted, the resultant sperm nuclei are infertile, even via intracytoplasmic sperm injection (ICSI) [28]. These results indicate that protamine 2 is required for the process of nuclear compaction during spermiogenesis. Many nuclear proteins are expressed systematically during the nuclear condensation period. Almost all of these proteins are derived from histone H1 [29] and undergo complex processes of modification in mammals. Although some aspects of chromatin structure in spermatozoa have been elucidated [30–32], the molecular mechanisms that contribute to chromatin restructuring are still under investigation. Identification and characterization of proteins specific to spermatid nuclei will be useful to further our understanding of the formation mechanisms of haploid cell-specific chromosomal structures.

Regulation of transcription

During spermiogenesis, nuclear chromatin is remodeled and somatic histones are replaced by protamine, resulting in a highly condensed nucleus. Gene expression may cease entirely during chromatin construction, or transcription factors may bind to and inhibit the expression of specific sequences. It is generally believed that transcription and translation are repressed during spermiogenetic differentiation processes [33]. The transcription that does take place during spermatogenesis commences in the almost-round spermatids, and these transcripts are believed to be translated in accordance with spermatid elongation. Several transcription factors have been demonstrated to be expressed specifically during spermiogenesis.

CREM (cAMP-responsive element modulator) has been shown to play an important role in the regulation of spermiogenesis by binding to CRE sequences [34] (fig. 3). Mice that are CREM deficient have reduced testis weights and a complete lack of mature spermatozoa in the seminal fluid. Histological analyses of CREM-deficient mice show that spermatogenesis is arrested at the early round spermatid stage [34]. Thus, CREM is an

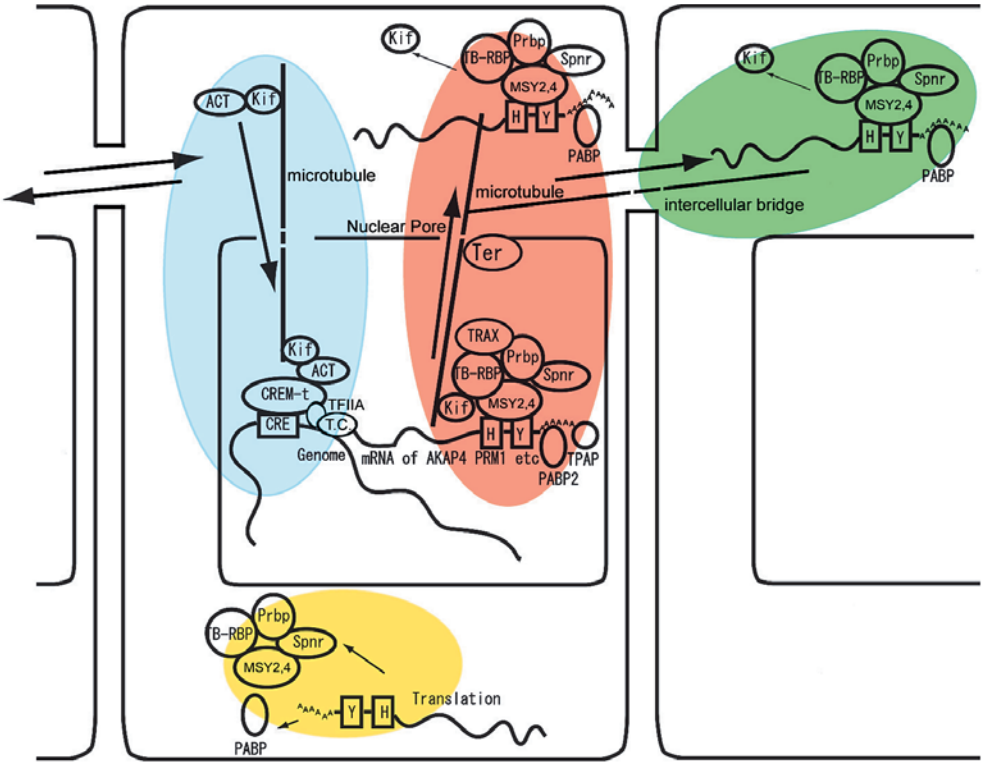

Figure 3. Schematic representation of regulatory pathways of gene expression during mouse spermatogenesis. Spermatids are connected by intercellular bridges. Boxes indicate cell membranes and nuclear membranes. The pathway of transcriptional control by CREM-t is shown in blue. RNA-binding proteins or poly(A) RNA polymerase modify the translation of transcribed mRNAs, which are then transferred to the cytoplasm along microtubules by the kinesin KIF17b (red). Some mRNAs are transferred to another cell through the intercellular bridge (green). The timing of translation is regulated by RNA-binding protein (yellow). Circles indicate proteins. TC indicates the transcriptional complex. Boxed Y and H indicate Y- and H-elements on the mRNA, respectively. Bars indicate microtubule networks; arrows show the direction of movement of protein and protein-mRNA complexes.

important transcription factor in spermiogenesis. In somatic cells, activation by CREM requires its phosphorylation at a unique regulatory site (Ser117) with the ubiquitous co-activator CBP (CREB-binding protein) [35]. In the testis, CREM transcriptional activity is controlled through an interaction with the tissue-specific activator of CREM in testis (ACT: activator of CREM in testis) protein [35, 36] (fig. 3), which has a phosphorylation-independent activation capacity. The function of ACT is regulated by the testis-enriched kinesin KIF17b [37] (fig. 3). However, some proteins that are specifically expressed in haploid germ cells do not contain CRE motifs in their promoter regions [38], which suggests that other regulatory mechanisms exist.

Homeobox genes whose expression is restricted to specific cell lineages are of particular interest as candidates for the regulation of differentiation. Several homeobox genes are reported to be expressed in testicular germ cells [39]. The TG-interacting factor (TGIF) subclass homeobox gene *Tex1* [40] and the homeobox-like gene *Rosbin* are specifically expressed in germ cells at the spermatid stage [41]. *Pbx4*, which is a Pbx family gene that is involved in axial patterning and organogenesis, is expressed in the testis, specifically in spermatocytes at the pachytene stage of the first meiotic prophase [42]. The Paired/Pax family homeobox gene *Tox* is expressed in the spermatid and spermatozoa of adult mice [43]. While studies have reported the presence of the messenger RNAs for these homeobox genes in germ cells, their functions in gene regulation are largely unknown.

Mice deficient in specific genes have been generated to evaluate the role of homeobox genes in spermatogenesis. *Hox-a4*, of the Hox gene family, is expressed at high levels in meiotic and postmeiotic male germ cells, as shown by Northern blot analysis and in situ hybridization [44, 45]. The homozygous knockout (KO) mutant of *Hox-a4* was viable and fertile [46]. *Nkx6.2* (originally named *Gtx*) has been identified as a novel homeobox gene expressed in brain glial cells and testicular germ cells [47]; null mutant *Nkx6.2* mice reproduced normally with a typical litter size, and were indistinguishable from their heterozygous and wild-type littermates [48]. *Esx1* is an X chromosome-linked homeobox gene whose expression is restricted to adult testis and extra-embryonic tissues [49, 50]; *Esx1* hemizygous mutant males were fertile, which demonstrates that Esx1 is not essential for spermatogenesis [51]. In contrast, Sperm 1 (Sprm-1) mutant mice exhibited normal testicular morphology and produced normal numbers of sperm, yet displayed subnormal fertility [52]. *Sprm-1* is a member of the POU domain family, which is specifically expressed in developing male germ cells immediately before the first meiotic division; *Sprm-1* is expressed in haploid spermatids after meiotic division [52, 53]. A novel protein that contains the plant homeodomain motif POG is implicated in spermatogenesis, as meiosis is impaired in POG-deficient mice [54]. The regulatory functions of these genes in spermatogenesis are still largely unknown.

Some fundamental transcription factors are expressed during spermiogenesis. For example, haploid gene expression occurs in the case of TATA-binding protein (TBP) that is expressed in haploid germ cells, and this protein accumulates at much higher levels in early haploid germ cells than in any other somatic cell type [55]. TF IIB and RNA polymerase II are also over-expressed in the testis [56]. Other fundamental transcriptional proteins that are known to be expressed specifically in the testis include testis-specific RNA polymerase II elongation factor (EEL3) [57], testis-specific Elongin (Elongin 2A) [58], testis-specific transcription factor IIA (TFIIAtau) [59], TBP-associated factor (TAF) [60, 61], TBP-related factor 2 (TRF2) [62, 63], a paralogue of transcription factor TFIID subunit (TAF7L) [64] and TFIIA alpha/beta-like factor (ATF) [65]. Although transcriptional activity is decreased with chromatin condensation, the machinery for transcription of spermatid-specific factors is not lost.

The expression of specific genes appears to be regulated by the alternative splicing of transcripts [66, 67]. The *CREM* gene is differentially regulated during spermatogenesis: repressors (α, β and γ) are expressed in premeiotic and early meiotic germ cells, whereas activators (τ, τ1 and τ2) are abundant in postmeiotic germ cells [68]. Low levels of CREM activator expression have also been detected in pachytene spermatocytes. Moreover, the expression of a truncated CREM isoform (an inducible cAMP early repressor) through the use of an alternative promoter has been described in Sertoli cells [69]. RNA-binding proteins may also play roles in germ-cell-specific mRNA splicing [70–72]. For example, SC35 is one component of a splicing factor that decreases in concentration during post-meiotic spermiogenesis [73]. Alternative expression patterns of SC35 splicing factor and spermatid-specific factors may be important in haploid germ cell development via mRNA regulation.

CREM is a critically important transcription factor in early, round spermatids. However, the isolation and characterization of haploid germ cell-specific genes has shown that, even in very late stages of spermiogenesis, the transcription of specific genes occurs from the highly condensed nucleus. A testis-specific homologue of succinyl coenzyme A (CoA): 3-Oxo acid CoA transferase (Scot-t) [74], and some of the haploid germ cell-specific cytoskeletal proteins are examples of late-transcribed gene products. Recent studies have shown that human ejaculated spermatozoa contain the transcription products of over 1000 genes [75]. Further detailed studies of the regulation of gene expression in elongated spermatids may elucidate some of the mechanisms of condensed chromatin-specific gene activity in elongated spermatids and mature spermatozoa.

Regulation of translation

Global transcription rates decline during spermiogenesis as the DNA condenses in the nucleus. However, de novo protein synthesis is necessary for the development of the specific sperm cell morphology. After meiosis, there is an intercellular bridge [11] (fig. 3) that enables cytoplasmic connection between the haploid spermatids, and certain mRNAs are known to be transported across this bridge [76]. RNA-binding proteins are critical in the regulation of translation and in the movement of mRNA between haploid germ cells. Protamine 1 and 2 genes are first transcribed in step-7 spermatids, but are not translated until about step 13 of spermiogenesis [17]. RNA-binding proteins are also important in the temporal regulation of translation through the addition of poly(A) sequences [77] (fig. 3) or via interactions with cis-acting elements in the 5′- and 3′-untranslated regions (UTRs) of certain genes [78] (fig. 3). H- and Y-elements are found in the 5′- and 3′-UTRs of genes expressed in spermatids. Although the specific proteins that recognize these mRNA sequences are unknown, many protein complexes have been found associated with mRNA sequences. For example, testis-brain RNA-binding protein (TB-RBP/translin) and translin-associated factor X (TRAX) bind to mRNA on protamines and to A kinase-anchoring protein 4 (AKAP4) [79, 80], and these protein-mRNA complexes are transported across the intercellular bridge between haploid spermatids. ACT combines with the germ cell-specific transcription factor CREM and is transported to the nucleus by the testis-enriched kinesin KIF17b. The transcription of mRNAs that contain H- or Y-elements, such as the mRNAs of protamines and AKAP4, may be controlled by CREM-ACT. TB-RBP/translin complexes bind to H- or Y-elements on the mRNA transcripts, and these protein-mRNA complexes, along with Ter ATPase [81], are transported by KIF17b through nuclear pores to polysomes, for storage in the cytoplasm [82]. These different mechanisms for transcriptional and post-transcriptional regulation may also act co-operatively.

mRNA-binding proteins, such as Prm-1 RNA-binding protein (Prbp) [83], MSY2 and 4 [84], a spermatid perinuclear RNA-binding protein (Spnr) [85], poly(A)-binding protein 2 (PABP2) [86], and TPAP [77] may serve to adjust the timing of translation. The length of the poly(A) tail of mRNA expressed in the testis changes during spermiogenesis [77], and PABPs are known to inhibit translation in vitro [87], which indicates that the length of the poly(A) tail may affect translation in spermatids. Recently, testis-specific PABP2 and TPAP were cloned and characterized [77, 86]. In the absence of TPAP-arrested spermiogenesis, TPAP-deficient mice display impaired expression of haploid-specific genes that are required for germ cell morphogenesis, and exhibit incomplete elongation of the poly(A) tails of particular transcription factor mRNAs. These results show that spermatogenesis requires the cytoplasmic elongation of mRNA poly(A) tails, which is catalyzed by TPAP, and imply the presence of regulatory mechanisms for the control of cytoplasmic mRNA polyadenylation.

Methylation

The methylation of genomic DNA is a central mechanism for regulating tissue-specific transcription [88]. Methylation induces heterochromatin condensation through histone acetylation, as well as the inactivation of the X chromosome [88] and the imprinting of chromosomes [88] during germ cell differentiation. De novo methyl ation and demethylation of chromosomal DNA are essential for these processes [89]. Methylation progresses globally in spermiogenesis after meiosis. Genes expressed during spermiogenesis avoid methylation in male germ cells, although they are methylated in somatic tissues. ALF is a germ cell-specific counterpart of the large (α/β) subunit of the general transcription factor TFIIA [90]. Promoter regions of ALF are methylated in somatic cells, in non-germ cells of the testis and in epididymal spermatozoa. ALF is demethylated, but not yet expressed, in spermatogonia, in which specific transcription factors are required to initiate expression. Interestingly, ALF regions of the genome of epididymal spermatozoa are methylated, although the sperm nuclei lack histones. In this case, DNA methylation may occur differently and have different effects than those known in histone-containing somatic cells. Spermiogenesis-specific gene sequences also contain CpG islands, which are unmethylated sequences that are often associated with housekeeping genes; these regions are probably deeply involved in the regulation of transcription and methylation.

Gene groups were identified that are expressed both in testes and cancerous tissues [91]. The expression of these genes is regulated by DNA methylation and is tightly repressed in non-testicular somatic tissue [92]. Tight repression of these genes is regulated by chromatin condensation via modification of histones. The mechanism of gene expression during changes in chromatin structure, during which protamine replaces histones in spermiogenesis, may be different from that in cells that contain histones. The DNA methylation statuses during spermiogenesis and in cancerous tissues are different from those of other cells. In certain respects, the methylation statuses in spermiogenesis and cancerous tissues may be similar. Although the silencing of parasitic sequences in cells generally occurs via methylation [93], specific expression of a gene in spermatids has been shown to require a small DNA element with specificity for the promoter, as shown in transgenic mice that carry promoter-fused reporter genes [38, 94]. Histone is re-

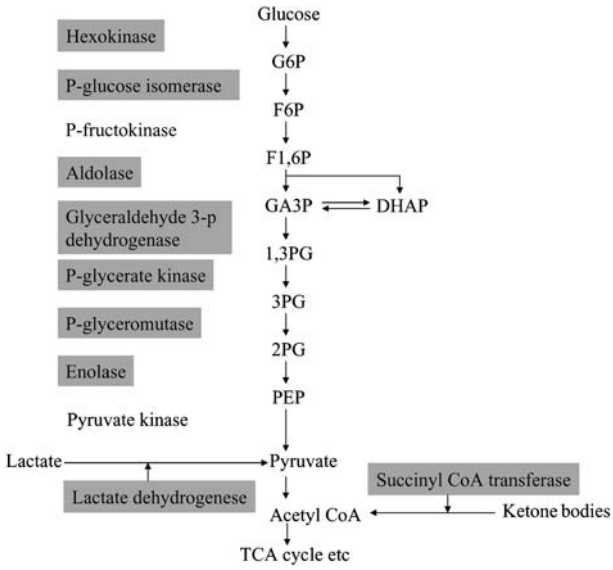

Figure 4. Metabolic pathways of glucose and ketone bodies for acetyl-CoA formation. Germ cell-specific enzymes that have been identified [95]. In germ cells, these steps would be catalyzed by germ cell-specific isozymes (shadowed) that are different from ubiquitously expressed somatic cell-type enzymes.

placed by protamine in spermiogenesis. Silencing of genes by DNA methylation do not function during spermiogenesis, and minimal promoter elements not included other regulatory elements of transcription. For example, enhancers may direct the transcription of mRNA in the testis.

Isozymes of energy metabolism

Germ cell-specific enzymes that differ from ubiquitously expressed somatic cell-type enzymes have been identified in almost all steps of glycolysis, from glucose to acetyl CoA [95]. In addition, SCOT-t has recently been identified in the ketone body metabolism pathway [74] (fig. 4). It is important to consider that at least some of the steps of glycolysis in germ cells may be catalyzed by germ cell-specific isozymes (fig. 4), and that functions during spermatogenesis and in spermatozoa may be supported by different, non-somatic metabolic enzyme pathways. It is possible that the movements of spermatozoa are facilitated by the use of ketone bodies as an energy source [96], and that these energy sources play important roles in sperm maturation in the female genital tract [96].

Genomic construction

Retroposition, which is an important mechanism of gene copying, produces a large number of functional genes in mammalian genomes. It has been reported that many genes located on the X chromosome are expressed in germ cells [97]. Genome projects indicate that there are approximately 100 functional retrotransposed genes on human and mouse chromosomes [98], and that gene loss and gain from X chromosomes by retroposition occur relatively easily compared with the retrotranspositional modification of autosomes [98]. Two intronless genes on autosomes, phosphoglycerate kinase-2 (PGK-2) [99] and pyruvate dehydrogenase subunit e2α [100], are believed to be derived from the transposon-mediated reverse transcription of ancestral genes on the X chromosome. Why do retrotransposition genes translocate to autosomes? One possible explanation is that translocation provides a means of escaping X chromosome inactivation during spermatogenesis [99].

Sex chromosomes may play important roles in the creation of new genes [101]. Since sex chromosomes do not have homologous chromosomes in males, homology between only a few regions of the X and Y chromosomes

Table 1. Male germ cell-specific intronless genes and putative original genes in mouse.

Gene Name	Chr. number	Protein	Putative origin gene	Chr. number	Ref.
PGK-2	17	phosphoglycerate kinase	PGK-1	X	99
Zfa	10	zinc-finger protein	Zfx	X	103
PDHA2	4	pyruvate dehydrogenase	PDHA1	X	100
PRM3	16	protamine	PRM	16	104
G6pd2	5	glucose-6-phosphate 1-dehydrogenase	G6pd1	4	105
Pabp2	18	poly(A) binding protein	Pabp1	15	86
Cetn1	18	centrin	Cent2	X	106
Gk-rs 1,2	18,5	glycerol kinase	Gyk	X	107
Gsg3	6	actin capping protein α	CPα	3	108
tPAP	5	poly(A) polymerase	PAPα	12	57
Scot-t	4	3-oxoacid CoA transferase	Scot	15	109
t-actin 1,2	4,4	actin-like protein	actin	8	110
Tssk 1,2	16,16	serine/threonine-protein kinase			111
Haspin	11	serine/threonine-protein kinase			112
Hils1	11	histone H1-like protein			21, 22
Hanp1	15	histone H1-like SR protein			23

can cause homologous recombination in males. Mutations on the X and Y chromosomes in a male germ cell will be transmitted to the next generation. Similar to mutations, retrotranspositions would be more easily retained on X and Y chromosomes than on autosomes. In females, the X chromosome has a homologous chromosome. However, since there is only one Y chromosome, males are vulnerable to the effects of mutations and recombination defects. The relationship between X chromosomes and retrotransposition may be partially explained by these observations.

Many germ cell-specific genes do not have ancestral counterpart genes on the X chromosome and are localized not only on X chromosomes but also on autosomes; these genes may have spread to different chromosomes by retrotransposition. Interestingly, many haploid germ cell-specific genes lack introns (some are shown in table 1).

Chromatin states can be distinguished by differential covalent modifications of histones or by utilization of histone variants. Chromatin associated with transcriptionally active loci becomes enriched for histones with lysine modifications [102]. Haploid germ cells do not have the usual histones and cannot regulate genes by the conventional histone code, whereby histone modifications in some chromatin states regulate gene expression. It is likely that there are specific mechanisms in haploid germ cell-specific genes that act to conserve an unusually large number of intronless genes. Further detailed studies of other haploid germ cell-specific genes might resolve these issues.

In spermiogenesis, the content of nuclear basic proteins in chromatin is mainly replaced by a protamine from a histone. The genomic DNA in haploid germ cells is folded by specific chromatin constructs. These specific mechanisms maintain the life cycle via germ cell differentiation, fertilization and development, which are not passed along parthenogenetical development. Further analysis of the molecular mechanism of spermiogenesis is needed to understand the overall processes in living organisms.

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