Role of microRNAs in mammalian spermatogenesis and testicular germ cell tumors

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Abstract

microRNAs (miRNAs) are a class of small endogenous RNAs, 19–25 nucleotides in size, which play a role in the regulation of gene expression at transcriptional and post-transcriptional levels. Spermatogenesis is a complex process through which spermatogonial stem cells (SSCs) proliferate and differentiate into mature spermatozoa. A large number of miRNAs are abundantly expressed in spermatogenic cells. Growing evidence supports the essential role of miRNA regulation in normal spermatogenesis and male fertility and cumulative research has shown that this form of regulation contributes to the etiology of testicular germ cell tumors (TGCTs). In this review, we addressed recent advancements of miRNA expression profiles in testis and focused on the regulatory functions of miRNA in the process of SSC renewal, spermatogonial mitosis, spermatocyte meiosis, spermiogenesis, and the occurrence of TGCTs.

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microRNA overview

microRNAs (miRNAs), small non-coding RNAs, play regulatory roles by repressing translation or cleaving RNA transcripts (He *et al.* 2009, Mclver *et al.* 2012a,b). The first miRNA gene, *lin4*, was discovered in 1993 and is involved in the normal temporal control of diverse postembryonic developmental events in *Caenorhabditis elegans* (Lee *et al.* 1993, Wightman *et al.* 1993). Subsequently, several hundred miRNAs have been identified in animals and plants. Most of our knowledge of miRNAs comes from the study of canonical miRNAs. In brief, miRNAs are transcribed as longer stem-loop precursors, termed pri-miRNAs, which are further recognized and cleaved by two endonucleases, Drosha/DGCR8 and Dicer, to yield a double-stranded RNA of 21–22 nucleotides (Hutvagner *et al.* 2001, Carmell & Hannon 2004, Kim *et al.* 2009). Subsequently, one strand of the duplex is incorporated into the RNA-induced silencing complex, which contains argonaute proteins, and is guided to target sequences by base-pairing (Liu *et al.* 2004), resulting in degradation of target mRNAs or repression of their translation (Lai 2002, Carrington & Ambros 2003, Kim *et al.* 2009). The nuclear biogenesis of non-canonical microRNAs appears to bypass Drosha cleavage; rather, upstream processing is performed by splicing machinery and the lariat-de-branching enzyme, which yields pre-miRNA-like hairpins that are directly suitable for Dicer cleavage

without further processing (Fig. 1; Okamura *et al.* 2007, Babiarz *et al.* 2008, Rosa & Brivanlou 2011, Havens *et al.* 2012). Increasing evidence has revealed that miRNAs are involved in various biological processes including embryonic development (Yi *et al.* 2008, Foshay & Gallicano 2009), cell proliferation (Brennecke *et al.* 2003, Lee *et al.* 2005), cell differentiation (Chen *et al.* 2004, Foshay & Gallicano 2009), apoptosis (Ambros 2003, Xu *et al.* 2003), fat metabolism (Xu *et al.* 2003), and oncogenesis (Calin *et al.* 2002, 2004, Esquela-Kerscher & Slack 2006, Blakaj & Lin 2008, Manikandan *et al.* 2008).

Introduction to spermatogenesis

Mammalian spermatogenesis starts from the self-renewal and differentiation of spermatogonial stem cells (SSCs). SSCs can divide into either new stem cells (As) or Apaired (Apr) spermatogonia that are committed to differentiation. Apr spermatogonia produce Aaligned (Aal) spermatogonia mitotically, which then give rise to several generations of spermatogonia, including A1–A4, intermediate, and type B spermatogonia (He *et al.* 2009). Type B spermatogonia divide by mitosis to form preleptotene spermatocytes, which initiate the long-lasting meiosis I, in which homologous recombination between sister chromatids occurs before they separate. In meiosis II, spermatocytes undergo a reduction division to split the sister chromosomes into two cells to generate

Figure 1 Biogenesis of canonical miRNAs and non-canonical miRNAs.

secondary spermatocytes, which divide without replicating their DNA to form haploid round spermatids. The round spermatids commence the differentiation phase (spermiogenesis) to develop into mature spermatozoa (Kotaja 2014). Throughout spermatogenesis, germ cells maintain cytoplasmic bridges to facilitate synchronized cell division and differentiation (McIver *et al.* 2012a). Besides, spermatogenesis is regulated by hypothalamic–pituitary–gonadal axis. Gonadotropin-releasing hormone from the hypothalamus regulates the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary gland. Then FSH and LH stimulate the Leydig cells to release testosterone (Kotaja 2014).

miRNAs and spermatogenesis

Spermatogenesis is a complex and highly regulated process that supports the daily production of millions of sperm, which takes place within the seminiferous tubules of the testis of the sexually mature male. This developmental process requires the coordination of both somatic and germ cells through the phases of proliferation, meiosis, and differentiation to generate mature spermatozoa that are responsible for delivery of the paternal genome (Grimaldi *et al.* 2013, Fok *et al.* 2014, Goudarzi *et al.* 2014).

Emerging evidence has shown that miRNAs are essential for spermatogenesis and may play an important role during mitotic, meiotic, and post-meiotic stages of spermatogenesis by regulating the expression of the target gene (Tang *et al.* 2007, Hayashi *et al.* 2008, Bouhallier *et al.* 2010). Dicer, an RNase III endonuclease, is essential for the biogenesis of miRNAs. Several studies have shown that testes were reduced in size and the process of spermatogenesis was disrupted after ablation of Dicer (Romero *et al.* 2011). In addition, these studies demonstrated that removal of Dicer1 at the early onset of male germ cell development led to infertility, due to multiple cumulative defects in the meiotic and post-meiotic stages (Wu *et al.* 2012). Specifically, the progression of spermatocyte meiosis

and spermiogenesis was delayed, the number of haploid cells decreased, the number of apoptotic spermatocytes increased, and the low number of mature sperm in epididymides exhibited abnormal morphology and motility. In addition, sperm lacking Dicer1 were rarely able to fertilize WT eggs to generate viable offspring (Maatouk *et al.* 2008, Korhonen *et al.* 2011, Romero *et al.* 2011).

Expression profile of miRNA in the testis

Over the past several decades, a number of expression profile studies using miRNA microarrays, RT-PCR, or small RNA sequencing have demonstrated that numerous miRNAs are exclusively or preferentially expressed in the testis or germ cells of humans and mice (Barad *et al.* 2004, Ro *et al.* 2007, Yan *et al.* 2007, Buchold *et al.* 2010, Smorag *et al.* 2012). A total of 770 known and five novel human miRNAs were detected in normal human testes by Solexa sequencing technology (Yang *et al.* 2013b).

The global miRNA expression in cell populations from different stages of spermatogenesis, such as spermatogonia, spermatocytes, and spermatids, was conducted by microarray analysis. The results showed that most miRNAs are preferentially expressed in meiotic germ cells (Ro *et al.* 2007, Marcon *et al.* 2008). Some studies have demonstrated that miRNA expression patterns differ between immature and mature testes in human. For example, Yan *et al.* (2007) found 14 up-regulated and five down-regulated miRNAs in immature testes compared with adult testes. Subsequently, the differences in miRNA expression between immature and mature rhesus monkey (Yan *et al.* 2009) and porcine testes (Luo *et al.* 2010) were found. The expression profile of miRNAs in testes is given in Table 1.

miRNA and spermatogonial stem cell renewal

Within the testis, the SSCs reside in a unique micro-environment, or ‘niche’, which includes the surrounding somatic cells. Spermatogenesis originates from SSCs,

Table 1 Expression profile of miRNAs in testes.

Origin	Expression	References
Mouse testis	141 miRNAs from the mouse testis were detected, of which 29 were novel	Ro <i>et al.</i> (2007)
Human testis	770 known and five novel human miRNAs were detected by Solexa sequencing technology	Yang <i>et al.</i> (2013a)
Saanen dairy goat testis	91 novel paired miRNAs were found	Wu <i>et al.</i> (2014b)
Immature and mature testes (mouse)	14 up-regulated and five down-regulated miRNAs were found in the immature testis compared with the adult testis	Yan <i>et al.</i> (2007)
Immature and mature testes (pig)	51 miRNAs were significantly up-regulated and 78 miRNAs were down-regulated in mature testes	Luo <i>et al.</i> (2010)
Testis tissue from the immature rhesus monkey, mature rhesus monkey, and mature human	26 miRNAs were shared by the IR and MR, and the IR and MH, although miRNA levels in each pair of samples differed by more than threefold	Yan <i>et al.</i> (2009)
Mouse spermatogonia	The miR17–92 and miR290–295 clusters are enriched in the spermatogonia of neonatal mice	Hayashi <i>et al.</i> (2008)
Human spermatozoa	68 small RNAs have been identified in human spermatozoa	Ostermeier <i>et al.</i> (2005)
Spermatozoa of patients with varicocele	miR15a was significantly decreased in patients with varicocele compared with the control	Ji <i>et al.</i> (2014)
Testes of patients with NOA and normal human testes	154 differentially down-regulated and 19 up-regulated miRNAs were found in testes from NOA patients	Lian <i>et al.</i> (2009)
Human spermatozoa in patients with different spermatogenic impairments	50 up-regulated and 27 down-regulated miRNAs were found in asthenozoospermic males. 42 up-regulated and 44 down-regulated miRNAs were found in oligoasthenozoospermic males compared with normozoospermic males	Abu-Halima <i>et al.</i> (2013)
Cryptorchid and normal testes	miR135a was expressed at a lower level in cryptorchid testes, which contributes to the maintenance of spermatogonial stem cells by regulating <i>Foxo1</i>	Moritoki <i>et al.</i> (2014)
Human testicular tissues of infertile men with different histopathological patterns	A total of 197, 68, and 46 miRNAs were found to be differentially expressed when comparing the samples from Sertoli cell only, mixed atrophy, and germ cell arrest groups, respectively, with normal spermatogenesis	Abu-Halima <i>et al.</i> (2014)

NOA, non-obstructive azoospermia.

which have the dual property of continually renewing and undergoing differentiation into a spermatogonial progenitor that expands and further differentiates (Hess *et al.* 2006, Ventela *et al.* 2012, Dovere *et al.* 2013, Silvan *et al.* 2013, van den Driesche *et al.* 2014, Guo *et al.* 2014). In the rodent testis, SSCs are among undifferentiated spermatogonia that include Asingle (As), Apaired (Apr), and Aaligned (Aal) spermatogonia (Dym 1994). The regulation of the balance between self-renewal and differentiation of SSCs determines the lifelong supply of spermatozoa by maintaining a population of undifferentiated spermatogonial stem cells and ensuring that adequate numbers of spermatogonia undergo spermatogenesis (van den Driesche *et al.* 2014). miRNAs, as critical endogenous regulators in mammalian cells, play important roles in the regulation of the fate of SSCs. A large number of miRNAs, such as the miR17–92 cluster (Tong *et al.* 2012), miR290–295 cluster (McIver *et al.* 2012a,b), miR146 (Huszar & Payne 2013), miR20 (He *et al.* 2013), miR21 (Niu *et al.* 2011), miR106a (He *et al.* 2013), miR221, and miR222 (Yang *et al.* 2013b), are highly expressed in THY1-enriched undifferentiated spermatogonia. These miRNAs are largely down-regulated during retinoic acid-induced

spermatogonial differentiation both *in vitro* and *in vivo*, suggesting that they are potentially involved in the regulation of proliferation and differentiation of SSCs during spermatogenesis (Kotaja 2014). For example, miR20 and miR106a promote renewal of SSCs at the post-transcriptional level via targeting *Stat3* and *Ccnd1* (He *et al.* 2013). miR135a contributes to SSC maintenance through modulation of *Foxo1* activity (Moritoki *et al.* 2014), and miR21, which is present in SSC-enriched germ cells and is regulated by the transcription factor ETV5, is important in maintaining the SSC population (Niu *et al.* 2011). The list of miRNAs implicated in maintenance of pluripotency in germ cells is given in Table 2.

The role of miRNAs in spermatocyte meiosis and spermiogenesis

Spermatocyte meiosis and spermiogenesis are unique cellular processes to germ cells in the male. The meiotic phase (chromosomal replication, recombination, and two consecutive meiotic cell divisions of spermatocytes) and haploid phase (also called spermiogenesis, differentiation of spermatids into spermatozoa) of

Table 2 miRNAs implicated in maintenance of pluripotency in germ cells.

Name of miRNA	Expression	Targets (predicted and confirmed)	References
miR17–92 cluster	THY1 ⁺ -enriched undifferentiated spermatogonia	<i>Bcl2l11</i> , <i>Kit</i> , <i>Socs3</i> , and <i>Stat3</i>	Tong <i>et al.</i> (2012)
miR106b-25	THY1 ⁺ -enriched undifferentiated spermatogonia	<i>Bcl2l11</i> , <i>Kit</i> , <i>Socs3</i> , and <i>Stat3</i>	Tong <i>et al.</i> (2012)
miR290–295 cluster	Highly enriched in the germ cell population of the 6-day-old testis, multipotent adult germ cells, and embryonic stem cell	<i>Stat3</i> and <i>Ccnd1</i>	Zovoilis <i>et al.</i> (2008, 2010) and Mclver <i>et al.</i> (2012a,b)
miR146	Highly expressed in undifferentiated spermatogonia	<i>Med1</i>	Huszar & Payne 2013)
miR221 and miR222	THY1 ⁺ -enriched undifferentiated spermatogonia		Yang <i>et al.</i> (2013b)
miR20 and miR106a	SSCs		He <i>et al.</i> (2013)
miR21	THY1 ⁺ -enriched undifferentiated spermatogonia	P12	Niu <i>et al.</i> (2011) and Zheng <i>et al.</i> (2011)
miR135a	SSCs	<i>Foxo1</i>	Moritoki <i>et al.</i> (2014)
miR302–367 cluster	Primordial germ cells, ES cells	NR2F2	Hayashi <i>et al.</i> (2008), Dyce <i>et al.</i> (2010) and Rosa & Brivanlou (2011)
miR376a	Down-regulated in mature mouse testes	CDK2 and AGO2	Wang <i>et al.</i> (2011)
miR302 cluster	Overexpressed in adult (undifferentiated) and pediatric germ cell tumors	NR2F2	Lian <i>et al.</i> (2009), Murray <i>et al.</i> (2010), Palmer <i>et al.</i> (2010) and Rosa & Brivanlou (2011)
miR335	Down-regulated in mature mouse testes	CCNT2, CCD2, RSNB1, and RUNX2	Yan <i>et al.</i> (2007) and Tome <i>et al.</i> (2011)
miR367	Embryonal carcinoma, ES cells	LATS2, KLF4, RUNX1, SYNJ1, and SMAD6	Li <i>et al.</i> (2009)

SSCs, spermatogonial stem cells.

spermatogenesis are characterized by high transcriptional activity but suppressed translational activity. Post-transcriptional control of gene expression in these phases is a significant feature of mammalian spermatogenesis (Kotaja 2014). A large number of miRNAs are preferentially expressed in spermatocytes and spermatids and are involved in the regulation of meiotic and post-meiotic gene expression (Kotaja 2014). Expression of the miR449 cluster is drastically up-regulated upon meiotic initiation during testicular development and in adult spermatogenesis, both of which are regulated by testes-specific transcription factors, CREM τ and SOX5, through binding to two highly conserved *cis*-elements of the Cdc20b/miR449 cluster (Bao *et al.* 2012). miR34c is highly expressed in isolated pachytene spermatocytes and round spermatids (Zhang *et al.* 2012, Li *et al.* 2013), and *Tgif2* and *Notch2* (important in spermatogenesis) are the direct targets of miR34c (Bouhallier *et al.* 2010). Tgfb signaling inhibits the second meiotic division in spermatogenesis (Damestoy *et al.* 2005), whereas notch signaling promotes spermatogonia differentiation (Kostereva & Hofmann 2008). miR449 and miR34b/miR34c regulate germ cell differentiation and survival by targeting NOTCH1 and DAZL, as well as germ cell apoptosis by targeting BCL2 and ATF1 (Yan *et al.* 2009, Luo *et al.* 2010, Liang *et al.* 2012). miR214 is preferentially expressed in pachytene spermatocytes and plays a key role in meiosis by targeting heat shock proteins (Marcon *et al.* 2008, Dai *et al.* 2011). miR320, which is expressed in all germ cells, is predicted to target

protocadherins that is important in cell adhesion (Marcon *et al.* 2008, Dai *et al.* 2011). Transition protein 2 (TNP2) and protamine 2 (PRM2) are key chromatin remodelers that facilitate the compaction and condensation of chromatin during spermiogenesis. miR122a reduces the expression of TNP2 to regulate chromatin remodeling during mouse spermatogenesis (Yu *et al.* 2005, Yu & Hecht 2008, Liu *et al.* 2013b). miR469 represses TNP2 and PRM2 protein expression at the translation level through binding to the coding regions of TNP2 and Prm2 mRNAs and then affects normal sperm production (Dai *et al.* 2011). miR355, miR181b, and miR181c are all up-regulated in the adult testis and are involved in transcriptional regulation in haploid germ cells by targeting Rsb1 (Yan *et al.* 2007, Mclver *et al.* 2012a). miR184 may be involved in the post-transcriptional regulation of mRNAs, such as *Ncor2*, in mammalian spermatogenesis (Wu *et al.* 2011). One miRNA can target various mRNAs and one mRNA can be targeted by multiple miRNAs (Peter 2010). Ablation of a single miRNA or miRNA cluster rarely leads to a discernable phenotype in mice under non-stress conditions, in some cases because of compensatory effects by other functionally related miRNAs (Wu *et al.* 2014a). For example, although no discernable phenotype has been observed in miR449 cluster-knockout mice, studies have suggested that miR34b/miR34c may compensate for the absence of miR449, as both miRNA families function redundantly by targeting the E2F–pRb pathway (Bao *et al.* 2012). Wu *et al.* (2014a) constructed the

Mir34b/Mir34c and Mir449 double knockout (dKO) mice and demonstrated again that these two miRNAs were functionally redundant. The dKO mice were found with severely disrupted spermatogenesis and sterility. The miRNAs that play a regulatory role in spermatocyte meiosis and spermiogenesis can be found in Table 3.

Role of miRNA expression in Sertoli cells

Sertoli cells play a central and essential role in coordinating spermatogenesis by structurally and nutritionally supporting germ cells and secreting factors that control the survival and progression of germ cells, without which the production of normal sperm would be hindered (Papaioannou *et al.* 2011, Panneerdoss *et al.* 2012). *Dicer* is absolutely essential for Sertoli cells to mature, survive, and ultimately sustain germ cell development. In fact, ablation of *Dicer* leads to infertility suggesting that miRNA expression in Sertoli cells is important for supporting germ cell development (Nicholls *et al.* 2011, Papaioannou *et al.* 2011). Panneerdoss *et al.* (2012) had identified miRNAs as one group of testosterone-dependent trans-acting factors in postnatal Sertoli cells that may play a crucial role in androgen-mediated events during spermatogenesis by targeting Sertoli cell/germ cell-specific genes. A large number of miRNAs (e.g., miR471, miR470, miR463, miR465, miR743a/miR743b, miR883, miR880, miR201, and miR547) expressed in Sertoli cells have been demonstrated to play a coordinated role in androgen-dependent spermatogenic events (Panneerdoss *et al.* 2012). In addition, a subset of miRNAs expressed in Sertoli cells, including miR23b, miR30c, miR30d, and miR690, are stimulated upon FSH and androgen suppression, which are critical regulators of target proteins associated with junction restructuring and spermiation (Nicholls *et al.* 2011). Thus, FSH and androgens act on Sertoli cells at stage VIII to regulate the expression of miRNAs that operate in a coordinated manner to control cell adhesion pathways and male fertility.

miRNA and germ cell tumors

miRNAs are essential for cell proliferation and differentiation by regulating cell cycle-related factors. A growing number of studies have demonstrated that miRNAs may play a similar role as proto-oncogenes, as both are involved in occurrence and evolution of tumors (Gregory & Shiekhattar 2005, Calin & Croce 2006, Costinean *et al.* 2006, Hwang & Mendell 2007, Manikandan *et al.* 2008). A high-throughput microarray showed that miRNA expression profiles in tumor tissue significantly differed from the normal tissue obtained from the same source (Murakami *et al.* 2006, Wang & Wang 2006, Hwang & Mendell 2007), and as such, could assist in the diagnosis

and treatment of cancer according to this differential expression pattern (Mattie *et al.* 2006, Volinia *et al.* 2006, Yanaihara *et al.* 2006). Human germ cell tumors comprise a heterogeneous group of neoplasms, all with a defined histological appearance. They have specific epidemiological characteristics, clinical behavior, and pathogenesis (Looijenga *et al.* 2014). In the testis, there are three types of testicular germ cell tumors (TGCTs) that occur at distinct ages in men, namely, the teratomas–yolk sac tumors of the infantile testis (type I), the seminomas and non-seminomas of adolescents and adults (type II), and the spermatocytic seminomas of the elderly (type III) (Oosterhuis & Looijenga 2005, Looijenga *et al.* 2006, 2014, Gillis *et al.* 2007). The expression profiles of miRNAs in these different types of TGCTs vary. For example, Gillis found that the miR302 cluster, which plays a role in the maintenance of embryonic stem cells pluripotency, is elevated in seminoma tumors. miR21 and miR155, as oncogenic miRNAs, are highly expressed in seminomas and spermatocytic seminomas. The up-regulation of miR19a and miR29a and down-regulation of miR133a and miR145 are found in seminomas and spermatocytic seminomas. miR146 expression is lower in seminomas, spermatocytic seminomas, and even different type II tumors such as embryonic carcinomas and teratomas compared with normal testes (Gillis *et al.* 2007). Analysis of the 156 miRNAs expressed in normal human testes, type II and type III TGCTs, and cell lines derived from TGCTs, verified that terminally differentiated histological subgroups (e.g., normal testicular tissue) expressed most discriminating miRNAs at a higher level than poorly differentiated tumor tissue subpopulations, such as seminoma, dysgerminoma, and embryonal carcinomas (Gillis *et al.* 2007). These results support the model that miRNAs are involved in the regulation of differentiation of stem cells retained in germ cell tumor (GCTs). *Lin 28* is thought to be involved in the maintenance of pluripotency. Let7, miR125a, and miR9 are implicated in the formation of testicular teratomas by targeting *Lin28* (Zhong *et al.* 2010). miR199a expression is significantly lower in TGCTs compared with normal testicular germ cells. The v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B (MAFB) transcription factor was identified as a putative target of miR199a-5p in TGCTs and mediates the tumor suppression activity of miR199a (Gu *et al.* 2013). miR372 and miR373 neutralize p53-mediated cyclin-dependent kinase (CDK) inhibition, possibly through direct inhibition of expression of the tumor suppressor LATS2 (Voorhoeve *et al.* 2006, 2007, Gillis *et al.* 2007). Studies have demonstrated that these miRNAs are potential novel oncogenes that participate in the development of human TGCTs by inhibiting the Trp 53 pathway, thus allowing tumorigenic growth in the presence of WT Trp 53 (Costinean *et al.* 2006). The hsa-miR371–373 cluster is involved in counteracting cellular

Table 3 miRNAs that play a regulatory role in spermatocyte meiosis and spermiogenesis.

Name of MiRNA	Expression	Targets (predicted and confirmed)	Functions	References
miR449	Up-regulated in mature rhesus monkey and mouse testis, preferentially expressed in mouse testes and localized to spermatocytes and spermatids	MECP2, ASB1, BCL2, NOTCH1, CASP2, KITLG, VCL, FOXJ2, INHBB, SOX11, CCNE2, GMFB, and DLL1	Represses the proliferation of a germ cell line, GC-1spg	Bao <i>et al.</i> (2012)
miR34a	Up-regulated in mature mouse testis, up-regulated from day 7 to day 14 in mouse testis	CCND2, BLC2, GMFB, and SIRT1	Represses proliferation, promotes apoptosis	Yan <i>et al.</i> (2007), Buchold <i>et al.</i> (2010) and Ito <i>et al.</i> (2010)
miR34b	Up-regulated in mature rhesus monkey testis, up-regulated from day 7 to day 14 in mouse testis	NOTCH1, LGR4, VEZT, MAN2A2, and FOXJ2	Regulates the germ cell proliferation and survival	Yan <i>et al.</i> (2009), Buchold <i>et al.</i> (2010) and Vogt <i>et al.</i> (2011)
miR34c	Highly expressed in isolated pachytene spermatocytes and round spermatids	CCND3, CCNG1, CCNB1, CCNC, CCNE1, CDK4, CDK6, E2F5, FOS, CDC2, TGIF2, NOTCH2, STRBP LGR4 KLF4, NOTCH1 PPP1CC, GALT, KITLG, SPAG4, CCNL, ZFP148, and GMFB	Cycle regulator, promoted mGSC apoptosis, SSC differentiation, enhances the germinal phenotype of cells already committed to this lineage	Yan <i>et al.</i> (2009), Buchold <i>et al.</i> (2010), Li <i>et al.</i> (2013) and Yu <i>et al.</i> (2014)
miR184	Localized to the germ cells of mouse testis	<i>Ncor2</i>	Promotes the proliferation of a germ cell line, GC-1spg	Marcon <i>et al.</i> (2008) and Wu <i>et al.</i> (2011)
miR24	Pachytene spermatocytes	MBD6 and H2AX	Potential role in meiosis	Marcon <i>et al.</i> (2008)
miR214	Mainly expressed in pachytene spermatocytes	WDTC1, heat shock proteins	Potential role in meiosis	Marcon <i>et al.</i> (2008) and Dai <i>et al.</i> (2011)
miR320	Expressed in all germ cells	Protocadherins	Cell adhesions	Marcon <i>et al.</i> (2008) and Dai <i>et al.</i> (2011)
miR18	Highly expressed in spermatocytes	HSF2	Male germ cell maturation	Bjork <i>et al.</i> (2010)
miR122a	Enriched in late-stage male germ cells	TNP2	Regulates the chromatin remodeling, plays a role in spermiogenesis	Yu <i>et al.</i> (2005), Yu & Hecht (2008) and Liu <i>et al.</i> (2013b)
miR469	Pachytene spermatocytes and round spermatids	TP2 and PRM2	Regulates the chromatin remodeling, plays a role in spermiogenesis	Dai <i>et al.</i> (2011)
miR355	Up-regulated in adult testis	<i>Rsb1</i>	Transcriptional regulation in haploid germ cells	Yan <i>et al.</i> (2007) and McIver <i>et al.</i> (2012a)
miR181b	Up-regulated in adult testis	<i>Rsb1</i>	Transcriptional regulation in haploid germ cells	Yan <i>et al.</i> (2007) and McIver <i>et al.</i> (2012a)
miR181c	Up-regulated in adult testis	<i>Sox5</i> , <i>Sox6</i> , and <i>Rsb1</i>	Transcriptional regulation in haploid germ cells	Yan <i>et al.</i> (2007) and McIver <i>et al.</i> (2012a)
miR185	Preferentially expressed in pachytene spermatocytes	RHOA and CDC42	Cell cycle regulator	Marcon <i>et al.</i> (2008)
miR191	Highly expressed in testis, preferentially expressed in beta pachytene spermatocytes, down-regulated in teratozoospermia	BNC2	Required for normal sperm morphology	Marcon <i>et al.</i> (2008)

GC, germ cell; mGSCs, male germline stem-cell; SSCs, spermatogonial stem cells.

senescence induced by oncogenic stress, allowing cells to become malignant (Gillis *et al.* 2007). Protein tyrosine phosphatase non-receptor type 23 (PTPN23) is one of the important tumor suppressor candidates and is involved in the tumorigenesis of various organs. Relatively higher miR142-3p expression can be found in the absence of PTPN23 protein expression in human TGCTs, suggesting that miR142-3p plays an important role in the pathogenesis of TGCTs by repressing PTPN23 expression (Tanaka *et al.* 2013). Together, the data presented in this review indicate that aberrant miRNA expression is related to human germ cell tumor occurrence. The miRNAs implicated in the development of TGCTs are shown in Table 4.

Epigenetics of miRNA in spermatogenesis and TGCTs

Epigenetics is the study of heritable changes in gene expression that occur without changes in DNA sequence and is an important mechanism that underlies the ability of environmental chemicals to influence health and disease (Singh & Li 2012). The association of DNA methylation, histone modification, and miRNA with environmental exposure and heritable phenotypes has been widely established. Cigarette smoke, one of the most prevalent and significant global chemical carcinogens, induces differential miRNA expression in the spermatozoa of smokers compared with non-smokers. These altered miRNAs predominantly mediate pathways

Table 4 miRNAs implicated in the development of TGCTs.

Name of miRNA	Expression	Targets (predicted and confirmed)	Functions	References
miR301 miR302 cluster	SS, YST, teratoma SE, YST	NR2F2		Gillis <i>et al.</i> (2007) Gillis <i>et al.</i> (2007), Palmer <i>et al.</i> (2010) and Rosa & Brivanlou (2011)
miR302d	SE, EC, up-regulated in YST	LATS2, LEFTY1, MLL3, DAZAP2, TNFA1P1, BCL11B, and PLAG1	Oncogene, suppressor of apoptosis	Gillis <i>et al.</i> (2007) and Murray <i>et al.</i> (2010)
miR21 miR155 miR19a miR29a miR133a miR133b	Highly expressed in SE and SS Highly expressed in SE and SS Overexpressed in SE and SS Overexpressed in SE and SS Down-regulated in SE and SS Teratomas	P12 POU4F1, MEIS2, and FSCN1	Oncogene Oncogene Tumor suppressor	Gillis <i>et al.</i> (2007) Gillis <i>et al.</i> (2007) Gillis <i>et al.</i> (2007) Gillis <i>et al.</i> (2007) Gillis <i>et al.</i> (2007) Gillis <i>et al.</i> (2007), Kano <i>et al.</i> (2010) and Qiu <i>et al.</i> (2014)
miR140	Teratomas	BCL11A, SOX4, and CXCL12	Skeletal development and cartilage maintenance	Nicolas <i>et al.</i> (2008) and Miyaki <i>et al.</i> (2010)
Let7 miR9 miR125a miR145	Teratomas Teratomas Teratomas Down-regulated in SS	Lin28 <i>Lin28</i> <i>Lin28</i> PLAGL2, E2F3, SOX9, OCT4, SOS2, KLF4, and FSCN1		Zhong <i>et al.</i> (2010) Zhong <i>et al.</i> (2010) Zhong <i>et al.</i> (2010) Gillis <i>et al.</i> (2007), Kano <i>et al.</i> (2010), Port <i>et al.</i> (2011) and Qiu <i>et al.</i> (2014)
miR146	Down-regulated in SE, SS, and even certain type II tumors, such as EC and teratomas			Gillis <i>et al.</i> (2007)
miR199a	Expressed at low levels in TGCTs	MAFB	Tumor suppressor	Gu <i>et al.</i> (2013)
miR200c	Expressed in EC, SE, and various elements of NS	ZEB1 and TRKB	Tumor suppressor, inhibitor of cell invasion/migration, control of apoptosis	Radisky 2011)
miR367	Expressed at low levels in human ES and EC cells	LATS2, KLF4, RUNX1, SYNJ1, and SMAD6	Maintenance of pluripotency, regulation of gene transcription	Gillis <i>et al.</i> (2007) and Li <i>et al.</i> (2009)
miR368 miR371–373	SE, EC SE, EC, overexpressed in malignant pediatric germ cell tumors and cisplatin-resistant cell lines derived from GCTs	LATS2, ZIC4, LEFTY1, DAZAP, TNFA1P1, PLAG2, and MLL3	Involved in over-ruling cellular senescence	Gillis <i>et al.</i> (2007) Voorhoeve <i>et al.</i> (2006, 2007) and Gillis <i>et al.</i> (2007)
miR375	Overexpressed in pediatric YSTs	IGR1R	Tumor suppressor, inhibits metastasis	Murray <i>et al.</i> (2010) and Hudson <i>et al.</i> (2013)
miR142-3p	TGCT cell lines		Represses PTPN23 expression	Tanaka <i>et al.</i> (2013)

SE, seminomas; NS, non-seminomas; EC, embryonal carcinoma; YST, yolk sack tumors; SS, spermatocytic seminoma; TGCTs, testicular germ cell tumors; GCT, germ cell tumor.

vital for healthy sperm and normal embryonic development, particularly in cell death (Marczylo *et al.* 2012). Epigenetics can also affect miRNA expression through DNA methylation and histone modifications and can inversely regulate epigenetic inheritance through DNA methylation transferase, thereby maintaining DNA methylation levels and altering histone modifications (Sato *et al.* 2011, Liu *et al.* 2013a, Udali *et al.* 2013, Nana-Sinkam & Choi 2014). For example, expression of miR34b and let-7a-3 is regulated by DNA methylation (Lu *et al.* 2007, Toyota *et al.* 2008), and miRNAs with tumor suppressor functions are often silenced by DNA hypermethylation, leading to tumor formation. miR199a expression, which is significantly lower in TGCTs compared with normal testicular germ cells, is controlled by epigenetic changes such as DNA methylation

and histone modification; demethylation with 5-aza can restore miR199a expression (Gu *et al.* 2013).

Future perspectives

The most current miRNA research has demonstrated the biological significance of these molecules in the regulation of spermatogenesis and TGCTs. There are a large number of miRNAs and their expression during spermatogenesis is phase or cell specific. In other words, some miRNA expression differs from SSCs, and pre-meiotic, meiotic, post-meiotic, and spermatid cells. The differential expression pattern of these miRNAs demonstrates that they play a regulating role in a specific phase during spermatogenesis. In addition, studies demonstrate that some serum miRNAs could act as biomarkers to detect germ cell tumors. However, future studies

concerning the stage- and cell-specific expression of miRNAs during spermatogenesis and the potential roles of miRNA in TGCT occurrence are still required for addressing more thoroughly. The in-depth study of miRNAs in testes would lead to a better understanding of the etiology of male infertility and testicular cancer and would probably result in the design and development of a number of biological agents for the diagnosis and treatment of male infertility and testicular cancer. This would lead to an improved quality of life, ultimately resulting in enormous economic and social benefits.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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