miRNA and mammalian male germ cells

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Background: Achieving the correct spatial and temporal expression of germ-cell-specific genes is fundamental to the production of viable healthy spermatozoa. Notably, post-transcriptional gene regulation resulting in the repression of protein translation is central to many embryonic processes, and is particularly active during spermatogenesis. In this review, we discuss microRNA (miRNA) regulation of target gene expression in relation to mammalian spermatogenesis, the establishment of testicular germ cell tumours (TGCT) and the potential use of miRNA manipulation for cancer therapy and fertility regulation.

Methods: Journal databases such as PubMed were searched using key words, including miRNA, testis, spermatogenesis, germ cell, testicular cancer and cancer.

Results: In the past decade, the deployment of small non-coding RNA molecules, including miRNA, by the cell, has been recognized as among the most important mechanisms of fine-tuning translational regulation in differentiating cell types. For key regulators of male gametogenesis, high levels of gene expression do not always correspond to elevated levels of protein expression. Cumulatively this indicates that enhancement and repression of post-transcriptional regulatory mechanisms are essential to the success of spermatogenesis. There is also growing evidence that this form of regulation contributes to the aetiology of both TGCT and spermatocytic tumours.

Conclusions: miRNA plays an essential role in regulation of genes during the process of spermatogenesis. Disruption of this regulation has the ability to contribute to the neoplastic development of germ cell tumours. However, targeted knockdown of specific miRNA molecules has the potential to form both anti-oncogenic reagents and underpin the basis for novel contraceptive technologies.

Key words: non-coding RNA / primordial germ cell / differentiation / meiosis / spermatogenesis
Introduction

MicroRNA (miRNA) molecules are short non-coding single-stranded RNA molecules that bind specifically, in conjunction with a protein complex, to several messenger RNA (mRNA) molecules to control their translation (Thomas et al., 2010). miRNA was first identified in 1993 when scientists studying the development of Caenorhabditis elegans discovered a short non-coding RNA (lin-4) that contained sequences complementary to the 3’ untranslated region (UTR) of the lin-14 mRNA, which was known to encode a developmentally important temporal control protein. It was hypothesized that lin-4 controlled the expression of lin-14 through RNA:RNA interactions. However, it was not until the discovery of a second miRNA let-7 in 2000, which controls developmental timing and differentiation of C. elegans in a manner similar to that proposed for lin-4 that the scientific community acknowledged the importance of miRNA in controlling gene expression. Since 2000, research on short non-coding RNA molecules has increased exponentially (Zhang et al., 2007). The expression of miRNA is highly regulated and essential for normal development (Blakaj and Lin, 2008). However, dysregulation of miRNA expression has been linked to the development of several types of cancer, including testicular cancer. For example, the miR-17–92 cluster, which promotes cell survival and proliferation, is up-regulated in most cancer types, and considered oncogenic (DeSano and Xu, 2009).

During spermatogenesis, the spatial and temporal regulation of gene expression is of vital importance. In particular, post-transcriptional regulation is essential due to the fact that, during spermatogenesis, germ cells are periodically transcriptionally silenced (Papaioannou and Nef, 2010).

In the last 50 years, the rate of type II testicular cancer has been increasing in developed countries, making it the most common form of cancer in young men (Baade et al., 2008). Testicular germ cell cancer is considered a developmental disease as both seminoma and non-seminoma develop from a precursor lesion, Carcinoma in situ (CIS), shown to be dysfunctional gonocytes left over from fetal development (Sonne et al., 2009). Testicular cancer has a unique miRNA expression profile, and several miRNA molecules have been implicated in its neoplastic development, e.g. miR-372 and miR-373 (Voorhoeve et al., 2006; Gills et al., 2007).

In this review, we discuss miRNAs regulation of mRNA as having a key role in spermatogenesis and the development of testicular cancers. We also examine the potential role for miRNA therapies in cancer therapy and novel contraceptives.

Methods

Journal databases including PubMed, Science Direct, Ovid, Wiley Online Library, Oxford and Google Scholar were searched using key words, including miRNA, testis, spermatogenesis, spermatogonia, germ cell, testicular cancer and cancer. The most commonly used search included miRNA and varying combinations of these key words. Journal articles were included based on their quality and relevance. The reference list of selected journals was examined for relevant articles to include.

Results

miRNA synthesis and specific targeting

miRNA are short single-stranded non-coding RNA molecules of ~21 base pairs. Coding sequences for miRNA are found in all regions of the genome; however, approximately half (40–50%) are located in the introns of genes where they are subject to the same transcriptional regulation as their host gene (Shomron and Levy, 2009). Usually miRNA genes are transcribed by RNA Polymerase II either as monocistronic primary transcripts (pri-miRNA), or as polycistronic pri-miRNA, when miRNA coding sequences are clustered together in the genome (Kim et al., 2009). Pri-miRNA form specific stem loop structures that undergo cleavage in the nucleus by the RNase Drosha, to form isolated hairpin loops (pre-miRNA) (Kim et al., 2009), and are then transported into the cytoplasm (Fig. 1). Once in the cytoplasm the loop is cleaved by the endoribonuclease Dicer, forming double-stranded mature miRNA. One strand of the miRNA is preferentially loaded into the effector miRNA-induced silencing complex (miRISC); however, the complementary (or Star *) strand can also be loaded (Mah et al., 2010). The miRISC complex comprising Argonaute (AGO) proteins mediates the post-translational regulation of mRNA targets of the loaded miRNA (Shomron and Levy, 2009).

Most commonly miRNA bind to target sequences in the 3’UTR of mRNA. However, miRNA have also been shown to bind to the 5’UTR and open reading frame of a subset of target mRNA (Moretti et al., 2010). A short seed region, nucleotides 2–7, on the miRNAs is the most influential factor for target binding (Bartel, 2009). Sequences outside this region (such as nucleotides 13–16) as well as AU-rich sequences and the position of binding on the 3’UTR (e.g. close to the stop terminus) can influence how the miRNA regulates the gene expression (Bartel, 2009; Zhao and Liu, 2009).

The fate of the targeted miRNA depends both on the complementarity of the miRNA to its mRNA partner and on the catalytic activity of the AGO proteins within the RISC complex. AGO2 is the only AGO protein with the ability to cleave RNA in mammals (Liu et al., 2004). High complementarity base pairing between the target and miRNA combined with the presence AGO2 causes mRNA degradation (Thomas et al., 2010). Mismatched pairing between the target and the miRNA causes mRNA sequestering in cytoplasmic granules or translational repression via a number of mechanisms (Thomas et al., 2010). First, miRNA prevents the formation of the functional 80S subunit. The RISC complex has been shown to bind to ElF6, a factor that inhibits the interaction of the 40S and 60S ribosomal subunits and thus prevents the formation of a functional ribosome (Liu, 2008). Second, AGO2 has been shown to bind to the m7G 5’ cap of mRNA and thus competes with the initiation factor eIF4E, which normally binds to the cap during translation (Fig. 2). Third, miRNA mediates the deadenylation of the polyA tail of miRNA molecules, rendering it too short to support the binding of the PolyA-Binding Protein (PABP) required for effective translation (Liu, 2008; Liu et al., 2008).

Under certain conditions, miRNA binding has been shown to activate translation. Translational activation depends on the presence of AU-rich elements within the 3’UTR of the mRNA target as well as the proliferation state of the cell. For example, upon cell cycle arrest in HEK293 cells, miR-369–3 expression is up-regulated and binds to the 3’UTR of tumour necrosis factor alpha (TNFαx). Once bound, the miRNA recruits AGO2 and fragile X mental retardation related protein I (FXR1), leading to enhanced translation of TNFαx. However, when arrested cells are stimulated to grow, the presence of miR-369–3 and AGO2 on the 3’UTR of TNFαx causes translational repression (Vasudevan et al., 2007).
Epigenetic modifications (DNA methylation and histone modifications) also affect miRNA expression, and miRNA in turn can affect the epigenetic status of the cell. Epigenetic modifications play important roles in tumourigenesis of most cancer types by silencing tumour suppressor genes and activating oncogenes (Chuang and Jones, 2007; Valeri et al., 2009). For example, when cancer cell lines are treated with agents removing DNA epigenetic modifications, 5% of all miRNAs in the cells increased expression—indicating that some miRNA molecules may be controlled by epigenetic changes (Valeri et al., 2009). In particular, miR-127 expression is suppressed by both DNA methylation and histone modifications, and miR-127 is a tumour suppressor that directly targets the oncogene BCL6, a key modulator of bladder carcinogenesis (Valeri et al., 2009).

In contrast, a specific subgroup of miRNAs (known as epi-miRNA) has been demonstrated to both directly and indirectly regulate the epigenetic machinery of the cell. For example, the miR-29 family, miR-290 cluster, Mir-148, Mir-152 and Mir-301 control the expression either directly or indirectly of both the de novo DNA methyl transferases DNMT3a and 3b, as well as the maintenance DNA methyl transferase DNMT1. Histone methylation (miR-101) and acetylation (miR-1, MiR-140 and miR449a) are also under the control of epi-miRNAs (Chuang and Jones, 2007; Valeri et al., 2009; Iorio et al., 2010).

Therefore, through the deployment of cell- and tissue-specific miRNAs, with multiple roles in negative and positive regulation of gene expression, miRNAs are likely to be critically involved in most biological processes, including mammalian germ cell development.

**Origin of mammalian male germ cells**

Primordial germ cells (PGCs) arise as a small cohort of cells in the early embryo, which proliferate on their migratory route from the hindgut into the developing gonads (Tarbashevich and Raz, 2010). PGCs are first identifiable at embryonic Day 6.25 in mice when a group of *fragilis* positive cells begin to express *blimp* (Chang et al., 2002). This protein suppresses the expression of somatic genes, thus allowing the up-regulation of germ-cell-specific markers (*stella* and *nanos3*) and pluripotency genes (*sox2, oct4* and *nanog*), resulting in the formation of the germ cell lineage (Sage, 2008a, b).
At embryonic Day 7.5, PGCs are in the posterior region of the primitive streak, easily identifiable by their alkaline phosphatase expression, and shortly afterwards become motile (Culty, 2009). PGCs migrate into the hindgut during its anterior extension (E8–9.5); they then move into the mesoderm (E9.5) and bilaterally migrate to the genital ridges to contribute to the formation of the gonads (E10.5–11.5) equivalent to embryonic Day 33–37 in humans (Bendel-Stenzel et al., 1998; Richardson and Lehmann, 2010).

Steel factor (kit-ligand) has been identified as a key survival and proliferative signal as well as acting to guide PGCs along the hindgut and towards the genital ridges (Farini et al., 2007; Gu et al., 2009). The movement of PGCs out of the hindgut and into the gonads (E9.5) is dependent on E-cadherin (CDH1) and β1-integrin (Itgb1) (Richardson and Lehmann, 2010) and is directed by cxcl12 (Molyneaux et al., 2003). On reaching the genital ridges at around Day 11–11.5 the PGC’s proliferate and form gonocytes (Culty, 2009).

Male sex determination is triggered by the expression of Sry (Sex-determining region on the Y chromosome), a high-mobility group (HMG) transcription factor that activates sox9 (Sry-related HMG box 9)—another transcription factor that in itself is sufficient for sex determination (Kashimada and Koopman, 2010; Sekido, 2010). Sox9 positive pre-Sertoli cells recruit cells from the mesonephros and the coelomic epithelium to form the testicular cords (Kanai et al., 2005; Barsoum and Yao, 2006), which occurs in concert with the commitment of male germ cells to the prespermatogonia cell fate (Kocer et al., 2009).

miRNA has been implicated in the regulation of several pluripotency genes required for germ cell specification. For example, miR-145 has been found to totally suppress the expression of OCT4, and partially repress the expression of SOX2 in human embryonic stem (ES) cells, thereby promoting their differentiation (Xu et al., 2009). Additionally NANOG, SOX2 and OCT4 are regulated by miR-134, 296 and 470 in ES cells (Tay et al., 2008). The pluripotency markers NANOG, OCT4 and SOX2 bind directly to mRNA promoters (e.g. OCT4 to ES cell-specific miR-302 cluster) and collaborate with these molecules to regulate cell fate (Rosa and Brivanlou, 2011). Studies in murine early germ cells have demonstrated that transcriptional repression of nanog and sox2 occurs, while oct4 is post-transcriptionally repressed, demonstrating the importance of translational repression possibly by miRNA in germ cell development (Western et al., 2010).

Male germ cells are maintained in mitotic arrest within the seminiferous tubules by the presence of cyp26b1, which degrades retinoic acid, preventing the expression of stra8 (stimulated by retinoic acid 8) and hence entry into meiosis (Bowles and Koopman, 2007). After the

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**Figure 2** Translational repression of mRNA by miRNA. miRISC usually binds to the 3’UTR of mRNA molecules (**A**), where it binds to the cap, therefore preventing the binding of initiation factor 4 (eIF4) required for translation. (**B**) miRISC also can facilitate the degradation of the mRNA molecule or (**C**) the degradation of the poly A tail to prevent the binding of the PABP also required for efficient translation. (**D**) The miRISC complex binds to initiation factor (eIF6) that is known to prevent the formation of a functional ribosome, therefore preventing the formation of a novel polypeptide chain.
expression of cyp26b1 is decreased at embryonic Day 13.5, the RNA-binding protein nanos2 maintains mitotic arrest in male germ cells (Saga, 2008a, b). Shortly after birth, gonocytes (prospermatogonia) migrate from the centre to the basement membrane of the seminiferous tubules and by post-natal Day 6, they have begun to divide and are designated single spermatogonia or spermatogonial stem cells (Oatley and Brinster, 2006) and spermatogenesis is initiated.

Overview of spermatogenesis

The spermatogonial stem cells divide, allowing both self-renewal (A single) and daughter cells (A paired) that are committed to differentiation. Secreted factors such as gdnf (glial cell line derived neurotrophic factor) in combination with transcription repressor plzf (promyelocytic leukaemia zinc finger), and transcription factor etv5 (ETS variant gene 5) promote self-renewal, while steel factor and notch signalling promote differentiation (Kostereva and Hofmann, 2008; de Rooij, 2009; Hermo et al., 2010a, b). Paired spermatogonia divide mitotically to become aligned (A aligned) differentiating spermatogonia, which then mature into intermediate and type B spermatogonia before entering meiosis to become spermatocytes. Throughout spermatogenesis, germ cells maintain cytoplasmic bridges to facilitate synchronized cell division and differentiation (Oatley and Brinster, 2006; Hess and de Franca, 2008; Hogarth and Griswold, 2010).

Type B spermatogonia divide by mitosis to form preleptotene spermatocytes. Spermatocytes then divide in meiosis I, and homologous recombination between sister chromatids occurs before they separate. After separation of the sister chromatids, the cells become secondary spermatocytes, which divide without replicating their DNA to form haploid spermatids (Fig. 3; Hess and de Franca, 2008; Hermo et al., 2010a, b).

Haploid cells undergo a differentiation process known as spermiogenesis to develop into mature spermatozoa. Spermiogenesis involves four phases referred to as the Golgi, capping, acrosomal and maturation stages (Hermo et al., 2010a, b). The initial Golgi stage involves the formation of the proacrosomal vesicle containing a suite of enzymes. During capping, the proacrosomal vesicle interacts with the nuclear envelope and flattens to cover the nuclear surface as it starts to elongate. In the acrosomal stage, the vesicle migrates over the ventral surface of the nucleus and, due to a remarkable repacking of the genome, the nucleus also condenses to a third of its original size. This phase also sees the mitochondria migrate to the posterior of the cell and the initiation of tail formation. During the final maturation phase of spermiogenesis, the residual cytoplasm, containing some of the Golgi complex and other cytoplasmic constituents, is removed from the spermatid and phagocytosed by the Sertoli cells. The spermatozoa are then positioned ready for release into the rete testis and the epididymis where a suite of additional maturational events take place (Kerr, 1991; Holstein et al., 2003; Hess and de Franca, 2008).
Role of miRNA in germ cell development

Disruption of the miRNA synthesis pathways (Fig. 1) has demonstrated that miRNA is essential for germ cell survival during the colonization of the gonads. The germ cell-specific dicer knockout mouse (generated using the cre-flox system under the direction of the trap promoter which is active from E7.5) displayed normal germ cell numbers until E11.5, at which time the PGCs are colonizing the gonad, then declined sharply. Continued observation concluded that female germ cell numbers recovered while male germ cells did not, and neonatal null mice had half the spermatogonial population of their wild type brothers (Hayashi et al., 2008).

The presence of miRNA is also necessary for the later stages of spermatogenesis. Studies of dicer null mice at 3, 4, 8 and 16 weeks as well as mice over 8 months of age found active spermatogenesis in a few tubules; however, the older mice experienced a dramatic loss of fertility (Hayashi et al., 2008; Maatouk et al., 2008). Many tubules had either a Sertoli cell only phenotype or germ cell defects. Older mice displayed an increased number of Sertoli cell only tubules with a few tubules containing round spermatids; however, a decreased proportion of tubules with elongating spermatids were observed (Maatouk et al., 2008). Hayashi et al. (2008) examined the genomic status of the offspring of dicer null males and determined that most of the pups had an intact dicer gene and thus the germ cells producing spermatozoa escaped repression in the younger mice. However, it was concluded that the infertility observed in older mice may have been attributable to the excision of dicer during the aging process.

Maatouk et al. (2008) observed sperm in the epididymis of dicer germ cell knockout mice and used a reporter system for the cre recombinase to demonstrate that the knockout germ cells were progressing to elongating spermatid stage. The dicer null spermatozoa had relatively normal head structures but the tail was often orientated incorrectly, contained residual cytoplasm and the mitochondria were abnormal leading to pronounced motility defects. Loss of sperm propulsion could explain the reduced fertility in the knockout mice (Maatouk et al., 2008). On the basis of these results, the authors concluded that deletion of dicer caused defects in spermatogonial proliferation and early spermatogenesis.

Despite the inefficiency of the trap promoter, collectively these results demonstrate that dicer, and hence miRNA, are essential for early male germ cell proliferation and late spermatogenesis in order to form functional sperm (Hayashi et al., 2008; Maatouk et al., 2008). Thus research has now focused on the identification and characterization of the molecular function of specific miRNAs in germ cells.

What is known about miRNA expression in the testis?

In normal and neoplastic tissues, the miRNA expression and the corresponding protein expression is not always consistent, highlighting the possible role of small RNA molecules in the control of post-transcriptional regulation (Novotny et al., 2007a, b). Expression levels of miRNA are high in PGCs, germ cells and germ line stem cells, when compared with somatic cells (Buchold et al., 2010).

The miR17–92 cluster are thought to promote survival and proliferation of the germ cells, given that their expression is down-regulated in female germ cells following meiotic arrest (Hayashi et al., 2008). The expression of several other miRNAs is dependent on the developmental stage of the germ cells. In mice, the expression of miR141, 200a, 200c and 323 was down-regulated gradually in both male and female germ cells throughout their development (Hayashi et al., 2008). During male germ cell development, the expression of the let7 miRNA family increased along with miR-125a and miR-9 families; meanwhile, the expression of these molecules remained unchanged in the female germ cells (Hayashi et al., 2008). MiR-125a and the let 7 miRNA family are both tumour suppressors. Let 7 regulates both the RAS oncogenic pathway (Hayashi et al., 2008) and the Fas-regulated apoptosis while miR-125a binds to HuR and suppresses cell growth in breast cancer (Guo et al., 2009). Most importantly for testicular function, let7, miR-125a and miR-9 have all been found to regulate the expression of LIN28, a key controller of stem cell pluripotency that has been implicated in the formation of testicular teratomas (Zhong et al., 2010). In addition, germ cells and ES cells have some similarities in miRNA expression profiles. Studies comparing ES cells and adult germ cells have identified miR-290 and the miR-302 cluster as highly expressed and involved in the maintenance of pluripotency in both populations (Zovolli et al., 2008).

Post-transcriptional control of gene expression is highly active during spermatogenesis. Large-scale gene transcription occurs in two phases: before the cells are rendered quiescent during the process of meiosis (e.g. pachytene spermatocytes), and again post-meiotically prior to nuclear silencing. Up to two-thirds of these transcripts are then stored for translation later in spermatogenesis (Papaioannou and Nef, 2010). Increased miRNA levels coincide with both of these waves of active gene transcription (Ro et al., 2007; Yan et al., 2007), thus highlighting their potential role in the post-transcriptional regulation of genes during spermatogenesis. Despite the potential importance of miRNA in spermatogenesis, there have been limited studies on the changes in the miRNA expression profile of germ cells during spermatogenesis. This is especially true of the very early stages of spermatogenesis, e.g. from gonocytes to spermatogonia.

In the majority of cases, the changing testicular miRNA levels have been examined over developmentally significant periods in whole testicular cell lysates. Yan et al. (2007) investigated immature and mature mouse testis and identified 19 differentially expressed miRNA molecules (5 higher and 14 lower in adult testis compared with neonatal testis). Similar studies in rhesus monkey (Yan et al., 2009) and porcine testis (Luo et al., 2010) have consistently shown differential miRNA expression between mature and immature testis with the majority of significant molecules down-regulated in adult tissue. However, with the targets of differentially expressed miRNA molecules remaining largely unknown, insight into their function is hard to determine. To circumvent this limitation, the authors employed a variety of in silico target prediction analyses to identify potential targets involved in spermatogenesis. However, many predictive software programs deliver a significant number of false positives, up to 20% of the predicted targets. In addition, predictive software has also been known to overlook miRNA targets with poorly conserved binding sites. These bioinformatic tools are being continually updated and improved as laboratory research uncovers more about the properties of miRNA binding. Therefore, despite our enhanced knowledge of miRNA binding sites, at present any predicted target must be experimentally confirmed for its validity (Huang et al., 2010; Thomas et al., 2010; Witkos et al., 2011). Using this approach, Yan et al. (2007, 2009) identified several important genes in spermatogenesis as potential
targets for testicular miRNAs. For example, sox5 and sox6 are presumed targets of miR-181c, and rbnl1 (a gene postulated to be involved in transcriptional regulation in haploid germ cells) is putatively targeted by miR-355, 181c, 181b, all of which are up-regulated in adult testis (Yan et al., 2007). In rhesus monkey testes, predicted targets of miRNA include NOTCH1 (targeted by miR-34b, 34c and 449, more highly expressed in mature testis), a key regulator of germ cell differentiation and survival, as well as BCL2 (miR-449 highly expressed in mature tissue), a key regulator of germ cell apoptosis (Yan et al., 2009). Using a porcine testis model, Luo et al. (2010) went a step further by examining the expression levels of putative targets of the differentially expressed miRNA molecules. This analysis revealed candidates such as dazl (deleted in azoospermia like gene), which is known to be essential for germ cell differentiation in mice, as a likely target of miR-34b and 34c.

In addition to known somatic cell miRNA molecules, cloning experiments have identified miRNA species that are either preferentially or exclusively expressed in testis, as well as a suite of novel miRNA molecules (Ro et al., 2007). Deep sequencing analysis of Day 7 and Day 14 murine testis has identified unique splice variants, novel miRNA species and confirmed miRNA expression changes (Buchold et al., 2010). This approach also led to the identification of miRNA clusters on chromosomes 2 and X which are up-regulated in both 14 day testis and neonatal ovary. Considering that both these tissues harbor meiotic cells, these miRNA clusters may play a role in germ cell development (Buchold et al., 2010).

Studies conducted with whole testes clearly demonstrate that testicular expression of miRNA molecules changes depending on the stage of spermatogenesis. However, determining the relevance of these changes is complicated by the presence of germ cells at multiple stages of differentiation as well as significant populations of somatic cells. Therefore, studies in purified populations of germ cells at different stages of maturation are required. However, there has only a few studies, one small scale detailed analysis of 28 testis expressed miRNA molecules in isolated testicular cell populations, i.e. Sertoli cells, spermatogonia, pachytene spermatocytes, round and elongating spermatids as well as spermatoza (Ro et al., 2007). Microarray analysis has been performed on purified spermatogonia, spermatocytes and spermatids to examine global miRNA expression in these cell populations (Marcon et al., 2008). However, there were issues of cell purity with the spermatogonia and spermatid samples that were contaminated with germ cells of different stages to 30 or 40% of the total cell population, respectively (Marcon et al., 2008). These two studies on isolated germ cells identified that most miRNA were preferentially expressed in the meiotic germ cells. Marcon et al. (2008) examined some potential roles for highly expressed miRNA in the germ cells. For example, miR-320 (expressed in all germ cells) is predicted to target protocadherins, which play a role in cell adhesions; miR-214 (mainly expressed in pachytene spermatocytes) is predicted to target heat shock proteins, which have a role in meiosis.

**Can germ cell miRNA molecules be assigned a function in spermatogenesis?**

Specific roles for a few miRNA molecules during spermatogenesis have been proposed (Table I). For example, miR-34c is highly expressed in isolated pachytene spermatocytes and round spermatids. The miR-34 family has been shown to target many cell cycle regulators (e.g. notch1, cdk4 and myc) and further study identified two genes important in spermatogenesis (tgif2 and notch2) as direct targets of miR-34c (Bouhali et al., 2010). Notch signalling promotes germ cell differentiation from spermatogonia (Kostereva and Hofmann, 2008). In contrast, tgif2 is a negative regulator of tgfβ signalling and thus inhibits the second meiotic division in spermatogenesis (Damestoy et al., 2005). Overexpression of miR-34c along with vasa in HeLa cells promoted the expression of germ cell markers leading the authors to hypothesize that miR-34c down-regulates somatic genes and enhances germ cell characteristics (Bouhali et al., 2010). Additionally miR-18a directly targets HSF2 (heat shock factor 2), a transcription factor that controls the expression of many genes required for successful spermatogenesis. HSF2 knockout mice have small, morphologically abnormal testes with fewer spermatids and severe sperm abnormalities (Bjork et al., 2010). TNP2 (Transition protein 2) is one of the key transition proteins that replaces histones during the early phase of chromatin condensation that accompanies spermiogenesis (Yu et al., 2005) and miR-122a directly binds to and reduces the expression of TNP2 miRNA. In summary, miRNAs are emerging as key players in germ cell function and cell fate determination and act to interpret and transduce cellular signals to allow the maintenance of the undifferentiated stem cell population as well as allowing cell differentiation during spermatogenesis. However, further study is required to assign specific roles to all miRNA species implicated to have a role in spermatogenesis. These fundamental roles for miRNAs in germ cell development have implications for normal and disease states such as infertility and germ cell tumours in young men.

**Testicular germ cell tumours**

Testicular germ cell cancer can affect men throughout life; however, there are three types of testicular germ cell tumours (TGCT) that occur at distinct ages in men. Type I germ cell tumours consisting of benign mature teratomas or malignant yolk sac tumours occur in young children usually before the age of four and always become apparent before puberty (Looijenga and Oosterhuis, 1999; Bahrami et al., 2007; Kristensen et al., 2008). In contrast, type III TGCT also known as spermatocytic seminomas usually occur in older men (over 50). These tumours are usually benign and slow growing, with genetic markers in common with type B spermatogonia. The incidence of type I and III TGCT is very low and has remained steady over the last 30 years (Looijenga and Oosterhuis, 1999; Bahrami et al., 2007; Kristensen et al., 2008). In contrast, there has been an alarming increase in the incidence of type II germ cell tumours in the developed world during the last 50 years (Bahrami et al., 2007; Huyghe et al., 2007; Kristensen et al., 2008). Type II TGCT or seminoma and non-seminoma are the most common cancers in men in their 30s to 40s. These tumours are associated with a preinvasive lesion C or undifferentiated intratubular germ cell neoplasia. As dysregulated miRNA action is associated with the aetiology of many cancers, the presence and influence of miRNA in the development of type II germ cell tumours is reviewed later (Table II).

**Carcinoma in situ**

First identified in 1972, half of all patients diagnosed with CIS develop overt TGCT within 5 years with the remainder expected to develop
Table I miRNA molecules implicated in germ cell development including their expression pattern, proposed function, predicted and confirmed targets.

<table>
<thead>
<tr>
<th>Name</th>
<th>Expression</th>
<th>Proposed function</th>
<th>Predicted and confirmed targets involved in spermatogenesis</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Let-7 family</td>
<td>Male PGC’s, Female oocytes, highly expressed in the testis</td>
<td>Differentiation into mature gametes, tumour suppressors</td>
<td>LIN28, NR6A1, TAF5, FASL, EIF4G2, SUV39H2, DZIP1, DDX19B, MYCN, NCOR2</td>
<td>Dyce et al. (2010), Luo et al. (2010), Zhong et al. (2010)</td>
</tr>
<tr>
<td>Let-7e</td>
<td>Down-regulated in mature mouse and porcine testis</td>
<td>Tumour suppressor</td>
<td>FRAP1/mTOR, TBP11, RSBN1, AMD, DAZAP2, PIK3R3, RNF6, BCL2</td>
<td>Yan et al. (2007), Luo et al. (2010), Buechner et al. (2011)</td>
</tr>
<tr>
<td>miR-10a</td>
<td>Enriched in the spermatogonial cell population compared with somatic cells of day 6 testis</td>
<td>Cellular differentiation</td>
<td>PPP1CC, PCNA, AQP9, HMG2A, STAT3, E2F1, PTEN (19a, 19b)</td>
<td>Williams et al. (2007), Yan et al. (2009)</td>
</tr>
<tr>
<td>miR-100</td>
<td>In day 7–10 mouse testis</td>
<td>Tumour suppressor, ovarian cancer</td>
<td>KPNB1, NR6A1, SOX6, RAD21, CREB1, SOX5, RSBN1, AMD, TNPO1, DAZAP2, NOTCH4, KRAS</td>
<td>Novotny et al. (2007a, b), Hayashi et al. (2008), Marcon et al. (2008), Marcon et al. (2008), Liu et al. (2011a, b, c)</td>
</tr>
<tr>
<td>miR-122a</td>
<td>Up-regulated in mature mouse testis</td>
<td>Later stage germ cell maturation</td>
<td>TIMP3, RNF6, KPNB1, BCL2</td>
<td>Yan et al. (2009), Zhu et al. (2010)</td>
</tr>
<tr>
<td>miR-124a</td>
<td>Up-regulated in mature rhesus monkey testis, ES cells</td>
<td>Suppression of cell migration, pluripotency</td>
<td>QKI, MYO10, SP3, MITF, FGGR2, CDK4, KLF4, SLUG, IQGAP1, ITGB1</td>
<td>Yan et al. (2009), Lee et al. (2010), Hunt et al. (2011)</td>
</tr>
<tr>
<td>miR-125a</td>
<td>Later male PGC’s</td>
<td>Control of differentiation</td>
<td>LIN28, BRD2, SIRT1, ZEB2</td>
<td>Hayashi et al. (2008), Zhong et al. (2010)</td>
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<tr>
<td>miR-127</td>
<td>Down-regulated in mature mouse testis</td>
<td>Lung development</td>
<td>SIP1</td>
<td>Hayashi et al. (2008), Buchold et al. (2010), Hu et al. (2010)</td>
</tr>
<tr>
<td>miR-141</td>
<td>Early PGC’s, up-regulated from day 7 to day 14 in mouse testis</td>
<td>Inhibits cell migration and invasion</td>
<td>HSF2, RHOA, CDC42</td>
<td>Williams et al. (2007), Yan et al. (2009)</td>
</tr>
<tr>
<td>miR-154</td>
<td>Down-regulated in mature rhesus monkey testis</td>
<td>Lung development</td>
<td>PPP1CC, PCNA, AQP9, HMG2A, STAT3, E2F1, PTEN (19a, 19b)</td>
<td>Novotny et al. (2007a, b), Hayashi et al. (2008), Marcon et al. (2008), Liu et al. (2011a, b, c)</td>
</tr>
<tr>
<td>miR-17–92 cluster</td>
<td>PGC’s, ES cells, some members down-regulated in non-obstructive azoospermia</td>
<td>Regulator of differentiation, proliferation and apoptosis</td>
<td>KPNB1, NR6A1, SOX6, RAD21, CREB1, SOX5, RSBN1, AMD, TNPO1, DAZAP2, NOTCH4, KRAS</td>
<td>Bjork et al. (2010)</td>
</tr>
<tr>
<td>miR-18</td>
<td>Spermatocytes</td>
<td>Oncogenic</td>
<td>BNC2</td>
<td>Marcon et al. (2008), Hayashi et al. (2008), Marcon et al. (2008), Liu et al. (2011a, b, c)</td>
</tr>
<tr>
<td>miR-181b</td>
<td>Down-regulated in mature mouse and porcine testis</td>
<td>Tumour suppressor</td>
<td>RB1, RB2, RB3</td>
<td>Hayashi et al. (2008), Bucák et al. (2011)</td>
</tr>
<tr>
<td>miR-181c</td>
<td>Down-regulated in mature rhesus monkey and mouse testis</td>
<td>Tumour suppressor</td>
<td>AKT2</td>
<td>Hayashi et al. (2008), Bucák et al. (2011)</td>
</tr>
<tr>
<td>miR-181d</td>
<td>Down-regulated in mature rhesus monkey testis</td>
<td>Drug resistance, regulation of apoptosis</td>
<td>TIMP3, RNF6, KPNB1, BCL2</td>
<td>Hayashi et al. (2008), Marcon et al. (2008), Teng et al. (2011)</td>
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<tr>
<td>miR-184</td>
<td>Highly expressed in GC1 cells, Enriched in the spermatogonial cell population compared with somatic cells of day 6 testis, preferentially expressed in late stage spermatocytes and spermatids</td>
<td>Tumour suppressor</td>
<td>RHOA, CDC42, BNC2</td>
<td>Marcon et al. (2008), Liu et al. (2011a, b, c)</td>
</tr>
<tr>
<td>miR-185</td>
<td>Preferentially expressed in pachytene spermatocytes</td>
<td>Tumour suppressor, cell cycle regulator</td>
<td>RHOA, CDC42, BNC2</td>
<td>Marcon et al. (2008), Liu et al. (2011a, b, c)</td>
</tr>
<tr>
<td>miR-191</td>
<td>Highly expressed in tests, preferentially expressed in beta pachytene spermatocytes, down-regulated in teratozoospermia</td>
<td>Required for normal sperm morphology</td>
<td>RHOA, CDC42, BNC2</td>
<td>Marcon et al. (2008), Grinchuk et al. (2010)</td>
</tr>
<tr>
<td>miR-191*</td>
<td>Up-regulated in mature rhesus monkey testis</td>
<td>Indirect regulation of Dicer1 and BNC2</td>
<td>RHOA, CDC42, BNC2</td>
<td>Marcon et al. (2008), Liu et al. (2011a, b, c)</td>
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<tr>
<td>miR-200a</td>
<td>Early PGC’s</td>
<td>Tumour suppressor, Inhibitor of cell invasion/migration</td>
<td>SIRT1, ZEB2</td>
<td>Hayashi et al. (2008), Eades et al. (2011), Wu et al. (2011)</td>
</tr>
<tr>
<td>Name</td>
<td>Expression</td>
<td>Proposed function</td>
<td>Predicted and confirmed targets involved in spermatogenesis</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>miR-200c</td>
<td>Early PGC’s</td>
<td>Tumour suppressor, Inhibitor of cell invasion/migration, control of apoptosis</td>
<td>ZEB1, TRKB</td>
<td>Hayashi et al. (2008), Radisky (2011)</td>
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<tr>
<td>miR-21</td>
<td>Spermatogonial stem cells</td>
<td>Spermatogonial self-renewal, anti-apoptosis, oncogene</td>
<td>P12</td>
<td>Niu et al. (2011), Zheng et al. (2011)</td>
</tr>
<tr>
<td>miR-214</td>
<td>Pachytene spermatocytes</td>
<td>Potential role in meiosis</td>
<td>WDTC1</td>
<td>Marcon et al. (2008)</td>
</tr>
<tr>
<td>miR-214</td>
<td>Down-regulated in mature mouse testis</td>
<td>Cell survival, cell migration and invasion</td>
<td>HSDTC1, TEX27, ADCYAP1R1, HBPI1, APIG1, SRR1, PTEN, AP-2Y ITGA3</td>
<td>Yan et al. (2007), Yang et al. (2008), Bar-Eli (2011)</td>
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<tr>
<td>miR-22</td>
<td>Up-regulated in sheep fetal testis development from D42 to D75</td>
<td>Regulation of estrogen signalling</td>
<td>ESR1</td>
<td>Torley et al. (2011)</td>
</tr>
<tr>
<td>miR-24</td>
<td>Pachytene spermatocytes</td>
<td>Potential role in meiosis</td>
<td>MBD6, H2AX</td>
<td>Marcon et al. (2008)</td>
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<tr>
<td>miR-27b</td>
<td>Down-regulated in sheep fetal testis development from D42 to D75</td>
<td>Cell differentiation (cardiac and adipocyte)</td>
<td>PPARY</td>
<td>Karbiener et al. (2009), Busk and Cirera (2010), Torley et al. (2011)</td>
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<tr>
<td>mir-290-295</td>
<td>PGC’s, ES cells</td>
<td>G1 to S phase cell cycle control</td>
<td>WEE1, FBXL5</td>
<td>Hayashi et al. (2008), Dyce et al. (2010), Lichner et al. (2011)</td>
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<tr>
<td>miR-296</td>
<td>Up-regulated in mature rhesus monkey testis</td>
<td>Tumour suppressor, cell motility repressor</td>
<td>NCA1D, SCRIB, CREB5, BAK1, USP42, MLF1, HBPI1, SNX24, PTEN</td>
<td>Yan et al. (2009), Vaira et al. (2011)</td>
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<tr>
<td>miR-29b</td>
<td>Up-regulated in mature mouse testis</td>
<td>Oncogene, represses apoptosis promotes cell motility</td>
<td></td>
<td>Yan et al. (2007), Wang et al. (2011a, b)</td>
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<tr>
<td>miR-30a</td>
<td>Down-regulated in non-obstructive azoospermia</td>
<td>Tumour suppressor, inhibits metastasis</td>
<td>SNAI1</td>
<td>Lian et al. (2009), Kumarswamy et al. (2011)</td>
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<tr>
<td>miR-30e</td>
<td>Up-regulated in sheep fetal testis development from D42 to D75</td>
<td>Tumour marker</td>
<td></td>
<td>Yang et al. (2010), Torley et al. (2011)</td>
</tr>
<tr>
<td>miR-302–367</td>
<td>PGC’s, ES cells</td>
<td>Transcription, cell growth and metabolism, maintenance of pluripotency</td>
<td>NR2F2</td>
<td>Hayashi et al. (2008), Dyce et al. (2010), Rosa and Brivanlou (2011)</td>
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<td>miR-31</td>
<td>Enriched in the spermatogonial cell population compared with somatic cells of day 6 testis</td>
<td>Promotes cell migration and invasion</td>
<td>TIAM1</td>
<td>Cottonham et al. (2010), Niu et al. (2011)</td>
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<tr>
<td>miR-320</td>
<td>Spermatogonia, beta Pachytene and spermatids</td>
<td>Adhesions between Sertoli and germ cells</td>
<td>Protocadherin family</td>
<td>Marcon et al. (2008)</td>
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<tr>
<td>miR-323</td>
<td>Early PGC’s</td>
<td>Inhibits H1N1 virus replication</td>
<td>PBI</td>
<td>Hayashi et al. (2008), Song et al. (2010)</td>
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<tr>
<td>miR-335</td>
<td>Down-regulated in mature mouse testis</td>
<td>Maintenance of pluripotency in mesenchymal stem cells</td>
<td>CCNT2, CCD2, RSBN1, RUNX2</td>
<td>Yan et al. (2007), Tome et al. (2011)</td>
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<td>miR-337</td>
<td>Down-regulated in mature mouse testis</td>
<td></td>
<td>TBX, APIG1, TAF5, TAF12, CREB1, CCNL1</td>
<td>Yan et al. (2007)</td>
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<tr>
<td>miR-34a</td>
<td>Up-regulated in mature mouse testis, up-regulated from day 7 to day 14 in mouse testis</td>
<td>Repression of cell proliferation, Inducer of apoptosis</td>
<td>NOTCH1, LGR4, VEZT, MAN2A2, FOXJ2</td>
<td>Yan et al. (2007), Buchhold et al. (2010), Ito et al. (2010)</td>
</tr>
<tr>
<td>miR-34b</td>
<td>Up-regulated in mature rhesus monkey testis, up-regulated from day 7 to day 14 in mouse testis</td>
<td>Tumour suppressor, promotes apoptosis, cell cycle arrest and senescence</td>
<td></td>
<td>Yan et al. (2009), Buchhold et al. (2010), Vogt et al. (2011)</td>
</tr>
<tr>
<td>miR</td>
<td>Remarks</td>
<td>Genes</td>
<td>References</td>
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<tr>
<td>--------------</td>
<td>-------------------------------------------------------------------------</td>
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<tr>
<td>miR-34c</td>
<td>Pachytene spermatocytes and round spermatids, up-regulated in mature rhesus monkey and mouse testis, up-regulated from day 7 to day 14 in mouse testis</td>
<td>CCND3, CCNG1, CCNB1, CCNC, CCNE1, CDK4, CDK6, E2F5, FOS, CDC2, TGIF2, NOTCH2, STRBP LGR4 KL4, NOTCH1 PPPI1 CC, GALT, KITLG, SPAG4, CCNL, ZFP1148, GMFB</td>
<td>Yan et al. (2007), Yan et al. (2009), Bouhallier et al. (2010), Buchold et al. (2010)</td>
<td></td>
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<tr>
<td>miR-361</td>
<td>Down-regulated in mature mouse testis</td>
<td></td>
<td>Yan et al. (2007)</td>
<td></td>
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<tr>
<td>miR-375</td>
<td>Up-regulated from day 7 to day 14 in mouse testis, enriched in the spermatogonial cell population compared with somatic cells of day 6 testis, Highly expressed in newborn epididymis</td>
<td>KPNB1, ZFP1148, BMPR2, CALM2, IGR1R</td>
<td>Buchold et al. (2010), Zhang et al. (2010), Kong et al. (2011), Niu et al. (2011)</td>
<td></td>
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<tr>
<td>miR-376a</td>
<td>Down-regulated in mature mouse testis</td>
<td>Maintenance of pluripotency, and cell cycle arrest</td>
<td>Yan et al. (2007), Wang et al. (2011a, b)</td>
<td></td>
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<tr>
<td>miR-379</td>
<td>Down-regulated in mature mouse testis</td>
<td>Membrane transport</td>
<td>Yan et al. (2007), Haenisch et al. (2011)</td>
<td></td>
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<tr>
<td>miR-411</td>
<td>Down-regulated in mature mouse testis</td>
<td></td>
<td>Yan et al. (2007)</td>
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<tr>
<td>miR-434-5p</td>
<td>Down-regulated in mature mouse testis</td>
<td></td>
<td>Yan et al. (2007)</td>
<td></td>
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<tr>
<td>miR-449</td>
<td>Up-regulated in mature rhesus monkey and mouse testis, up-regulated from day 7 to day 14 in mouse testis (449a and c)</td>
<td>MECP2, ASB1, BCL2, NOTCH1, CASP2, KITLG, VCL, FOXJ2, INHBB, SOX11, CCNE2, GMFB, DLI</td>
<td>Yan et al. (2007), Yan et al. (2009), Buchold et al. (2010), Marcet et al. (2011)</td>
<td></td>
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<tr>
<td>miR-487b</td>
<td>Down-regulated in mature rhesus monkey testis</td>
<td></td>
<td>Yan et al. (2009)</td>
<td></td>
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<tr>
<td>miR-509-3</td>
<td>Expressed in human testis</td>
<td></td>
<td>Novotny et al. (2007a, b)</td>
<td></td>
</tr>
<tr>
<td>miR-557</td>
<td>Up-regulated in mature rhesus monkey testis</td>
<td>EIF4G2, EDN1, RNF6, ABCC2, USP42</td>
<td>Yan et al. (2007)</td>
<td></td>
</tr>
<tr>
<td>miR-702</td>
<td>Up-regulated in mature rhesus monkey testis</td>
<td>SBF1, MMP14</td>
<td>Yan et al. (2009)</td>
<td></td>
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<tr>
<td>miR-709</td>
<td>Male and female germ line, up-regulated in mature rhesus monkey and porcine testis</td>
<td>ETS1</td>
<td>Tamminga et al. (2008), Yan et al. (2009), Luo et al. (2010)</td>
<td></td>
</tr>
<tr>
<td>miR-714</td>
<td>Up-regulated in mature rhesus monkey testis</td>
<td></td>
<td>Yan et al. (2009)</td>
<td></td>
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<tr>
<td>miR-715</td>
<td>Up-regulated in mature rhesus monkey testis</td>
<td></td>
<td>Yan et al. (2009)</td>
<td></td>
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<tr>
<td>miR-9</td>
<td>Later male PGC’s, up-regulated from day 7 to day 14 in mouse testis</td>
<td></td>
<td>Yan et al. (2009), Hayashi et al. (2008), Buchold et al. (2010), Zhong et al. (2010)</td>
<td></td>
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<tr>
<td>miR-99a</td>
<td>Enriched in the spermatogonial cell population compared with somatic cells of day 6 testis, down-regulated in cisplatin resistant germ cell tumour cell lines</td>
<td>FRAP1/mTOR, SMARCA5, SMARCD1</td>
<td>Niu et al. (2011), Port et al. (2011), Sun et al. (2011)</td>
<td></td>
</tr>
</tbody>
</table>
cancer over a longer period (Reuter, 2005). CIS cells are large atypical germ cells that are found between the thickened basement membrane and the Sertoli cell layer within the seminiferous tubules. Tubules containing CIS cells do not usually have active spermatogenesis and contain mostly Sertoli cells (Looijenga and Oosterhuis, 1999; Reuter, 2005). In addition to their abnormal size and shape, CIS cells express PGC markers such as placental-like alkaline phosphatase (PLAP) and c-KIT. CIS cells also express many pluripotency genes (such as NANOG and OCT 3/4) that are normally found only in early embryonic development, germ cells, and in stem cells responsible for maintaining a pool of undifferentiated cells for tissue generation (Rajpert-De Meyts et al., 2006). Recently an additional pluripotency marker LIN28 has been identified. LIN28 is expressed in PGC, gonocytes and prespermatogonia as well as CIS cells. It is believed that LIN28 has a role in maintaining the cells in an undifferentiated state up stream but linked to both NANOG and OCT 3/4 (Gillis et al., 2011). CIS cells express other genes common to ES cells, including DDX4 (vasa) (Rajpert-De Meyts et al., 2006).

A recent study, using microdissected germ cell populations and microarray analysis, identified CIS as arrested gonocytes, with both sharing a common expression profile of the majority of genes. The only differentially expressed genes were; DEFB119 (an antimicrobial peptide) and NMNAT1 (NAD biosynthesis) which were up-regulated in CIS, and three that were down-regulated in CIS were PTPRZ1 (a tyrosine phosphatase receptor involved in cancer), ASZL3 (a predicted cancer associated gene-homologue of Drosophila additional sex combs gene) and an unannotated gene (Sonne et al., 2006). The confirmation of CIS cells as arrested gonocytes indicates that the transition from gonocytes into spermatogonia is an essential area of study in order to understand the aetiology of TGCT.

### Table II miRNA molecules implicated in the development of testicular cancer including their expression pattern, proposed function, predicted and confirmed targets.

<table>
<thead>
<tr>
<th>miRNA name</th>
<th>Expression</th>
<th>Function</th>
<th>Predicted and confirmed targets</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-133b</td>
<td>Teratoma</td>
<td>Tumour suppressor</td>
<td>POU4F1, MEIS2, FSCN1</td>
<td>Gillis et al. (2007), Kano et al. (2010)</td>
</tr>
<tr>
<td>miR-140</td>
<td>Teratoma</td>
<td>Skeletal development, and cartilage maintenance</td>
<td>BCL11A, SOX4, CXCL12</td>
<td>Gillis et al. (2007), Nicolas et al. (2008), Miyaki et al. (2010)</td>
</tr>
<tr>
<td>miR-145</td>
<td>Down-regulated in seminoma and embryonal carcinoma, down-regulated in non-obstructive azoospermia, down-regulated in cisplatin resistant germ cell tumour cell lines</td>
<td>Represses pluripotency, tumour suppressor</td>
<td>PLAGL2, E2F3, SOX9, OCT4, SOS2, KLF4, FSCN1</td>
<td>Gillis et al. (2007), Lian et al. (2009), Xu et al. (2009), Kano et al. (2010), Port et al. (2011)</td>
</tr>
<tr>
<td>miR-152N</td>
<td>Teratoma</td>
<td>Tumour suppressor</td>
<td>ZEB1, TRKB</td>
<td>Gillis et al. (2007)</td>
</tr>
<tr>
<td>miR-200c</td>
<td>Seminoma</td>
<td>Inhibitor of cell invasion/ migration, control of apoptosis</td>
<td></td>
<td>Gillis et al. (2007), Radisky (2011)</td>
</tr>
<tr>
<td>miR-302 Cluster</td>
<td>Overexpressed in adult (undifferentiated) and paediatric germ cell tumours</td>
<td>Maintenance of pluripotency</td>
<td>NR2F2</td>
<td>Gillis et al. (2007), Lian et al. (2009), Palmer et al. (2010), Murray et al. (2010), Rosa and Brivanlou (2011)</td>
</tr>
<tr>
<td>miR-302d</td>
<td>Seminoma, embryonal carcinoma, up-regulated in yolk sack tumours</td>
<td>Oncogene, suppressor of apoptosis</td>
<td>LATS2, LEFTY1, MLL3, DAZAP2, TNFAIP1, BCL11B, PLAG1</td>
<td>Gillis et al. (2007), Murray et al. (2010)</td>
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<tr>
<td>miR-34aN</td>
<td>Teratoma</td>
<td></td>
<td></td>
<td>Gillis et al. (2007)</td>
</tr>
<tr>
<td>miR-367</td>
<td>Embryonal carcinoma, ES cells</td>
<td>Pluripotency, regulation of gene transcription</td>
<td>LATS2, KLF4, RUNX1, SYNJ1, SMAD6</td>
<td>Gillis et al. (2007), Li et al. (2009a, b)</td>
</tr>
<tr>
<td>miR-371-373 cluster</td>
<td>Seminoma, embryonal carcinoma, overexpressed in malignant paediatric germ cell tumours, down-regulated in non-obstructive azoospermia, up-regulated in cisplatin resistant germ cell tumour cell lines</td>
<td>Inhibits apoptosis</td>
<td>ZIC4, LATS2, LEFTY1, DAZAP2, TNFAIP1, PLAG2, MLL3</td>
<td>Voorhoeve et al. (2006), Gillis et al. (2007), Lian et al. (2009), Port et al. (2011)</td>
</tr>
</tbody>
</table>

### Seminoma and non-seminoma

Two types of germ cell tumours develop from CIS cells; these are seminoma and non-seminoma, which have in common an amplification of the short arm of chromosome 12. Seminomas account for 50% of the type II germ cell tumours and have a peak incidence in men from their mid 30s to mid 40s, about 10 years later than non-seminomas. Seminoma also closely resemble both CIS cells and normal spermatogonia; however they can metastasize. In common with CIS cells, seminomas are usually uniform tumours consisting of large cells that express PLAP, KIT and OCT 3/4, and most often presents as lumps in an enlarged testis. In contrast, non-seminoma tumours include several different forms of cancer, including embryonal carcinoma, yolk sac tumours, immature or mature teratoma and...
miRNA and TGCT

Intriguingly, type I and type III germ cell tumours and the histological subgroups of type II germ cell tumours (seminoma and non-seminoma) can be differentiated by their miRNA expression profiles (Gillis et al., 2007). The 302 cluster, which is highly expressed in ES cells and thought to be involved in the maintenance of pluripotency, is elevated in seminoma tumours but not in the other types of germ cell tumours that are more differentiated. Oncogenic miRNAs miR-21 (cell survival) and 155 (myc regulation) are over-expressed in seminomas and type III germ cell tumours. Other miRNA have been confirmed as tumour-specific—for example, over-expression of 19a is found in seminomas and type III tumours, over-expression of 29a occurs in type III tumours and under-expression of 133a and 145 in both seminomas and type III tumours. miR-146 expression is lower in seminomas and type III tumours compared with normal testis and lower still in the more differentiated type II tumours such as embryonic carcinoma and teratomas (Gillis et al., 2007).

miRNA also has an important role in testicular germ cell tumouriagnosis and survival, given that most germ cell tumours contain wild type p53. Voorhoeve et al. (2006) identified miR-371, -372 and -373 as over-expressed in TGCT expressing wild type p53. Over-expression of these miRNA molecules was found to down-regulate expression of Suv39-H1 and large tumour suppressor homolog 2 (LATS2), both of which are associated with the Ras oncopene pathway. LATS2 deletion causes cell proliferation and oncogenic transformation, while its over-expression was shown to prevent Ras-mediated transformation of cells. Upon further investigation, miR-371/2/3 was found to directly bind to the LATS2 3′UTR to control its translation and that over-expression of miR-371/2/3 allowed cells to proliferate regardless of negative signals originating from p53 and the cell cycle inhibitor p21 (Voorhoeve et al., 2006). More recently, miR-373 has been identified as a factor promoting cell migration and metastasis but not cell proliferation in breast cancer cells (Huang et al., 2008). Therefore, it is entirely possible that this miRNA could have a role in testicular cancer metastasis in addition to the roles identified by Voorhoeve et al. (2006). The miR371–373 cluster was up-regulated in addition to other miRNA molecules in several cisplatin-resistant germ cell tumour cell lines and may play a role in inhibiting cell death and promoting differentiation in response to cisplatin exposure (Port et al., 2011). Consequently, the manipulation of miRNA levels within tumours has emerged as an attractive option in the development of new treatments for testicular cancer (Bhardwaj et al., 2010).

Targeting miRNA for therapeutic purposes: cancer and fertility regulation

Many studies of miRNA expression in tumours and their corresponding normal tissue types have determined that cancerous tissue always has significantly different miRNA profiles than that of normal tissue (Gillis et al., 2007; Li et al., 2009a, b; Babashah and Soleiman, 2011). Of particular note is the finding that tumours that originate from the same or similar tissue types usually have consistent changes in their miRNA profile(s) and these may prove to be useful diagnostic and prognostic tools in cancer research (Li et al., 2009a, b). However, this is not the universal phenomena, as there have been changes in miRNA expression detected in subpopulations of cells obtained from the same lineage (Liu et al., 2011a, b, c). In addition, the consistent dysregulation of miRNA within cancer has identified them as promising targets for anti-cancer therapy only after the miRNA expression profile has been confirmed in the patient.

There are two main modes by which miRNA molecules can be targeted to treat cancer or other diseases. Either reduce the expression or effect of oncogenic miRNA or induce the expression of tumour suppressing miRNAs within tumour cells (Li et al., 2009a, b). Forced expression of let-7, miR-15, -16 and -34c pri-miRNA as well as double-stranded pre-miRNA constructs in cancer cell lines as well as mouse models have provided a proof of concept for miRNA up-regulation as a cancer therapeutic. For example, the over-expression of let-7 in lung cancer cell lines reduced their ability to form tumours in xenograft models, while injections of a miR-34c construct in mice retarded the growth of lung tumours. The over-expression of miR-15/16 has reduced the expression of the anti-apoptotic gene BCL2 in tumour cell lines, indicating that this construct could be used for the treatment of cancers expressing high levels of BCL2 such as leukaemia (Li et al., 2009a, b; Bader et al., 2010, 2011).

Alternative therapeutic strategies include reducing the expression or efficacy of miRNA molecule(s) that are essential for cancer development. This may be achieved by introducing artificial miRNA with multiple binding sites for the target miRNA and thus reducing the amount of miRNA available to bind endogenous targets. Another technique is to introduce a miR-Mask with 100% complementarity of the miRNA target, causing high affinity binding and thus preventing binding to endogenous targets. However, the use of chemically modified high stability anti-miRNA oligonucleotides is currently the most promising method under development (Bader et al., 2011). The most common modification employs locked nucleic acids (LNA), in which an extra methylene bridge is introduced between bases, thus securing them into a binding position and increasing the thermal stability when bound to miRNA targets. LNA binding permits RNA degradation along the siRNA pathway; also, LNA display low toxicity and high solubility. In some cases, the length and composition of the LNA molecule allow it to circulate systemically and enter the target tissue without a method under development (Bader et al., 2011). The most common modification employs locked nucleic acids (LNA), in which an extra methylene bridge is introduced between bases, thus securing them into a binding position and increasing the thermal stability when bound to miRNA targets. LNA binding permits RNA degradation along the siRNA pathway; also, LNA display low toxicity and high solubility. In some cases, the length and composition of the LNA molecule allow it to circulate systemically and enter the target tissue without a delivery system, overcoming the limiting step in miRNA therapy (Li et al., 2009a, b; Bader et al., 2011).

An example of how this technology could be applied was recently described in primates, using a LNA anti-MiR against miR-122, a molecule implicated in cholesterol synthesis and important for hepatitis...
infections (Petri et al., 2009). Intravenous injections of the LNA anti-MiR over 5 days resulted in lowering of plasma cholesterol in African green monkeys. Further tests indicated that the miR-122 was filtered out by, and found in high concentrations, in the liver. Lowering of the cholesterol levels was gradually reversed over 3 months during which time the LNA construct was degraded. No toxicity from the LNA construct was observed in the treated animals, indicating that anti-MiR treatment is a viable option in many diseases (Petri et al., 2009).

The ability of anti-MiR treatment to reversibly regulate a pathway (as described above) raises the possibility that it could be used to temporarily interfere with other pathways such as those essential to spermatogenesis. As previously described, many novel miRNA molecules are required for spermatogenesis, and in fact some pivotal steps of spermatogenesis rely on a single miRNA molecule (e.g. miR-122a, 18a and 34c). Thus, interfering with their function could in theory catastrophically disrupt spermatogenesis, thereby providing unique opportunities for the development for new male contraceptives. miRNA molecules whose primary role is to inhibit germ cell-specific genes such as miR-122a with TNP2 and miR-18a with HSF2 are not such promising targets for gene knockdown. miR-34c however has the ability to drive cells further towards a germ cell fate once they have been specified as germ cell like; therefore its knockdown could severely inhibit germ cells differentiation and eliminate sperm production.

Prior to exploiting the immense potential of miRNA as either contraceptives or cancer treatments, there are several problems that must be surmounted. One of the first obstacles is the delivery and significant expense of modified nucleic acids. While frequent injections are a viable option for the treatment of a disease, this is unlikely to be acceptable for use as a contraceptive in healthy individuals. Targeting of miRNA mimics and anti-MiRs remains the limiting factor in the viability of miRNA modification treatment, primarily due to miRNA molecules having different functions within various tissues. This problem is not so apparent when treating organs such as the liver, which cleans and filters the blood, leading to accumulation of blood born miRNA modifiers; however, accessing other organs without direct injection remains a challenge. There has been some success using nanoparticles, consisting of lipids, atelocollagen and artificial ligands to increase cell specificity and cell permeability, in allowing miRNA treatment to enter specific cell types, i.e. vascular endothelial cells (Bader et al., 2011; Liu et al., 2011a, b, c). The use of viral vectors (adenoviral or lentiviral) to deliver miRNA mimics and sponges has also provided encouraging results in payload delivery and long-term expression to specific cell types (e.g. lung) with limited toxicity (Bader et al., 2011; Liu and Berkhout, 2011; McDermott et al., 2011). There remains an additional obstacle for miRNA modulation to work in the testis, the blood testis barrier. Currently a similar physiological hurdle, the blood brain barrier, has proved to be impenetrable, limiting the effectiveness of miRNA modification in treating diseases and cancers of the brain. This has been circumvented by either performing intracranial injections, or providing a slow release pellet at the tumour site after surgical removal (Corsten et al., 2007; Mathupala et al., 2007; Roshan et al., 2009; McDermott et al., 2011).

In fact, the ability of miRNA modification therapy to limit tumour growth and inhibit metastasis opens up the possibility of using this treatment in conjunction with more traditional drugs to allow effective personalized treatment disease as well as contraceptive therapy (Mathupala et al., 2007; Liu and Berkhout, 2011; McDermott et al., 2011).

Concluding remarks

The characterization of the roles of the regulatory miRNAs in germ cell development and dysregulation of testicular function requires further investigation and consolidation in both animal models and humans. Diverse aspects of post-transcriptional regulation in these differentiating cell types lays open many options for contraceptive development and the investigation of male infertility. The advent of 21st century medicinal chemistry leading to the availability of personalized medicine coupled to long acting manipulation of cell-specific targets could realistically mean that miRNA become the methods of choice for the treatment of testicular germ cell cancer and non-hormonal male fertility regulation.

Authors’ roles

S.C.M. and E.A.M. was involved in the study design, conducting literature review, the analysis of identified data and manuscript and figure drafting; S.D.R. was involved in the study design, analysis of identified data and manuscript drafting; B.N. took part in the study design and manuscript and figure drafting.

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Conflict of interest

The authors declare that there is no conflict of interest as defined by the guidelines of the International Committee of Medical Journal Editors (ICMJE; www.icmje.org).

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