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Genome-wide microRNA expression profiling of males with different spermatogenic and testicular impairments

Dissertation zur Erlangung des Grades eines Doktors der Naturwissenschaften der Medizinischen Fakultät

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ABSTRACT

Checking for spermatogenesis abnormalities is usually done through a final examination of the end product. Basic sperm parameters like sperm count, motility and morphology are typically the means of determining whether the spermatogenesis process is being completed. Even a small interruption of the spermatogenesis process may create major difficulties for couples attempting to conceive. Without proper spermatogenesis occurring, a male human will be subfertile or infertile. In recent years, miRNAs (small RNAs of ~22 nucleotides in length), were found to be involved in several regulatory processes like cell growth, proliferation, differentiation and apoptosis, and play important roles in several diseases, including reproductive abnormalities like male infertility. The present thesis aimed to identify miRNAs with altered levels in the spermatozoa of males with different spermatogenic impairments (in asthenozoospermic and oligoasthenozoospermic patients), and in testes of patients showing different histopathological patterns (in Sertoli cell only, mixed atrophy and germ cell arrest). I employed a strategy consisting of initial screening by miRNA microarray then further validation with a quantitative RT–PCR assay. Validation of the profiling results was conducted by using a cohort of samples recruited for this purpose. The bioinformatics analysis identified 77 and 86 differentially expressed miRNAs in asthenozoospermic and oligoasthenozoospermic males, respectively as compared with the normozoospermic fertile control males. In addition, 197, 68, and 46 miRNAs were significantly deregulated in patients with Sertoli cell only, mixed atrophy, and germ cell arrest groups, respectively, as compared with normal control. Quantitative RT-PCR assays were used to confirm these expression patterns. Based on these data and published data of authors candidate miRNAs – hsa-miR-34b*, hsa-miR-34b, hsa-miR-34c-5p, hsa-miR-429, and hsa-miR-122 were selected for further validation using a set of patient samples with different forms of spermatogenic impairments and control males. These 5 miRNAs exhibited the highest fold change. Their diagnostic potential based on AUC value was suitable to be used as a novel biomarker for the diagnosis and assessment of male infertility. These miRNAs were also found to be involved in apoptosis, cell proliferation, differentiation and the spermatogenesis process. The alteration of miRNAs in infertile males compared to normal control have opened a new

door to unveil the causes of infertility and contributed to the identification of new genes correlated with the male factor infertility. Overall, these results provided strong evidence for the use of miRNA profiles as a future diagnostic tool for male infertility.

ZUSAMMENFASSUNG

Die Analyse von Anomalien in der Spermatogenese wird normalerweise durch die Untersuchung des Endproduktes der Spermatogenese durchgeführt. Grundlegende Parameter, wie die Anzahl der Spermien, die Beweglichkeit (Motilität) und die Morphologie sind typischerweise ein Maß dafür, ob der Prozess der Spermatogenese abgeschlossen wurde. Selbst eine geringe Störung in der Spermatogenese kann bei Paaren mit Kinderwunsch zu schwerwiegenden Problemen führen. Eine nicht korrekt ablaufende Spermatogenese wird bei einem Mann zwangsläufig zu einer Subfertilität oder Infertilität führen. Vor wenigen Jahren wurde für miRNAs (kurze RNA einer Länge von ca. 22 Nukleotiden) nachgewiesen, dass sie in verschiedenen regulatorischen Prozessen, wie beispielsweise dem Zellwachstum, der Zellproliferation, der Differenzierung oder der Apoptose involviert sind. Daneben spielen sie eine bedeutende Rolle bei der Entstehung von verschiedenen Krankheiten, einschließlich Reproduktionsanomalien wie der Unfruchtbarkeit des Mannes. Ziel der vorliegenden Dissertation war die Identifizierung von miRNAs, die in Spermatozoen von Männern mit unterschiedlichen Beeinträchtigungen in der Spermatogenese (Patienten mit Astenozoospermie und Oligozoospermie) und in Testes-Gewebe von Patienten mit verschiedenen histopathologischen Ausprägungen (Sertoli Cell only, mixed atrophy und germ cell arrest) in verändertem Maße vorliegen. Es wurde ein initiales Screening mittels miRNA Microarray durchgeführt, welches mittels quantitativer RT-PCR validiert wurde. Die Validierung der gemessenen Profile wurde an einer Patientenkohorte durchgeführt, die zu diesem Zweck rekrutiert wurde. Mit Hilfe bioinformatischer Analysen wurden, verglichen mit fruchtbaren normozoospermischen Männern 77 miRNAs bei Männern mit Asthenozoospermie und 86 miRNAs bei Männern mit Oligoasthenozoospermie als differentiell exprimiert identifiziert. Des Weiteren waren, verglichen mit gesunden Spendern, 197 miRNAs in der Gruppe der “Sertoli Cell Only” Patienten, 68 miRNAs in der Gruppe der “mixed atrophy” Patienten und 46 miRNAs in der Gruppe der Patienten mit “germ cell arrest” signifikant dereguliert. Mittels quantitativer RT-PCR wurden die gefundenen Expressionsmuster bestätigt. Basierend auf den in vorliegender Studie gefundenen Daten und Daten aus der Literatur wurden die miRNAs hsa-miR-34b*, hsa-miR-34b, hsa-miR-34c-5p, hsa-miR-429, and hsa-miR-122 als Kandidaten für weitere Analysen an Patienten mit beeinträchtigter Spermatogenese und an gesunden Kontrollprobanden ausgewählt.

Die ausgewählten Kandidaten-miRNAs zeigten, gemessen am “fold change” die größte Deregulation. Basierend auf dem AUC-Wert, scheinen diese miRNAs dazu geeignet zu sein als neue Biomarker für die Diagnose der männlichen Unfruchtbarkeit zu dienen. Diese miRNAs scheinen außerdem eine Rolle in der Apoptose, der Zellproliferation, der Differenzierung und der Spermatogenese zu spielen. Die veränderte Expression von miRNAs bei unfruchtbaren Männern im Vergleich zu gesunden Männern eröffnet die Möglichkeiten die Ursache der Unfruchtbarkeit zu identifizieren und trägt dazu bei, neue Gene zu identifizieren, die mit der männlichen Form der Infertilität in Zusammenhang stehen. Insgesamt deuten die hier dargestellten Ergebnisse darauf hin, dass sich miRNA Profile dazu eignen die Infertilität bei Männern zu diagnostizieren.

***Palestine*, the land of the three monotheistic faiths, is where the Palestinian people was born, on which it grew, developed, and excelled.**

SCIENTIFIC PAPERS

The thesis is a cumulative thesis based on the following three published papers. The papers bound in the thesis are identical to the published versions.

I. **Abu-Halima M**, Hammadeh M, Backes C, Fischer U, Leidinger P, Lubbad AM, Keller A, Meese E: A panel of five microRNAs as potential biomarkers for the diagnosis and assessment of male infertility. Fertility and sterility 2014. ;102(4):989-997.e1

II. **Abu-Halima M**, Backes C, Leidinger P, Keller A, Lubbad AM, Hammadeh M, Meese E: MicroRNA expression profiles in human testicular tissues of infertile men with different histopathologic patterns. Fertility and sterility 2014, 101(1):78-86 e72.

III. **Abu-Halima M**, Hammadeh M, Schmitt J, Leidinger P, Keller A, Meese E, Backes C: Altered microRNA expression profiles of human spermatozoa in patients with different spermatogenic impairments. Fertility and sterility 2013, 99(5):1249-1255 e1216.

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ABBREVIATIONS

3’-UTR	3-untranslated region
5’-UTR	5-untranslated region
Ago	Argonaute
ART	Assisted Reproductive Technology
AZF	Azoospermia Factor
bp	base pair
C. Elegans	Caenorhabditis elegans
cDNA	complementary DNA
CIS	Carcinoma In Situ
DAZ	Deleted in Azoospermia Gene
dazl	Deleted in Azoospermia–Like gene
DNA	Deoxyribonucleic Acid
E2F1	E2F transcription factor 1
fg	Femtogram
FSH	Follicle Stimulating Hormone
GA	Germ Cell Arrest
ICSI	Intra Cytoplasmic Sperm Injection
IUI	Intra Uterine Insemination
IVF	In Vitro Fertilisation
LH	Luteinizing Hormone
MA	Mixed Atrophy
MESA	Microsurgical Epididymal Sperm Aspiration
miRNA	microRNA
mRNA	messenger RNA
MSCI	Meiotic Sex Chromosome Inactivation
MVSA	Microscopic Vasal Sperm Aspiration
ncRNA	non-coding RNA
NOA	Non-Obstructive Azoospermia
nt	Nucleotides
OA	Obstructive Azoospermia
PCR	Polymerase Chain Reaction

PESA	Percutaneous Epididymal Sperm Aspiration
PGCs	Primordial Germ Cells
piRNA	Piwi-interacting RNA
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
qRT-PCR	quantitative Real Time Polymerase Chain Reaction
RISC	RNA-induced silencing complex
RNA	Ribonucleic Acid
RNAi	RNA interference
rRNA	ribosomal RNA
SCO	Sertoli Cell-Only
SCOS	Sertoli Cell-Only Syndrome
SD	Standard Deviation
SE	Standard Error
siRNA	small interfering RNA
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
SRY	Sex determination region of Y gene
SSCs	Spermatogonial Stem Cells
TEFNA	Testicular Epididymis Fine Needle Aspiration
TESA	Testicular Sperm Aspiration
TESE	Testicular Sperm Extraction
Tnp2	Transition Protein 2 gene
tRNA	transfer RNA
WHO	World Health Organization

1. Introduction

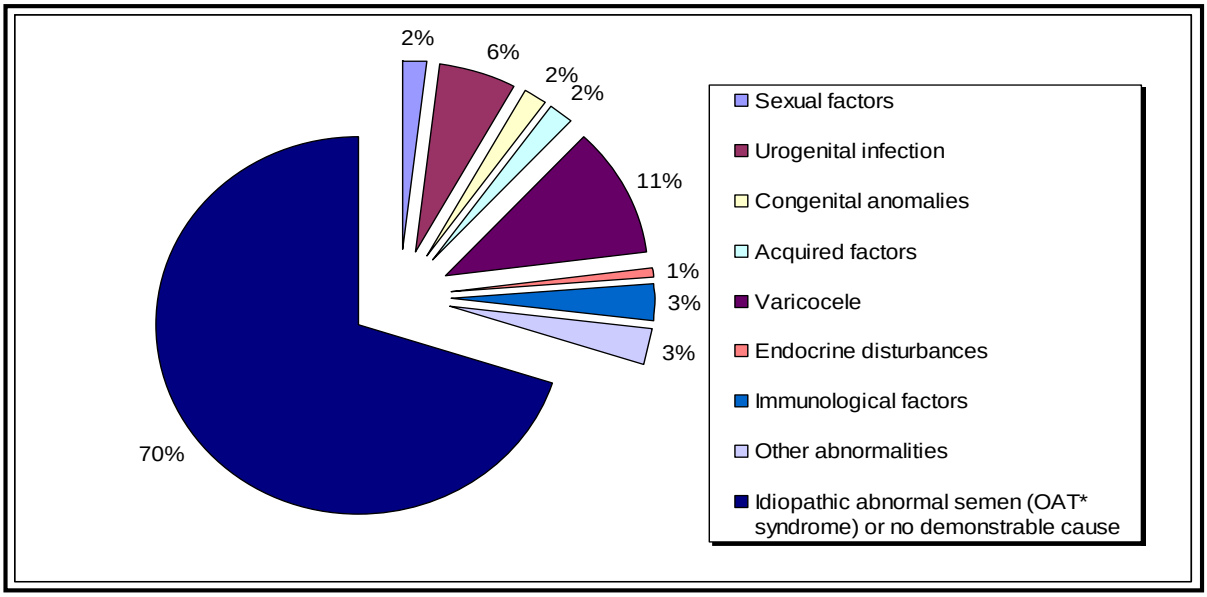
1.1. Overview

The inability to conceive naturally after at least one year of unprotected intercourse in the absence of contraceptives between married couples is defined as infertility by the world health organization (WHO). The WHO recognises infertility as a public health problem suggesting that having a child is a basic human right. Based on the Universal Declaration of Human Rights, Article 16.1, stating: “Men and women of full ages, without any limitation due to race, nationality or religion, have the right to marry and to found a family.” As many as 10-15% of couples have difficulties of conceiving, and seek medical care during their reproductive lifetime. Recent studies show that the number of infertile couples in the general population is growing (Dohle *et al.,* 2002; Feng, 2003; Hellani *et al.,* 2006). Most patients are subfertile, rather than sterile (infertile), but the degree of subfertility is difficult to predict (Baker, 2001). Male infertility is responsible for ~50% of these couples' inability to conceive and is the result of acquired and/or congenital abnormalities. Idiopathic male infertility occurs in ~60-75% of all cases in patients without previous fertility problems (Jungwirth *et al.,* 2013). These men usually have no previous history associated with fertility problems and have normal findings on physical examination. Genetic abnormalities contribute to a fair enough percentage of male infertility, in ~15% of cases either as chromosomal abnormalities or as genetic translations of the chromosome Yq (Chandley *et al.,* 1998; Fedder *et al.,* 2004; Gianotten *et al.,* 2004; Vicdan *et al.,* 2004; Ferlin *et al.,* 2006; Poongothai *et al.,* 2009). It is thought, however, that a substantial part of the remaining unexplained cases also have a genetic aetiology. Several lines of evidence indicate that reduced male fertility is genetically and epigenetically not just one disorder, but also a diverse group of disorders with a multi-factorial aetiology. It has been estimated that thousands of genes are involved in the genetic control of human spermatogenesis (Gianotten *et al.,* 2004), but the functional role of the majority of these genes in male infertility are still poorly understood. However, the biological functions of these genes and their underlying genetic drivers may be affected by various biological factors, including microRNAs (miRNAs). MiRNAs belong to a family of small non-coding RNAs (~22 nucleotides) that interact with their target mRNAs to inhibit translation by either degradation of the mRNAs or blocking translation. Since miRNAs have a significant

role in many biological processes and cellular dysfunctions, it is of interest to study and to characterize the miRNA expression profile in subferile and infertile males and to illustrate the functional role of expressed miRNAs in the male reproductive organs. Alteration expression of specific miRNAs is associated with certain male reproductive abnormalities. For this reason, evaluation of expression of such miRNAs may contribute to identification of new non-invasive molecular markers for the evaluation and assessment of male fertility status.

1.2. Aetiology of male subfertility and infertility

Reduced male fertility can be the result of congenital or acquired abnormalities like urogenital abnormalities, infections of the genital tract, varicocele, endocrine disturbances, genetic and immunological factors (Figure 1). However, no clear causal factor could be diagnosed in about 60-75% of cases coining the term "idiopathic male infertility". In most cases, genetic predisposition in combination with environmental factors plays a role in their hampered reproductive function (Skakkebaek *et al.,* 2001; Sharpe and Irvine 2004). Reduced male infertility is accompanied by quantitative (azoospermia, cryptozoospermia, and oligoasthenozoospermia) and/or qualitative (asthenospermia, teratozoospermia, and necrospermia) abnormalities (Ferlin *et al.,* 2007). According to these aforementioned causes, the reduced male fertility can be further divided into five major diagnostic categories: (i) disorders related to motility or sperm function; (ii) disorders related to obstructive lesions; (iii) disorders related to spermatogenic failure; (iv) sexual dysfunction disorders of erection and ejaculation; (v) endocrine dysfunction.



*OAT: Oligoasthenoteratozoospermia

Figure 1. Aetiology and distribution of male infertility Adapted from WHO (WHO, 2000).

1.2.1. Disorders related to sperm function

Sperm dysfunction is one of the most common causes of infertility. To date, many studies have been performed on human spermatozoa in order to investigate the causes of sperm dysfunction and male infertility. However, there is no drug a man can take to improve fertility. It was shown that 81% of spermatozoa had defective motility while concentration and morphology were not affected (Curi *et al.,* 2003). The only option is assisted reproductive technology (ART), which usually depends on severity of sperm dysfunction, i.e., intrauterine insemination (IUI) for males with mild, *in vitro* fertilisation (IVF) for males with moderate and intracytoplasmic sperm injection (ICSI) for males with severe sperm dysfunction. Sperm basic parameters such as sperm concentration, sperm motility and morphology are essential for normal fertilization and also play a crucial role in sperm function (Boatman and Robbins, 1991). Diagnostic assessment of sperm function is currently assessed using descriptive semen analysis. According to the WHO guidelines, semen analysis includes many parameters like volume (mL), pH, count/concentration (10^6/mL), motility (%-motile), vitality (eosin) (%) and morphology (%) (WHO, 2010). These parameters are used to classify male infertility such as decreased spermatozoa motility, (asthenozoospermia), decreased spermatozoa morphology (teratozoospermia) and decreased number of spermatozoa (oligozoospermia) or a combination of each. In case of complete absence of spermatozoa in the ejaculate in at least two separated centrifuged samples, the

abnormality is described as azoospermia. Any deviations from the reference semen variables (Table 1), considers a male as abnormal. However, descriptive semen analysis is a poor method of diagnosing male infertility and is only considered useful in cases where the concentration of motile sperm is low like in males with azoospermia or severe oligozoospermia (<5 million sperm/mL) (Tomlinson *et al.* 1999; Guzick *et al.* 2001; Lefièvre *et al.* 2007). This is due to an overlap by as much as 60% between the semen parameters of males with proven fertility and males with male factor infertility (Nallella *et al.* 2006). As a result, there is not a clear demarcation between populations of fertile and infertile males (Bartoov *et al.* 1993; Barratt *et al.* 1995; Nallella *et al.* 2006), making the finite diagnosis of infertility very difficult.

Table 1. Lower reference limits (5th centiles and their 95% CIs) for semen characteristics. Adapted from Jungwirth (Jungwirth *et al.,* 2013)

Parameter	Lower reference limit (range)
Semen volume (mL)	1.5 (1.4-1.7)
Total sperm number (106/ejaculate)	39 (33-46)
Sperm concentration (106/mL)	15 (12-16)
Total motility (PR + NP)	40 (38-42)
Progressive motility (PR, %)	32 (31-34)
Vitality (live spermatozoa, %)	58 (55-63)
Sperm morphology (normal forms, %)	4 (3.0-4.0)

CIs = confidence intervals; NP = non-progressive; PR = progressive.

1.2.2. Disorders related to obstructive lesions

Obstructive azoospermia (OA) is considered one of the most favourable prognostic conditions for male infertility since spermatogenesis is not disrupted, unlike in non-obstructive azoospermia. OA can result from epididymal, vasal and/or ejaculatory duct pathology. Severe genitourinary infections, surgical procedures and congenital anomalies are other common causes of OA. Although azoospermia has many causes, approximately 40% of cases result from obstruction in the ductal system (Jarow *et al.,* 1989). Azoospermia is diagnosed based on the absence of spermatozoa after centrifugation of complete semen specimens using microscopic analysis according to WHO guidelines (WHO, 2010). Azoospermia affects approximately 1% of the males' population and ranges between 10-15% among infertile males. History,

physical examination, semen analysis and hormone profile with serum FSH, LH and total testosterone are undertaken to define the cause of azoospermia (Figure 2). Together, these factors provide a >90% prediction of the type of azoospermia (obstructive azoospermia *v.* non-obstructive azoospermia). Males with diagnosed OA may conceive children by one of two ways: **1)** surgical correction of the obstruction, which allows the couple to conceive naturally and obviate the need for ART, or **2)** retrieval of spermatozoa directly from testis or epididymis, using sperm retrieval techniques like testicular sperm extraction (TESE), testicular sperm aspiration (TESA) and testicular epididymis fine needle aspiration (TEFNA), followed by IVF or ICSI. The use of these techniques in clinical practice revolutionized the treatment of patients with severe male factor of infertility (Palermo *et al.,* 1992).

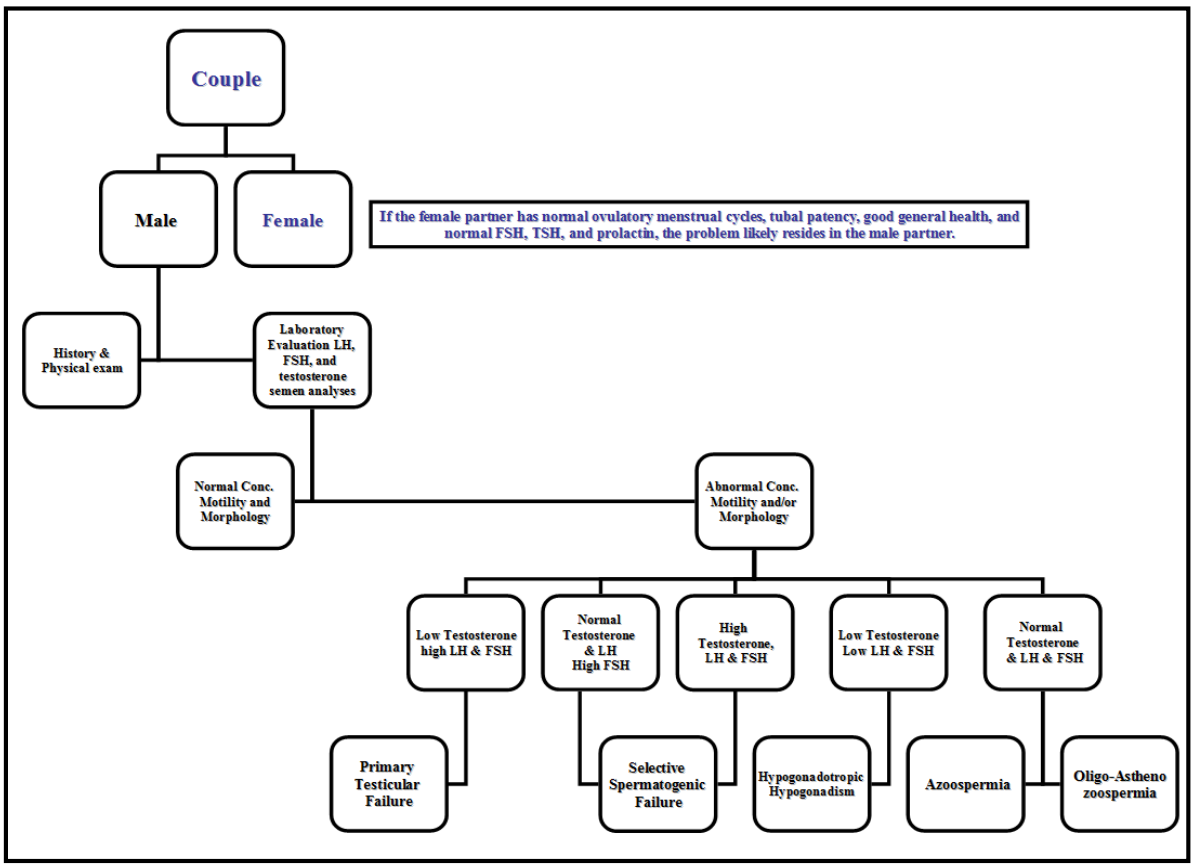


Figure 2. Algorithmic approach to the initial evaluation of patients with infertility. Adapted from Bhasin (Bhasin, 2007).

1.2.3. Disorders related to spermatogenic failure

Non-obstructive azoospermia (NOA) is a condition in which male partner have impaired production of spermatozoa. These males, who constitute up to 10% of all infertile males, have abnormal spermatogenesis as the cause of their azoospermia (Irvine, 1998). Approximately 60% of these cases are due to testicular failure (Willott, 1982). Unlike in obstructive azoospermia, the spermatogenesis in males with NOA is disrupted. Males with NOA actually have either atrophic and/or hyalinized seminiferous tubules with only Sertoli cells. These tubules with complete or incomplete spermatogenesis and markedly reduced spermatogenesis defined with hypospermatogenesis. Germ cell arrest at a particular stage, most often at the spermatogonial or primary spermatocyte stage and in rare cases at spermatid stage diagnosed with maturation arrest. The complete absence of germ cells or their products noted with Sertoli cell only syndrome (Vicdan *et al.* , 2004; Cerilli *et al.,* 2010; Gat *et al.,* 2010). The challenge, however, is to improve their spermatogenic function to enable the appearance of sperm in their ejaculate or to improve the chances of a successful retrieval from the testis for ICSI. As presented in Figure 1, the initial diagnostic evaluation is to confirm the cause of azoospermia and to exclude treatable conditions like hypogonadotropic hypogonadism and obstructive azoospermia, and to identify males who are candidates for ART, who are sterile and who should undergo genetic testing and counselling.

1.2.4. Disorders related to genetic disorders

Genetic abnormalities account for 10-15% of cases of infertility (Ferlin *et al*., 2006). A striking feature of these abnormalities is that they frequently affect the sex chromosomes. Between 10% and 15% of cases of azoospermia are caused by Y chromosome microdeletions (Foresta *et al.* 2001) and a further 10% are caused by chromosome translocations and aneuploidy (Ferlin *et al.* 2006). Numerous other genes are likely to be associated with male infertility. Initial reports from human studies have identified several candidate genes, including PRM1 and PRM2 genes, DAZL1, SPO11, EIF5A2, USP26, and others (De Kretser, 1997; Tsui *et al.,* 2000; Foresta *et al.,* 2002a; Christensen *et al.,* 2005; Carrell *et al.,* 2006; Gázquez *et al.,* 2008; Zhang *et al.,* 2011; Grassetti *et al.,* 2012; Ghalkhani *et al.,* 2014). Genetic anomalies are either detected at the cytogenetic and/or at the molecular levels. In addition to gene

mutations and polymorphisms, damage to the chromatin resulting in single and double strand DNA breaks affects male fertility (Enciso *et al.,* 2009; González-Marín *et al,* 2012). Epigenetic abnormalities such as gene imprinting may also contribute to male infertility (Dada *et al.,* 2012; Laurentino *et al.,* 2014). The two most common categories of genetic factors associated with male infertility and specifically with non-obstructive azoospermia are: **1)** numerical and structural chromosomal aberration resulting in impaired testicular function; and **2)** Y-chromosome microdeletions leading to spermatogenic impairments.

1.2.4.1. Numerical and structural chromosomal aberration

Numerical and structural chromosomal disorders interfere with spermatogenesis and the percentage of chromosomal abnormality increases with the decline in spermatozoa concentration (De Braekeleer and Dao, 1991; Escudero *et al.,* 2003; Nagvenkar et al., 2005). These abnormalities are found in about 14% of azoospermic males and 5% of oligozoospermic males (Johnson, 1998; Olesen *et al*., 2001; Foresta *et al*., 2002b; Vogt, 2004). Most of the aneuploidies causing male infertility involve both sex chromosomes as well as autosomes and can numerical or structural aberrations. Klinefelter's syndrome (47, XXY) is the most frequent chromosomal disorder associated with infertility (Chandley, 1979; Foresta *et al.,* 1999; Bojesen *et al.,* 2003). These infertile males are characterized by testicular hypotrophy, azoospermia and elevated FSH levels (Ferlin *et al.,* 2006). In 50% of cases, sperm found upon TESE (Rosenlend *et al.,* 2002; Greco *et al.,* 2013). Klinefelter patients with increasing number of X chromosomes, (48, XXXY) and (49, XXXXY), shift their sexual phenotype to the female side. This suggests an X-chromosome dosage effect on the males gonad development, and also shows that the balance between X and Y chromosomes is crucial (Egozcue *et al.,* 2000; Rives *et al.,* 2005; Moretti *et al.,* 2007; Abdel-Razic *et al.,* 2012). For double Y syndrome males, spermatogenesis ranges from normal to severely impaired, and if they are fertile, their phenotype is usually oligozoospermia (Vogt, 2004). Studies have increasingly reported an association between 47, XYY and fertility problems (Speed *et al.,* 1991; Lim *et al.,* 1999; Gonzalez-Merino *et al.,* 2007; Wong *et al.,* 2008). Structural abnormality of autosomal chromosomes in the male partner with azoospermia and oligozoospermia with Robertsonian translocation t (14; 21) and t (13; 14) is also reported (Vogt, 2004;

Nagvenkar *et al.,* 2005, Ferlin *et al.,* 2006). Other autosomal chromosomal anomalies, like aneuploidies, balanced Robertsonian translocations, balanced reciprocal translocations, balanced inversions, and deletions were found less frequently than sex chromosome abnormalities (Vogt, 2004; Ferlin *et al*., 2006). Furthermore, most studies on X-gene expression have focused on protein-coding genes. Recent studies have now demonstrated the expression of miRNAs during spermatogenesis (Ro *et al.* 2007; Song *et al.* 2009). Interestingly testis-expressed miRNAs also preferentially map to the X chromosome (Ro *et al.* 2007) and, more strikingly, around 80% of these X-linked miRNAs are expressed during pachytene spermatocytes and participate in a critical function during spermatogenesis (Song *et al.* 2009). This finding is interested, as it represents the first example of a class of genes that are able to evade meiotic sex chromosome inactivation (MSCI). These genes are proposed to play roles in either late meiosis, early post-meiotic stages of spermatogenesis and/ or in MSCI itself (Song *et al.* 2009). Ghorai and Ghosh observed that certain chromosomes are having higher miRNA genes through evolution and X chromosomes have high number of miRNA genes whereas Y chromosomes have least or no miRNA gene(s) in all the studied species (Ghorai and Ghosh, 2014).

1.2.4.2. Microdeletion of Y chromosome

The Y chromosome contains many testis-specific genes necessary for spermatogenesis (Skaletsky *et al.,* 2003; Hawley, 2003). Most deletions occur in non-overlapping regions of the long arm of the Y chromosome (Yq11) that contain multiple genes important for spermatogenesis (Tiepolo and Zuffardi, 1976; Vogt *et al.,* 1996; Repping *et al.,* 2002). These deletions are considered one of the most frequent genetic causes of severe oligozoospermia (<5 million spermatozoa/ml) and azoospermia and can be detected in 10-15% of azoospermic males and 3-10% of oligozoospermic males with normal karyotype (Kent-First *et al,* 1996; Pryo *et al.,* 1997; Vogt, 1998; Krausz *et al.,* 2003; Simoni *et al.,* 2004; Cram *et al.,* 2006; Ferlin *et al.,* 2007). *Vogt et al.* and others classified Yq microdeletions into three groups depending on the location of microdeletion: AZFa, AZFb, and AZFc. Subsequently, three additional types of microdeletions were recognized: gr/gr deletion, AZFbc, and AZFabc. These six types of microdeletions account for nearly all the Yq microdeletions that have been associated with infertility (Vogt, 1998; Cram *et al.,* 2006; Ferlin *et al.,* 2007). The

specific location of any Y chromosome microdeletion may determine the extent to which spermatogenesis is affected. For instance, deletions removing the entire AZFa or AZFb regions (complete deletions) are associated with Sertoli Cell Only Syndrome (SCOS) and germ cell arrest (GA) at the spermatocyte stage and/or at spermatid stage (Krausz *et al.,* 2000; Kamp *et al.,* 2001). Partial deletions at the spermatocyte stage are associated with variable phenotypes ranging from hypospermatogenesis, oligozoospermia, to complete absence of germ cells (SCOS). A possible explanation for such a variable phenotype is a progressive regression of the germinal epithelium over time, which has been reported in patients with AZFc deletions (Calogero *et al.,* 2001).

1.3. Human spermatozoal RNAs

Over the past decade, it has been documented that the nucleus of mature spermatozoa contains a complex population of RNAs that are transcriptionally inert (Kierszenbaum and Tres, 1975). Findings from several studies support the conclusion that spermatozoa contain a complex repertoire of mRNAs (Kumar *et al.,* 1993; Miller *et al.,* 1994; Wykes *et al,* 1997; Miller *et al.,* 1999; Ostermeier *et al.,* 2002; Dadoune *et al.,* 2004). These mRNAs are thought to provide an insight into past events of spermatogenesis (Kramer and Krawetz, 1997; Miller, 1997; Rockett and Dix, 2000). The majority of sperm transcripts, which have been detected in sperm, had also been detected previously in testes (Miller *et al.,* 2005; Sendler *et al.,* 2013). Although these transcripts were well described, their role in male spermatogenesis remains unclear. Irregularities in the levels of sperm RNAs have been recognized as markers and potential effectors of human male infertility (Miller, 2000; Yatsenko *et al.,* 2006; Platts *et al.,* 2007; Zhao *et al.,* 2007; Steger *et al.,* 2008). Krawetz *et al.* suggested that some of these mRNA, which were detected in the sperm nucleus, were delivered into oocytes during fertilization (Krawetz, 2005). In human, the total amount spermatozoal RNA per mature sperm is estimated to be 10–400 fg (Goodrich *et al.,* 2007; Lalancette *et al.,* 2009). Sperm retain specific coding (Miller *et al.,* 1997; Wykes *et al.,* 1997; Ostermeier *et al.,* 2002; Wykes and Krawetz, 2003; Ostermeier *et al.,* 2004; Miller and Ostermeier, 2006) and non-coding RNAs (ncRNAs) (Ostermeier *et al.,* 2005; Krawetz *et al.,* 2011). These small RNAs regulate gene expression at different levels, and have essential roles in nearly all biological processes. Krawetz *et al.* were the first to

identify the presence of multiple classes of small RNAs in human spermatozoa by bioinformatics analysis (Figure 3). These subclasses are microRNA (miRNAs) (6.9%), Piwi-interacting piRNAs (piRNA) (16.9%) and repeat-associated small RNAs (65%). While each of these RNAs has distinct functions, the knowledge and understanding of miRNA function is a rapidly expanding area in biology (Krawetz *et al., 2011*).

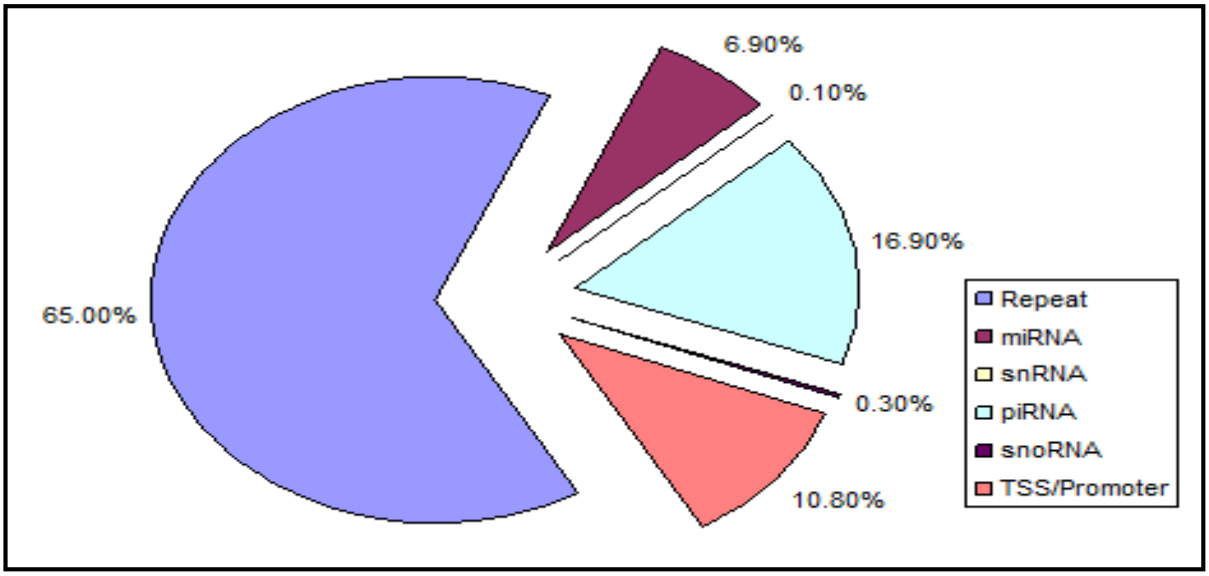


Figure 3: Overview of all the classes of small RNAs found in the human spermatozoa. Adapted from Krawetz (Krawetz *et al.,* 2011).

1.4. MicroRNAs (miRNAs)

MicroRNAs (miRNAs) are a class of RNA molecules that have recently received a considerable amount of attention due to their remarkable impact on gene regulation. MiRNA molecules are non-coding RNAs that are ~22 nucleotides in length, which act to bind target mRNA to regulate gene expression (Lau *et al.,* 2001; Lim *et al.,* 2003). MiRNAs are considered negative regulators of gene expression and have been found to act on vast number of genes (Ambros, 2004; Farh *et al.,* 2005; Filipowicz *et al.,* 2008). During the past decade, miRNAs have emerged as key post-transcriptional regulators of gene expression via sequence-specific interaction with the 3'untranslated region (3' UTR) of target mRNAs, resulting in inhibition of translation and/or mRNA degradation (Bartel, 2004; Pasquinelli, 2012). A large number of studies have indicated that miRNAs have been implicated in nearly all biological processes, such as development (Alvarez-Garcia and Miska, 2005; Tang *et al.,* 2007; Zhao and Srivastava, 2007), cell proliferation and differentiation (Carleton *et al.,* 2007;

Lakshmipathy *et al.,* 2007; Enerly *et al.,* 2011), apoptosis (Hwang and Mendell, 2006) and tumorigenesis (Ventura and Jacks, 2009). MiRNAs belong to the class of small RNAs, which are non-coding RNAs with a length of less than 300 nt. Small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs), short interfering RNAs (siRNAs) and Piwi-interacting RNAs (piRNAs) also belong to this class. Until recently, small RNAs were regarded as evolutionary trash and RNAs that do not encode protein are often considered as results of "leakage" of the transcription machinery. However, present researches have highlighted that ncRNAs can have a wide range of functions and can be divided into different classes by sizes or functions (Figure 4). The regulatory functions of small RNAs in gene expression and their pivotal roles in physiological as well as pathological processes revealed them to be vital components of the genome (Kutter and Svoboda, 2008).

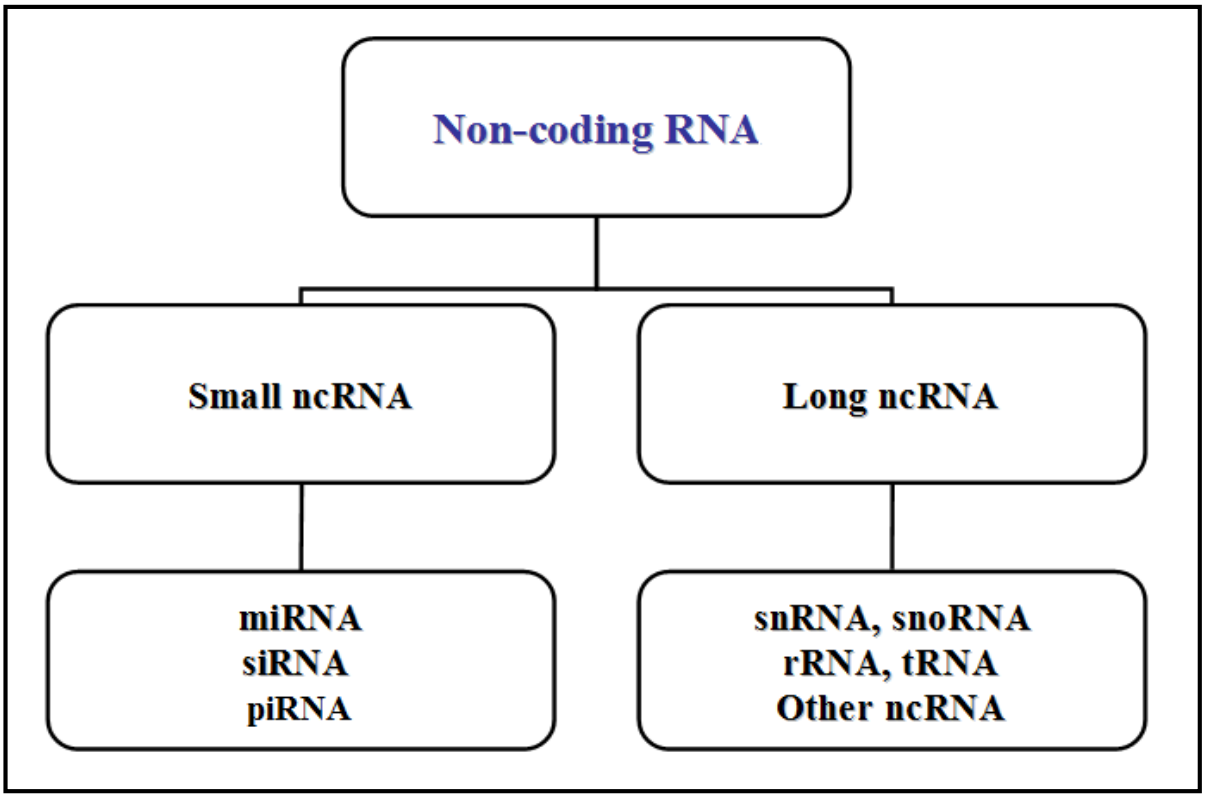


Figure 4. Classification of non-coding RNA species by size. Small ncRNA is below 30 bp while long ncRNA is above 200 bp.

1.4.1. History of miRNAs

The first non-coding RNA (ncRNA), miRNA, was discovered in the *Ambros* and *Ruvkun* labs in 1993 when a gene, lin-4, crucial for nematode Caenorhabditis elegans (*C.elegans*) development, was found to not encode a protein but to give rise to a small 22-nucleotide RNA. In 1993, Lee *et al.* and Wightman *et al.* discovered that the gene lin-4 is transcribed into a 22 nt long RNA that inhibits lin-14 by RNA interference in the nematode *Caenorhabditis elegans* (Lee *et al.,* 1993; Wightman *et al.* 1993). The authors concluded that lin-4 might belong to a class of small, regulatory non-translated RNA, albeit for years there was no evidence for other lin-4-like RNAs in *C.elegans* or other organisms. The identification of a second miRNA, let-7 in *C. elegans*, drosophila and humans was identified (Reinhart *et al.,* 2000; Pasquinelli *et al.,* 2000; Basyuk *et al.,* 2003). The actual miRBase database Release 21 contains 28645 hairpin precursor miRNAs and 35828 mature miRNA products in 223 species (http://microrna.sanger.ac.uk) (Kozomara and Griffiths-Jones, 2014).

1.4.2. MiRNA genomic location, biogenesis and mechanism of function

MiRNAs can be transcribed from independent miRNA coding genes, which can be grouped into two classes, intergenic miRNAs and intragenic miRNAs (Garzon *et al.,* 2009; Wang *et al,* 2010). About 42% of miRNAs are intergenic, i.e., they are located between protein-coding genes (Wang *et al,* 2010). Intragenic miRNA-coding genes are located within their host protein-coding genes and can be further subdivided into four subclasses: **a)** intronic miRNAs, located within introns of their host protein-coding genes; **b)** exonic miRNAs, located within exons of host protein-coding genes; **c)** 3'UTR miRNAs, located within 3'UTR of host protein-coding genes; and **d)** 5'UTR miRNAs, located within 5'UTR of host protein-coding genes (Wang *et al,* 2010). The miRNA genes are typically transcribed within the nucleus by RNA Polymerase II, but can also be transcribed by Polymerase III generating the preliminary miRNAs (pri-miRNA) that can be a few hundred to a few thousand nucleotides in length and is typically capped and polyadenylated like mRNAs (Lee *et al.,* 2004, Borchert *et al,* 2006; Garofalo *et al.,* 2011). The RNase III enzyme *Drosha* in complex with DGCR8 cleaves the pri-miRNA into a short hairpin RNA around 60-100 nucleotides in length called the pre-miRNA (Figure 5). After this cleavage event, the pre-miRNA hairpins are exported to the cytoplasm by exportin5, a Ran-GTP dependent exporter (Lee *et*

al., 2003; Yi *et al.,* 2003; Han *et al.*, 2004; Filipowicz *et al.,* 2008). In the cytoplasm, pre-miRNAs are recognized and processed by RNase III *Dicer* into ~22-nt miRNA duplexes, consisting of the "guide" (miR) strand and the "passenger" (miR*) strand. The "guide" strand is incorporated into the RNA-induced silencing complex (RISC) and serves as a functional, mature miRNA, acting by two different mechanisms according to the complementarity with the target mRNA (Garofalo *et al.,* 2001; Lee *et al.*, 2002; Hutvagner and Zamore, 2002; Schwarz *et al.,* 2003; Meister *et al.*, 2004; Kim *et al.,* 2005; Jones-Rhoades *et al.,* 2006). Recently, deep sequencing data have shown that some miRNAs* are not degraded and they play a functional role in the regulation of miRNA and target different mRNA populations (Okamura *et al.,* 2008; Suzuki and Miyazono, 2011).

1.4.3. MiRNA regulatory interactions

MicroRNA regulate gene expression by either translational repression or degradation of target mRNA, or both, depending on the degree of sequence complementarity between the miRNA and parts of the 3'UTR of the target mRNA (Zhang *et al.,* 2007; Trang *et al.,* 2008). The guide strand incorporated into the RISC complex is the key element for the recognition of the target mRNAs (Figure 5) (Parker *et al.,* 2006). A miRNA-mRNA duplex with nearly perfect complementarity usually occurs in plants (Jones-Rhoades *et al.,* 2006), but infrequently occurs in vertebrates. In vertebrates, miRNAs rather exhibit complementarity of their "seed region", which is composed of nucleotides 2-8 of the miRNA´s 5'-end, to the 3'UTR of their target mRNA, but mismatches can be present in the central region of the miRNA-mRNA duplex (Doench and Sharp, 2004; Filipowicz *et al.,* 2008). The 3'-region of the miRNA exhibits good complementarity to the mRNA thereby providing the necessary stability for the miRNA:mRNA interaction. The degree of complementarity between the miRNAs and their target sequences is dependent on the number of miRNA binding sites in the 3'UTR and is also considered as a predictor for the respective regulatory mechanism. Complete or nearly complete complementarity labels the target mRNA for proteolytic degradation whereas a lower degree of complementarity labels the mRNA for processes resulting in decreased translation or mRNA destabilization (Zhou *et al.,* 2007; Bartel, 2009; Wang *et al.,* 2010). Nevertheless, there are some exceptions in which low complementarity also leads to degradation of the target mRNA (Bagga

et al., 2005). In addition, mRNA degradation is not the primary mechanism of miRNA-mediated gene silencing. Argonaute proteins (Ago1–4) are the primary miRISC component that directly recruit miRNAs and promote miRNA-mediated translational inhibition (Schmitter *et al.,* 2006; Hock and Meister 2008; Czech and Hannon 2011). Ago2 is the sole catalytic member of the Ago1-4 and is thus responsible for cleavage of highly complementary targets of miRNAs (Liu *et al.* 2004; Meister *et al.* 2004). Ago2 is unique, with slicer activity that is capable of cleaving of perfectly matched targets for miRNAs (Liu *et al.* 2004; Meister *et al.* 2004; Hock and Meister 2008; Czech and Hannon, 2011). The functional significance of Ago1, Ago3, and Ago4 for miRNA activity is poorly understood, although they at least partially influence efficiency of repression (Pillai *et al.,* 2004).

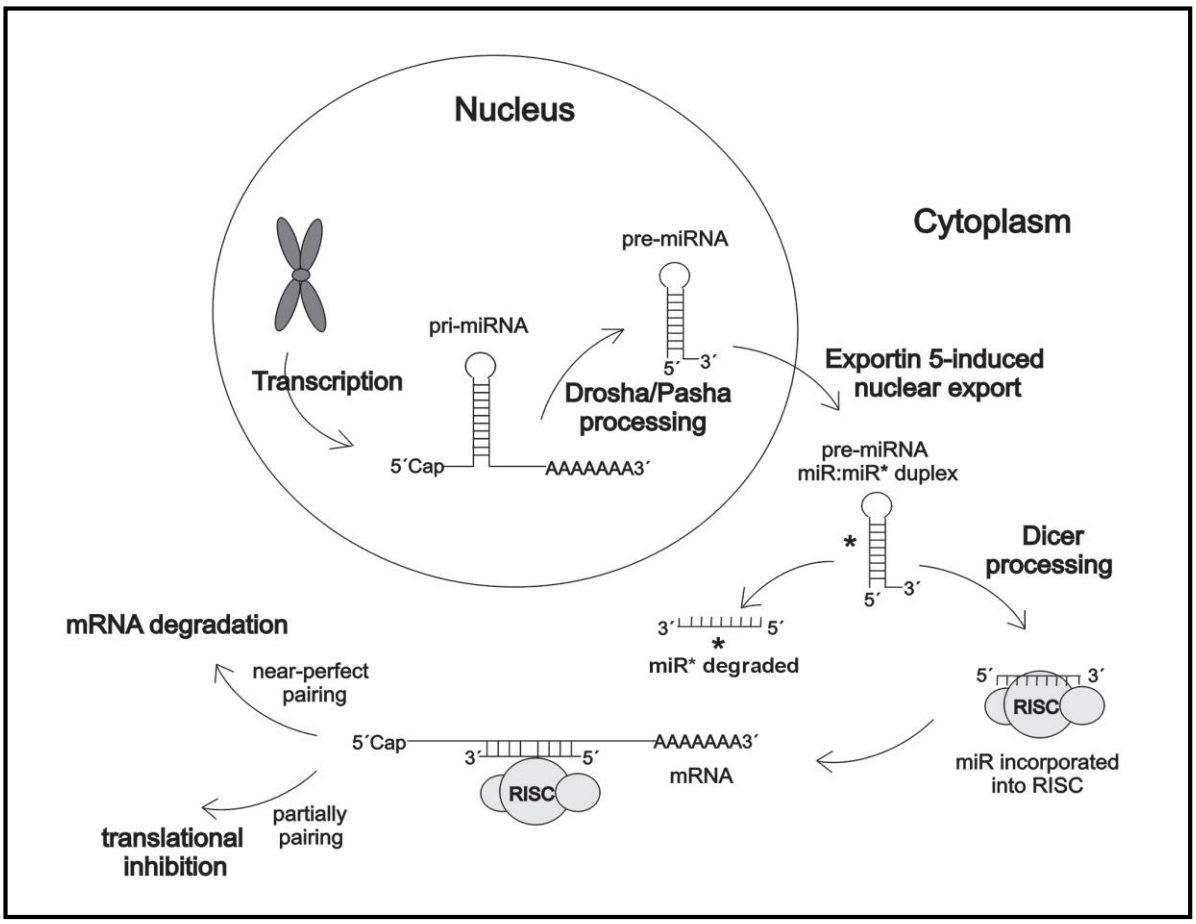


Figure 5: Schematic representation of miRNA biogenesis. MiRNAs are transcribed by RNA polymerase II from the genomic DNA as long (hundred or thousand nucleotides) primary miRNA transcripts (pri-miRNAs). A local stem-loop structure of pri-miRNAs is then cleaved in the nucleus by the ribonuclease *Drosha* to produce a 70 nucleotides long precursor miRNA (pre-miRNA). Pre-miRNAs in form of hairpins are then actively transported from the nucleus

to the cytoplasm. In the cytoplasm, pre-miRNAs are subsequently cleaved by RNase III *Dicer* into ~22-nt miRNA duplexes, consisting of the "guide" (miR) strand and the "passenger" (miR*) strand. The "guide" strand is incorporated into the RNA-induced silencing complex (RISC) and serves as a functional, mature miRNA, acting by two different mechanisms according to the complementarity with the target mRNA. Adapted (Tomankova *et al.,* 2010).

1.5. MiRNA and fertilization

Spermatozoa have been shown to contain a wide spectrum of RNAs, including miRNAs that are delivered from mammalian spermatozoa to the oocyte (Ostermeier *et al.,* 2004; Krawetz, 2005; Ostermeirer *et al.,* 2005; Lalancette *et al.,* 2008; *Yan et al.,* 2008). Thus, miRNAs play an important role in spermatogenesis as well as the fertilization process of oocyte, and they might even influence the phenotype of the offspring (Ostermeier *et al.,* 2004; Amana *et al.,* 2006; Rassoulzadegan *et al.,* 2006). The first evidence that miRNAs play a significant role during the fertilization and early embryogenesis events came with the finding that miR-430 expressed at the onset of zygote transcription and has been shown to target a large set of maternally derived mRNAs in zebrafish (Giraldez *et al.,* 2006). Amana *et al.* found that mature mouse spermatozoa contain a set of miRNAs; their potential mRNA targets are expressed in metaphase II oocytes. Furthermore, expression levels of sperm-borne miRNAs in fertilized oocytes were low relative to those in unfertilized oocytes. Thus, mouse spermatozoa miRNAs have very little, if any, influence on the fertilization process or early preimplantation development (Amana *et al.,* 2006). Liu and colleagues identified 25 miRNAs in the zona-bound sperm, 14 miRNAs (let-7d, miR-16, miR-19b, miR-200b, miR-214, miR-221, miR-25, miR-30b, miR-30c, miR-3-0d, miR-342, miR-34c, miR-93, and miR-99a) were found in a panel of 54 miRNAs identified in the epididymal sperm (Amana *et al.,* 2006: Liu *et al.,* 2012). This finding suggests that these miRNAs are likely to be more representative of the miRNAs delivered to the oocyte during fertilization because zona pellucida binding is the first step in fertilization. More recently, it has been shown that miRNAs are differentially expressed throughout embryonic cellular divisions and genome activation, and they are secreted from the embryo into the IVF culture media, and therefore they may play a role in the developmental competence of embryos (Kropp *et al.,* 2014; Rosenbluth *et al.,* 2014). These evidences suggest that many germ cell specific miRNAs, especially miRNAs that are highly expressed in sperm, not only play important roles in maintaining their

functions of producing a functional sperm during spermatogenesis, but also can be delivered into oocyte in order to complete the fertilization process of normal embryo development.

1.6. MiRNA in spermatogenesis

Spermatogenesis is the process of forming mature haploid spermatozoa or sperm from diploid spermatogonial stem cells. It is a highly complex, precisely organized and timely regulated developmental process. In mammals, spermatogenesis process is strictly regulated by transcriptional as well as post-transcriptional mechanisms (Cooke and Saunders, 2002; Bettegowda and Wilkinson, 2010). The transcriptional mechanisms are well described, however, the post-transcriptional regulation is still largely unknown (Pang *et al.,* 2003; Kimmins *et al.,* 2004; *Wu et al.,* 2004; MacLean and Wilkinson, 2005; Bettegowda and Wilkinson, 2010). In particular, the post-transcriptional regulation is essential due to the fact that, many unique genes are involved, the expressions of which are partially coordinated via miRNA suppression (Lee *et al.,* 2009; Papaioannou and Nef, 2010). In spermatogenesis, miRNAs have a significant impact on the development of spermatozoa, particularly in germ cells and somatic cells (Figure 6) (Papaioannou and Nef, 2010). It is conceivable that any deregulation in miRNA expression patterns significantly affects spermatogenesis pathway and leads to several types of reproduction abnormalities (He *et al.,* 2009; Bouhallier *et al.,* 2010). The mechanism of post-transcriptional regulation was first elucidated by conditional knock out mouse models in which *Dicer* or *Drosha* were specifically depleted in primordial germ cells (PGCs) or spermatogonia (Hayashi *et al.,* 2008; Maatouk *et al.,* 2008; Korhonen *et al.,* 2011). The lack of miRNAs resulted in infertility due to disruption of spermatogenesis. Although several miRNA microarray, qRT-PCR or small RNA sequencing studies have identified miRNAs that are highly, exclusively, or preferentially expressed in the spermatogenesis and their specific cell types, few functional studies were performed to explain the molecular function of these miRNAs. Therefore, the exact biological functions and the genetic factors driving their role and expression in the development of spermatogenic impairment disorders have been revealed for only a very limited number of miRNAs. To date, there are only few examples of miRNAs with well documented function in spermatogenesis.

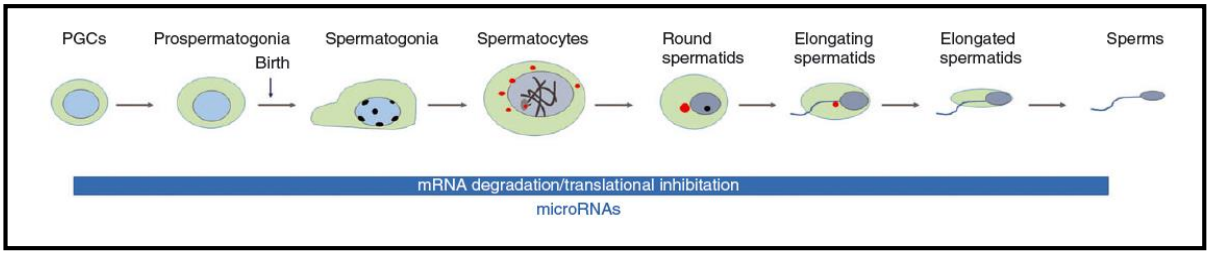


Figure 6: A schematic drawing of the expression and function of miRNAs during each stage of spermatogenesis. Adapted from Gou (Gou *et al.,* 2014).

1.6.1. Functional study of miRNAs in spermatogenesis

Until now, there are only few examples of miRNAs with well-documented function in spermatogenesis. For example, miR-122a is predominately expressed in post-meiotic male germ cells and participates in the posttranscriptional down-regulation of transition protein 2 (TNP2), a post-transcriptionally regulated testis-specific gene that involved in chromatin remodeling during spermatogenesis (Yu et *al.,* 2005). Two studies published shortly afterwards identified the miRNA profile using either microarray technology (Yan *et al,* 2007) or cloning analysis (Ro *et al,* 2007); in the first study, whole testis was used as starting material, whereas in the second, purified spermatogenic cells were isolated from various testicular cell populations, i.e., Sertoli cells, spermatogonia, pachytene spermatocytes, round and elongating spermatids, and spermatozoa. Those studies along with other studies, suggest that late meiotic stage of spermatogenesis is the main source of miRNA production during spermatogenesis (Hayashi *et al.,* 2008; Guo *et al.,* 2009; Bouhallier *et al.,* 2010). Recently, *Niu et al.* were able to demonstrate that miRNA-21, that is highly expressed in spermatogonial stem cells (SSCs), was involved in the regulation of expression of ets variant gene 5 (ETV5), an essential transcription factor for maintaining the self-renewal of SSCs, and plays an important role in regulating SSC homeostasis (Niu *et al.,* 2011). The high expression level of miR-34c was found in adult pachytene spermatocytes and round spermatids, and miR-34c is important to the first cell division by modulation of Bcl-2 expression (Bouhallier *et al.,* 2010; Liang *et al.,* 2012; Liu *et al.,* 2012). Likely targets of miR-34b and miR-34c include deleted in azoospermia–like (dazl) gene, which is involved in germ cell differentiation in mice (McIver *et al.,* 2012). MiR-34b was found to be deregulated from day 7 through day 14 in mouse and in mature rhesus monkey testes and to be involved in the cellular senescence, apoptosis, and control of the cell cycle (Corney *et al.,* 2007; Yan *et al.,* 2007;

Kumamoto *et al.,* 2008; Buchold *et al.,* 2010; Vogt *et al.,* 2011). Two studies reported that the miRNAs are temporally regulated during male germ cell development. In the first study, Hayashi *et al.* showed that the *miR-17-92* and miR-290-295 clusters were highly expressed in PGCs and spermatogonia, concluding that the level of miRNAs changes with the progression of PGCs development (Hayashi *et al.,* 2008). In the second study, Tong *et al.* showed that the role of *miR-17-92* cluster in the control of spermatogonial differentiation and their deletion leads to small testes and decreases the number of epididymal spermatozoa (Tong *et al.,* 2012). Another interesting study discussed the potential involvement of miRNAs in the regulation of meiotic and post-meiotic gene expression during spermatogenesis and reported that the miR-449a and miR-449b are drastically up regulated in spermatogonia, spermatocytes, and spermatids (Bao *et al.,* 2012). Despite a high expression level in male germ cells, miR-449 null male mice exhibit normal spermatogenesis (Bao *et al.,* 2012). The expression of miR-449a and miR-449b is positively regulated by the E2F transcription factor 1 (E2F1), which promotes cell cycle progression and induces apoptosis. Over-expression of E2F1 leads to increase the level of apoptosis in spermatocytes (Holmberg *et al.,* 1998; Marcet *et al.,* 2011). Depletion of E2F1 reduces spermatogonial proliferation and promotes testicular atrophy (Hoja *et al.,* 2004). Interestingly, miR-34 b/c levels were up regulated in miR-449 knockout testis, were shown to share some target genes that belong to the E2F1 gene (Bao *et al.,* 2012). Inactivation of miR-34b/c and miR-449 clusters disrupts their target genes involved in cell fate control, brain development, and microtubule dynamics and leads to severely disrupted spermatogenesis (Wu *et al.,* 2014). A very recently published article showed that the deletion of both miR34b/c and miR-449 loci resulted in oligoasthenoteratozoospermia in mice and the miR-34bc/449-deficiency impairs both meiosis and the final stages of spermatozoa maturation (Comazzetto *et al.,* 2014). Other miRNAs such as miR-469 (Dai *et al.,* 2011) and miR-184 (Wu *et al.,* 2011) have also been studied in spermatogenesis and their role during the various stages of spermatogenesis was considered crucial. MiR-372 and miR-373 have been proposed as a factor promoting tumorigenesis in germ cells, acting as oncogenes in testicular germ cell tumours (Voorhoeve *et al.,* 2006). Apart from their importance during the different stages of normal spermatogenesis, miRNAs have been also involved in many pathological aspects of spermatogenesis. Novotny *et al.* showed by comparing the expression of E2F1 mRNA in human testis with and without *carcinoma in situ* (CIS)

that the *miR-17-92* cluster is activated upon c-Myc expression and eventually leads to the translational repression of E2F1, thereby preventing apoptosis during meiotic recombination (Novotny *et al,* 2007). Additionally, results from other groups, have also indicated a reduced apoptosis in cancers, if they express the *miR-17-92* cluster (He L *et al*., 2005; Hayashita Y *et al*., 2005). Another interesting study reported that many X-linked miRNAs are transcribed and processed in pachytene spermatocytes. These X-linked miRNAs participate in a critical function during spermatogenesis, including the possibility that they contribute to the process of MSCI itself, and/or that they may be essential for post-transcriptional regulation of autosomal mRNAs during the late meiotic and early post-meiotic stages of spermatogenesis (Song *et al.,* 2009). Another approach more comprehensive, and more successful, is to validate the genes encoding proteins that are necessary for the biosynthesis of small ncRNAs. For instance, González-González *et al,* and Kotaja *et al.* described the expression patterns of several members of the miRNA pathway in the testis; *Drosha* and *Dicer* and the members of the Argonaute family Ago1, Ago2, Ago3 and Ago4, are all expressed in pachytene spermatocytes, round and elongated spermatids, and Sertoli cells (Kotaja e*t al.,* 2006; Gonzalez-Gonzalez *et al.,* 2008). The role of miRNAs and endo-siRNA during testicular development and spermatogenesis of mammals has been studied for several years and these studies confirmed that the ablation of *Dicer* affects the production of all *Dicer*-dependent small RNAs, whereas loss of *Drosha* affects largely the formation of pre-miRNAs and consequently mature miRNA production (Tomari and Zamore, 2005; Lee *et al.,* 2006). The importance of *Dicer* in early germ cell development was shown through its conditional ablation during early embryogenesis in PGCs (Hayashi *et al.,* 2008). This loss of *Dicer* results in proliferative defects in PGCs with either absent or retarded spermatogenesis in adult seminiferous tubules (Hayashi *et al.,* 2008). Maatouk *et al.* found that males lacking *Dicer* in germ cells were subfertile because of both a defect in the transition from round to elongating spermatids and production of sperm with abnormal motility. Shortly afterwards, another study reported that the selective ablation of *Dicer* in Sertoli cells leads to infertility due to complete absence of spermatozoa and progressive testicular degeneration, thereby suggesting an essential role of the *Dicer*-dependent miRNA/siRNA pathway in mammalian male germ cell development (Papaioannou *et al.,* 2009; Papaioannou and Nef, 2010). Moreover, Wu *et al.* demonstrated that *Drosha* is essential for the miRNA biogenesis, and *Drosha*-mediated miRNA

production is essential for normal spermatogenesis and male fertility (Wu *et al.,* 2012). Therefore, the selective inactivation of *Drosha* or *Dicer* in spermatogenic cells depletes spermatocytes and spermatids in the testes and leads to oligoteratozoospermia or azoospermia (Wu *et al.,* 2012).

1.6.2. MiRNA as potential biomarkers for male infertility

Growing evidence has shown that aberrant miRNA expression is associated with many diseases including human male infertility. Based on this observation, a number of genome-wide expression studies has been conducted to shed more light on the issues and defects that underlie this problem. Specifically, the expressions of miRNA as important biomarkers in male factor infertility were explored, and their deregulation patterns in the impaired spermatogenesis were identified. For instance, Lian *et al.* identified 154 differentially down-regulated and 19 up-regulated miRNAs between a non-obstructive azoospermia group and a control group (Lian *et al.,* 2009). Three studies published shortly afterwards reported the miRNA profile of the seminal plasma of patient with NOA using either microarrays and qRT-PCR (Wu *et al.,* 2012, Wu *et al.,* 2014) or next generation sequencing (Wang *et al.,* 2011). Wang *et al.* reported 7 miRNAs with altered expression in pooled seminal plasma samples obtained from infertile males and compared the results with normal fertile males as controls. The level of these 7 miRNAs was significantly lower in azoospermia and higher in asthenozoospermia compared to the control (Wang et al., 2011). Finally, they suggested that these 7 miRNAs might have confirmative molecular diagnostic value for male infertility. Wu *et al.* found that aberrant over-expression levels of miR-19b and let-7a might be an indicator of spermatogenic failure in idiopathic infertile individuals with NOA or oligozoospermia by quantitative RT-PCR. They showed that these two miRNAs distinctively expressed at higher levels in infertile cases compared with fertile individuals (Wu *et al.,* 2012). Subsequently, they found that miR-141, miR-429 and miR-7-1-3p were significantly increased in seminal plasma of patients with NOA compared with fertile controls. They concluded that these five miRNAs are good diagnostic molecular biomarkers for idiopathic infertile cases with NOA or oligozoospermia. Shortly afterwards, Liu *et al.* found 52 differentially expressed miRNAs by comparing the semen miRNA profiles of infertile and normal healthy males using miRNA microarray analysis (Liu *et al.* 2012*).* More recently, other miRNAs such

as miR-27b was found to be highly expressed in the ejaculated spermatozoa of patient with asthenozoospermia and it targets a Cysteine-Rich Secretory Protein 2 (CRISP2), which is highly associated with spermatogenesis and infertility (Zhou *et al.,* 2014). Similarly, Ji *et al.* found that the expression of miR-15a was significantly decreased in the spermatozoa of patients with varicocele compared with control males and it repressed the expression of Heat Shock 70kDa Protein 1B (HSPA1B), which exhibited an inverse correlation in spermatozoa (Ji *et al.,* 2014). A recent study by Hu *et al.* have identified 61 human seminal miRNAs predominately derived from human testis and epididymis. They suggested that levels of these miRNAs reflect their levels in these organs, where sperm production and maturation occurs. Consequently, these miRNAs could be used as reliable non-invasive biomarkers for further research on male infertility (Hu *et al.,* 2014).

2. Aims of the PhD thesis

As summarized in the introduction, miRNAs play a crucial role in regulating gene expression during sperm production and maturation in various stages of spermatogenesis starting from primordial germ cells to mature spermatozoa and in other cell types like Sertoli and Leydig cells. In patients with certain type of male infertility, miRNAs may function as potential biomarker for the diagnosis, and the diagnostic accuracy of conventional routine tests might be improved by the identification of new miRNAs biomarker.

1. The first aim of this PhD thesis was to determine whether miRNAs are differentially expressed in males with normal versus impaired spermatogenesis, and to find a biomarker for accurate diagnosis of male infertility.

2. The second aim was to define and compare the expression profiles of miRNA in human testes showing different histopathological patterns.

3. The third aim was to validate a set of five preferentially expressed miRNAs in spermatogenesis as specific novel biomarkers for the assessment of male infertility.

3. Results

The thesis is a cumulative thesis based on the following three published papers:

In this thesis, we determined the feasibility of miRNA expression profiles as a biomarker for accurate diagnosis of male infertility for 27 couples that were undergoing ART. There were 50 miRNAs up-regulated and 27 miRNAs down-regulated in asthenozoospermic males, while 42 miRNAs were regulated and 44 miRNAs were down-regulated in oligoasthenozoospermic as compared with the normozoospermic fertile control males. From the analysis, two miRNAs exhibiting expression changes in both oligoasthenozoospermic and asthenozoospermic males compared to control fertile males (miR-429 and miR-1973). Specifically, two miRNAs indicated slight differences in the asthenozoospermic/normal (miR-1274a and miR-4286) comparison, and a miRNA demonstrated the greatest expression change in the oligoasthenozoospermic/normal comparison (miR-34b*). The alteration in miRNA expression patterns has been also associated with different human testicular histopathologic patterns. For instance, a total of 197, 68, and 46 miRNAs were found to be differentially expressed when comparing the samples from SCO, MA, and GA groups, respectively, with normal spermatogenesis. Interestingly, four miRNAs namely miR-34b*, miR-34b, miR-34c-5p and mR-449a were highly expressed among the three tested groups compared to normal. Subsequently, we validated a set of five miRNAs, namely miR-34b*, miR-34b, miR-34c-5p, miR- 429 and miR-122 with altered expression in spermatogenic and histopathologic impairment patients. These five miRNAs have the potential to be used as biomarkers to diagnose males with infertility. These results provided strong evidence for the analysis of miRNA profiles as a future diagnostic tool for male infertility. Taken together, this thesis elucidates the miRNA expression profiles in different spermatogenic and histopathologic impairments and demonstrates that hsa-miR-34b*, hsa-miR-34b, hsa-miR-34c-5p, hsa-miR-429, and hsa-miR-122 may be useful as non-invasive molecular markers for the diagnosis and assessment of male infertility.

3.1. Altered microRNA expression profiles of human spermatozoa in patients with different spermatogenic impairments

Altered microRNA expression profiles of human spermatozoa in patients with different spermatogenic impairments

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Objective: To determine whether microRNAs are differentially expressed in men with normal versus impaired spermatogenesis, and to find a biomarker for accurate diagnosis of male infertility.
Design: Microarray with real-time polymerase chain reaction (RT-PCR) validation.
Setting: University research and clinical institutes.
Patient(s): Male partner of selected couples (n = 27) who were undergoing assisted reproduction techniques for infertility treatment.
Intervention(s): None.
Main Outcome Measure(s): Statistically significantly altered microRNA expression profiles in normozoospermic versus asthenozoospermic and oligoasthenozoospermic men.
Result(s): There were 50 miRNAs up-regulated and 27 miRNAs down-regulated in asthenozoospermic males. In oligoasthenozoospermic males, 42 miRNAs were up-regulated and 44 miRNAs down-regulated when compared with normozoospermic males. The miRNAs that exhibited the highest fold changes and area under the receiver operating characteristic curve were miR-34b, miR-122, and miR-1973 in samples from asthenozoospermic men and miR-34b, miR-34b*, miR-15b, miR-34c-5p, miR-122, miR-449a, miR-1973, miR-16, and miR-19a in samples from oligoasthenozoospermic men. Furthermore, quantitative RT-PCR assays on specific miRNAs, including miR-141, miR-200a, miR-122, miR-34b, miR-34c-5p, and miR-16, yielded results that were largely consistent with the microarray data.
Conclusion(s): Our results reveal an extended number of miRNAs that were differentially expressed in asthenozoospermic and oligoasthenozoospermic males compared with normozoospermic males. These data provide evidence for analysis of miRNA profiles as a future diagnosing tool for male infertility. (Fertil Steril® 2013;99:1249–55. ©2013 by American Society for Reproductive Medicine.)
Key Words: MicroRNA, small noncoding RNA, spermatozoa, spermatogenesis, male infertility

Infertility is estimated to affect 15% of couples worldwide (1, 2). Male infertility is responsible for ~50% of these couples' inability to conceive and is the result of acquired and/or congenital abnormalities. Idiopathic male infertility occurs in ~60%–75% of all cases in patients without previous fertility problems and normal findings on physical examination (1). A significant proportion of idiopathic male infertility is accompanied by quantitative (azoospermia, cryptozoospermia, and oligoasthenozoospermia) and/or qualitative (asthenospermia, teratozoospermia, and necrospermia) abnormalities (3, 4).

Deregulation of microRNAs (miRNAs) is likely to play an essential role in these processes leading to male infertility. miRNAs are a family of short (20–23 nucleotides), single-stranded noncoding RNA molecules that are required for regulating posttranscriptional gene silencing through base-pair

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E.M. and C.B. contributed equally to this work.
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binding on their target mRNAs, thereby inducing translational inhibition or repression (5, 6). During the past decade, miRNAs have been found to perform a pivotal role in various biologic processes, including development (7), cell growth (8), and differentiation (9). Several studies report testicular-expressed miRNA changes depending on the stage of spermatogenesis (10, 11). Those studies, which analyzed 28 of the commonly expressed testicular miRNAs isolated from various testicular cell populations, i.e., Sertoli cells, spermatogonia, pachytene spermatocytes, round and elongating spermatids, and spermatozoa, suggest that late meiotic and haploid germ cells are the main source of miRNA production during spermatogenesis (12–14). Nevertheless, little is known about the role of miRNAs in male infertility. Recently, Lian et al. identified 154 differentially down-regulated and 19 up-regulated miRNAs between a nonobstructive azoospermia group and a control group (15). Wang et al. reported 19 miRNAs with altered expression in the seminal plasma and suggested 7 miRNAs that could be used for diagnosing male infertility (16). The identification of 68 small RNAs by microarray technology in human spermatozoa indicates that profiling of spermatozoa could be used to specifically identify male infertility (17). Amanai et al. and Yan et al. reported the expression of deregulated miRNAs in mice spermatozoa using microarray and quantitative real-time polymerase chain reaction (qRT-PCR) (18, 19). More recently, 17 miRNAs have been detected in porcine spermatozoa with the use of a multispecies miRNA microarray with qRT-PCR verification and sequencing (20). Finally, Krawetz et al. was the first to identify 35 known and novel miRNAs found in mature spermatozoa of three fertile donors with the use of next-generation sequencing technology (21). Thus, miRNAs play an important role in spermatogenesis as well as the fertilization process of oocyte, and they might even influence the phenotype of the offspring (18, 22, 23). In the present study, we set out to contribute to a better understanding of the miRNA expression patterns in men with normal and impaired spermatogenesis. Our study aimed to further the understanding of the molecular role of miRNAs in male infertility.

MATERIALS AND METHODS

Study Population and Sample Collection

Institutional Review Board approval (no. 195/11) was obtained before initiation of this study, and informed consent was obtained from each the participants included. Sperm samples were obtained from the male partner of selected couples undergoing assisted reproduction techniques for infertility treatment at the Department of Obstetrics and Gynaecology, Saarland University. The samples were obtained from the participants by masturbation after 3 days of sexual abstinence, allowed to liquefy at 37°C for 30 minutes, and then processed immediately. All semen samples were analyzed for the primary semen parameters, such as liquefaction time, volume, pH, viscosity, agglutination, motility, viability, sperm density, and morphology according to World Health Organization (WHO) guidelines (24). These parameters, when taken together, determined our three tested subgroups. The semen samples were then loaded on to 45%–90% discontinuous Puresperm gradients (Nidacon International) and centrifuged at 500*g* for 20 minutes at room temperature. The pellet was washed again twice with Ham-F10 medium supplemented with human serum albumin (5 mg/mL) and penicillin G/streptomycin sulphate (0.1 mg/mL; PAN Biotech) and carefully overlaid with 0.75 mL of the same medium. Samples were then placed in an incubator at 37°C, and after 45 minutes the upper layer (supernatant) was aspirated from the lower layer (pellet). The supernatant was immediately assessed for sperm concentration, motility, and residual somatic contaminants with the use of a Makler counting chamber (Irvine Scientific).

Isolation of Total RNA, Including miRNAs, from Sperm Samples

Total RNA, including miRNAs, was purified from supernatant spermatozoa with the use of the Qiagen miRNeasy Mini Kit with slight modifications. Briefly, 200 μL culture media (PAN Biotech) containing $\sim 2 \times 10^6$/mL sperm was homogenized in 700 μL Qiazol Lysis reagent (Qiagen) mixed with DTT (80 mmol/L) (Sigma) for 2 hours to ensure complete lysis of sperm. Thereafter, the procedure was completed according to manufacturer's recommendations. Quantity and quality of RNA were assessed with the use of the Nanodrop ND-2000c spectrophotometer (Thermo Scientific).

miRNA Microarray Assay Analysis

miRNA expression profiles of 27 different supernatant sperm samples were established by applying the Sureprint G3 Human v16 miRNA, 8 × 60K (release 16.0) microarray platforms (Agilent Technologies). All procedures were carried out according to the manufacturer's recommendations.

Reverse Transcription and Quantitative Real-Time PCR

Relative quantitative real-time PCR was performed to confirm the array results on an ABI Prism 7500 Fast Detection System (Applied Biosystems) using the miScript PCR System along with the 10× miScript Primer Assays for hsa-miR-141, hsa-miR-200a, hsa-miR-122, hsa-miR-34c-5p, hsa-miR-34b, and hsa-miR-16 (Qiagen). The RNU6B snRNA primer assay (Qiagen) was chosen as an endogenous reference for normalization. All procedures were carried out according to manufacturer's recommendations.

Statistical Analysis

For this study, we used the freely available R software (25) to analyze the differences of miRNA expression among the three tested groups. After applying the Agilent Feature Extraction image analysis software on our hybridized microarray slides, we collected the computed total gene signal (TGS) for each miRNA and performed quantile normalization and a log (base 2) transformation of the TGS values. Different levels of miRNAs were analyzed with the use of an unpaired two-tailed *t* test for miRNAs that showed >2-fold differential

expression in the considered groups. The computed *P* values for the *t* test were adjusted with the use of Benjamini-Hochberg false-discovery rate (FDR) correction (26). *P* values $<.05$ were considered to be statistically significant. In addition, we computed area under the receiver operating characteristic curve (AUC) values for each miRNA to assess its potential as a single biomarker. An AUC value close to 0 (or 1) indicates a high diagnostic value, because the expression values of one group are in this case mostly higher (or lower) than in the compared group. In contrast, an AUC value of 0.5 for a miRNA indicates equal distribution of the expression values in both groups and thus that the miRNA can not be used to separate the groups. For validating the microarray results, we performed qRT-PCR. We used the relative quantitative method of $2^{-\Delta\Delta Cq}$ (27) to measure the dynamic change of specific selected miRNAs.

RESULTS

Patient Data Analysis and Spermatogenic Potential

Twenty-seven semen samples were collected from fertile and subfertile men classified according to the WHO guidelines into normozoospermia (N; n = 9), asthenozoospermia (A; n = 9), and oligoasthenozoospermia (OA; n = 9). Demographic information is presented in Supplemental Table 1 (available online at www.fertstert.org) with accompanying summary statistics for the comparison between the groups. There was no overall difference in the mean age, volume, or pH among the groups. However, there was a considerable difference in motility and sperm morphology (*P* values $<.001$ and $<.017$, respectively) between A and N groups; in sperm count, motility, vitality (eosin), and sperm morphology (*P* values $<.001$, $<.001$, $<.001$, and $<.019$, respectively) between OA and N groups; and in sperm count, vitality (eosin,) and membrane integrity (hypo-osmotic swelling) (*P* values $<.001$, $<.034$, and $<.015$, respectively) between A and OA groups. Spermatozoa were purified by centrifugation through discontinuous Pure-sperm density gradient (90:45, vol./vol.) and subsequent swim-up to ensure complete absence of seminiferous epithelium cells, somatic cells, and/or leukocytes (28–33). The purity of the spermatozoa was examined with the use of an optical microscope equipped with ×100 oil objective and by PCR of argonaute-2/eukaryotic translation initiation factor 2C 2, which is known to be expressed in seminiferous epithelium cell types (pachytene spermatocytes, round spermatids, elongating spermatids, and Sertoli cells), somatic cells (34), and leukocytes (35) but not in spermatozoa. The spermatozoa samples were found to be free of somatic cells, round cells, and leukocytes as shown by microscopic examination and by gel electrophoresis (Supplemental Fig. 1, available online at www.fertstert.org). The spermatozoa samples were then used for miRNA microarray and qRT-PCR analysis.

Differentially Expressed miRNAs between Fertile and Subfertile Groups

Using the high-throughput Sureprint G3 Human v16 miRNA microarray platform, we analyzed the level of 1,205 human miRNAs annotated in miRBase version 16.0. In total, we screened the miRNA level in spermatozoa cells from 27 different individuals, including A, OA, and N subjects (Supplemental Tables 2–4, available online at www.fertstert.org). By applying an unpaired two-tailed *t* test for miRNAs that showed >2-fold change in the considered groups, we found 77 (6.39%) and 86 (7.14%) significant differences in miRNA levels when comparing the samples from group A with group N and the samples from group OA with group N, respectively ($P<.05$; FDR adjusted). No significant differences were found between groups OA and A. In detail, 50 miRNAs were up-regulated and 27 were down-regulated in the A/N comparison; 42 miRNAs were up-regulated and 44 were down-regulated in the OA/N comparison (Supplemental Fig. 2; Supplemental Tables 5 and 6; available online at www.fertstert.org; $P<.05$). There were 34 miRNAs shared by A/N and OA/N comparisons, including 27 up-regulated and 7 down-regulated miRNAs (Supplemental Fig. 3, available online at www.fertstert.org). The greatest fold changes detected in A/N and OA/N miRNA comparisons ($P<.05$) are summarized in Tables 1 and 2. Among these highest-fold-change miRNAs, we identified eleven miRNAs that were common between OA/N and A/N comparisons, namely, miR-141, miR-193b, miR-26a, miR-29a, miR-429, miR-200a, miR-99a, miR-363, miR-34b, miR-197, and miR-122; seven miRNAs that were only in the OA/N comparison, namely, miR-200c, miR-34b*, miR-15b, miR-34c-5p, miR-449a, miR-16, and miR-19a; and four miRNAs that were only in the A/N comparison, namely, miR-30a, miR-24, miR-1274a, and miR-4286.

Using hierarchic clustering with the euclidian distance measure, we analyzed how the A, OA, and N samples relate to each other. For this task, we used the 50 miRNAs with the highest variance of miRNA levels out of the 1,205 miRNAs. Figure 1 shows the resulting heatmap of the hierarchic clustering. In general, we observed two distinct clusters. The first cluster contains mostly normozoospermic samples and the second most of the subfertile samples. A more detailed distinction between the A and OA groups based on the clustering dendrogram was, however, not possible.

Validation of Microarray Results Using Real-Time PCR

For validation purposes, we performed qRT-PCR for 6 selected miRNAs, namely, miR-141, miR-200a, miR-122, miR-34c-5p, miR-34b, and miR-16 for the A/N and OA/N comparisons with the same semen samples that were used for microarray analysis (Supplemental Table 1; $P<.05$). All qRT-PCR experiments were performed in triplicate and the results (Cq values) related to RNU6B. Melting curve analysis and agarose gel electrophoresis were used to control for the specificity of qRT-PCR products. The qRT-PCR fold change results for the tested miRNAs were largely concordant with the microarray data ($P<.05$; Supplemental Table 7, available online at www.fertstert.org). In detail, no overall significant differences were found for any of the selected miRNAs for the OA/A comparison. We found a significant difference for the four selected miRNAs (miR-122, miR-34b, miR-141, and

TABLE 1

The greatest fold change in miRNAs in spermatozoa samples from oligoasthenozoospermic patients compared with those from normozoospermic control subjects as determined by microarray (*t* test: >2.0-fold difference and 5% false-discovery rate).

miRNA	*P* value	Corrected *P* value	AUC	Fold change	Regulation	Sequence	Chr
hsa-miR-141	.00018	.00199	0.07407	8.21370	Up	CCATCTTTACCAGACAG	chr12
hsa-miR-193b	.00149	.00610	0.12346	7.44771	Up	AGCGGGACTTTGAGGG	chr16
hsa-miR-26a	.01788	.03252	0.11111	6.67219	Up	AGCCTATCCTGGATT	chr3
hsa-miR-200c	.00629	.01499	0.11111	6.50708	Up	TCCATCATTACCCGG	chr12
hsa-miR-29a	.00488	.01305	0.12346	6.50708	Up	TAACCGATTTCAGATGGTGC	chr7
hsa-miR-429	.00059	.00383	0.09877	6.06763	Up	ACGGTTTTACCAGACAGTA	chr1
hsa-miR-200a	.00071	.00420	0.07407	5.91279	Up	ACATCGTTACCAGACAGT	chr1
hsa-miR-99a	.00322	.00962	0.13580	5.60164	Up	CACAAGATCGGATCTACGG	chr21
hsa-miR-363	.00100	.00470	0.08642	5.42084	Up	TACAGATGGATACCGTGCA	chrX
hsa-miR-34b*	.00000381	.00011	0.98765	34.30493	Down	CAATCAGCTAATGACACTGCCT	chr11
hsa-miR-15b	.00000347	.00011	1.00000	20.10457	Down	TGTAAACCATGATGTGCTGC	chr3
hsa-miR-34c-5p	.00001902	.00031	0.98765	20.04993	Down	GCAATCAGCTAACTACACTG	chr11
hsa-miR-34b	.00000043	.00004	1.00000	18.80819	Down	ATGGCAGTGGAGTTAGT	chr11
hsa-miR-449a	.00001717	.00031	0.97531	15.50551	Down	ACCAGCTAACAATACACTGC	chr5
hsa-miR-1973	.00003654	.00053	1.00000	12.55374	Down	TATGCTACCTTTGCACG	chr4
hsa-miR-122	.00000056	.00004	1.00000	12.35956	Down	CAAACACCATTGTCACACT	chr18
hsa-miR-16	.00023	.00214	0.93827	10.70660	Down	CGCCAATATTTACGTGCTG	chr3
hsa-miR-19a	.00036	.00299	0.92593	9.85754	Down	TCAGTTTTGCATAGATTTGCA	chr13

Note: AUC = area under the receiver operating characteristic curve.
* miRNA on the opposite arm of the hairpin during processing.

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miR-200a) for the A/N comparison and no significant difference in the miR-34c-5p for the OA/N comparison ($P<.05$; Supplemental Table 7). In addition, the expression of miR-122, miR-34c-5p, miR-34b, and miR-16 were down-regulated and the expression of miR-141 and miR-200a up-regulated in the OA/N comparison of qRT-PCR and microarray assays. Also, the expression of miR-122 and miR-34b were down-regulated and miR-141 and miR-200a expression were up-regulated in the A/N comparison of qRT-PCR and microarray assays (Fig. 2).

DISCUSSION

We investigated the differentially expressed miRNAs between asthenozoospermic and oligoasthenozoospermic subfertile men compared with normozoospermic fertile control men by microarray analysis and qRT-PCR. Recent studies have identified a number of miRNAs that are enriched in the mammalian testis (14, 36–38), mouse and porcine spermatozoa (14, 18, 20, 39), and human testis and spermatozoa (15, 17, 21, 40, 41) and seminal plasma (16). Several of the miRNAs identified in our analysis have also been addressed in those previous studies. However, we identified five novel miRNAs that have not been detected before and may play a role during spermatogenesis steps. Two miRNAs showed expression changes in both OA/N and A/N comparisons (miR-429 and miR-1973), two miRNA exhibited differences in the A/N (miR-1274a and miR-4286) comparison, and one miRNA showed the greatest expression change in OA/N

TABLE 2

The greatest fold change in miRNAs in spermatozoa samples from asthenozoospermic patients compared with those from normozoospermic control subjects as determined by microarray (*t* test: >2.0-fold difference and 5% false-discovery rate).

miRNA	*P* value	Corrected *P* value	AUC	Fold change	Regulation	Sequence	Chr
hsa-miR-30a	.02030	.04946	0.23457	8.82776	Up	CTTCCAGTCGAGGATG	chr6
hsa-miR-363	.00240	.01677	0.13580	8.34870	Up	TACAGATGGATACCGTGCA	chrX
hsa-miR-26a	.00242	.01677	0.09877	8.29168	Up	AGCCTATCCTGGATT	chr3
hsa-miR-200a	.00281	.01728	0.11111	8.19739	Up	ACATCGTTACCAGACAGT	chr1
hsa-miR-141	.00142	.01677	0.08642	7.85796	Up	CCATCTTTACCAGACAG	chr12
hsa-miR-429	.00108	.01677	0.04938	7.20231	Up	ACGGTTTTACCAGACAGTA	chr1
hsa-miR-193b	.00530	.02174	0.13580	7.02336	Up	AGCGGGACTTTGAGGG	chr16
hsa-miR-29a	.00154	.01677	0.11111	6.67581	Up	TAACCGATTTCAGATGGTGC	chr7
hsa-miR-1274a	.01429	.04035	0.20988	6.14306	Up	TGGCGCCTGAACAG	chr5
hsa-miR-24	.000060	.00519	0.03704	6.02507	Up	CTGTTCCTGCTGAACTGA	chr9
hsa-miR-4286	.01827	.04620	0.20988	4.48740	Up	GGTACCAGGAGTGGG	chr8
hsa-miR-99a	.00245	.01677	0.08642	4.15290	Up	CACAAGATCGGATCTACGG	chr21
hsa-miR-1973	.00912	.03050	0.76543	12.43053	Down	TATGCTACCTTTGCACG	chr4
hsa-miR-34b	.01342	.03980	0.77778	12.16602	Down	ATGGCAGTGGAGTTAGT	chr11
hsa-miR-122	.00863	.03002	0.79630	8.43115	Down	CAAACACCATTGTCACACT	chr18

Note: AUC = area under the receiver operating characteristic curve.

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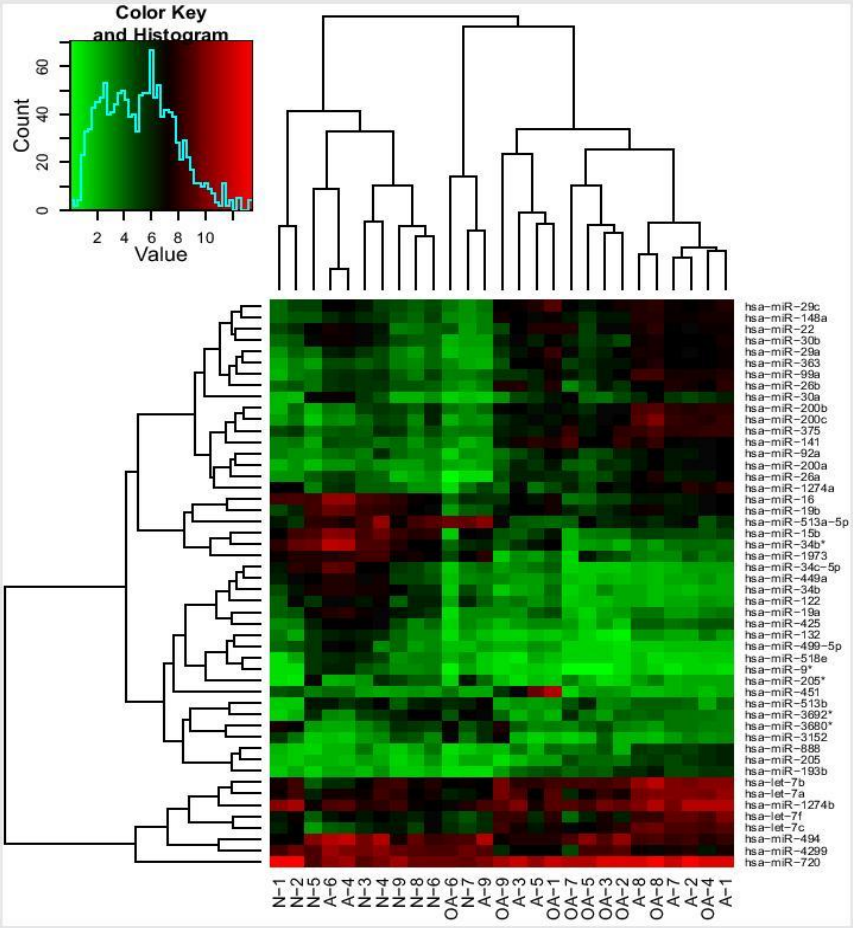
FIGURE 1



Clustering of 50 miRNAs with the highest variances. A = asthenozoospermic samples; OA = oligoasthenozoospermic samples; N = normozoospermic samples. Complete linkage hierarchic clustering was performed with the Euclidian distance measure. Subfertile patients and fertile healthy control subjects cluster separately with the exception of three samples (A-4, A-6, and N-7). The colors in the heatmap represent normalized values, with lower values being colored in shades of *green* and higher values in shades of *red*.

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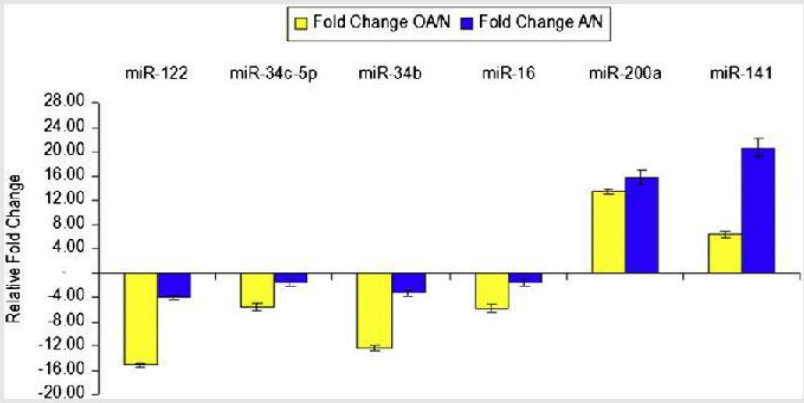
comparison (miR-34b*). We found that miR-122 was down-regulated in both OA and A groups compared with N group. Earlier studies show that miR-122 participates in the post-transcriptional down-regulation of transition protein 2 (TNP2) through targeting the 3′ untranslated region of TNP2 mRNA, which is synthesized only in round spermatids (38), suggesting that miR-122 plays an active and important function during testis development or spermatogenesis. MiR-34b is likewise down-regulated in both OA and A groups compared with N group and is significantly more highly expressed in adult than in prepuberal testis (42). Four of the miRNAs showing differences in the OA/N comparison, namely miR-34b, miR-15b, miR-16, and miR-34c were also identified to be differentially expressed in mouse testes with the use of conventional Northern blot analysis (12, 38). The putative target gene regulated by miR-34b and miR-34c is Notch gene homologue 1 (*NOTCH1*), which is highly expressed in mature rhesus monkey testes (19) and is requisite for differentiation and survival of germ cells in the rat testis (43). A high level of miR-34c was found in adult pachytene spermatocytes and round spermatids, and it is important to the first cell division via modulation of Bcl-2 expression, which is a direct target of miR-34c (12, 44). Expression level of miR-34c-5p was down-regulated in seminal plasma of azoospermic patients and increased in seminal plasma of asthenozoospermic patients, each compared with normozoospermic men (16). MiR-449a is predicted to target caspase-2 (45) and B-cell chronic lymphocytic leukemia/lymphoma-2 (*BCL2*) (46), both apoptosis-related genes; *NOTCH1*, a transcription-related gene (43); and inhibin beta B, a hormone-related

FIGURE 2



Verification of miRNA level by quantitative real-time polymerase chain reaction of six differentially expressed miRNAs in asthenozoospermia (A) and oligoasthenozoospermia (OA) versus normospermia (N) (>2.0-fold difference and 5% false-discovery rate). miRNAs are listed on the *x*-axis, and the *y*-axis refers to the relative expression levels.

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gene that is used as a potential marker for spermatogenesis and testicular function (47, 48). Bcl-2 plays a critical role in male germ cell development by mediating spermatogonial apoptosis, and its imbalance in transgenic animals affects spermatogenesis and subsequently causes male subfertility and infertility (19, 46). MiR-15b is predicted to target the mRNA coding region for isocitrate dehydrogenase 3 (NAD^+) alpha (*IDH3A*). Low expression of IDH3A disrupts sperm motility by altering sperm energy metabolism (39). The predicted target gene for miR-26a that showed significant expression in both OA/N and A/N comparisons is estrogen receptor 1, which is known to affect spermatogenesis (32, 49). Estrogen receptors were also associated with sperm motility and fertilizing ability (50, 51). In addition, miR-26a targets EH domain–containing 1, which is important for pre- and postnatal development and spermatogenesis (52). Moreover, miR-30a was highly up-regulated in the A/N comparison, but strongly down-regulated in nonobstructive azoospermia (15), suggesting that it plays an essential role in spermatogenesis.

Our microarray data also indicated altered miRNA levels of the Let-7 family in A and OA male. Similar alterations of the expression levels have been found in porcine testes, porcine sperm, and spermatogonial stem cell–enriched germ cell cultures (39, 53, 54). Let-7d and Let-7e were predicted to target high-mobility group AT-hook 2 (55), which is important to the spermatogenesis process (56). In general, Let-7 miRNAs were highly expressed in testis and are involved in spermatogenesis (41).

In conclusion, we demonstrated the validity of miRNA microarray and qRT-PCR analysis to identify miRNAs with altered levels in the spermatozoa of asthenozoospermic and oligoasthenozoospermic patients. The identified miRNAs that were shown in these groups compared with normozoospermic fertile control men will help us to better understand the mechanisms involved in spermatogenesis and may lay the groundwork for the development of novel biomarkers for male infertility.

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2.2. MicroRNA expression profiles in human testicular tissues of infertile males with different histopathological patterns

MicroRNA expression profiles in human testicular tissues of infertile men with different histopathologic patterns

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Objective: To investigate the expression profiles of microRNA (miRNA) in human testes showing different histopathological patterns.
Design: Microarray with quantitative real-time polymerase chain reaction (qRT-PCR) validation.
Setting: University research and clinical institutes.
Patient(s): Azoospermic men who underwent testicular biopsy for sperm recovery in preparation for intracytoplasmic sperm injection.
Intervention(s): Testicular biopsies.
Main Outcome Measure(s): Statistically significantly altered miRNA expression profiles among the testicular histopathologic patterns groups compared with normal pattern group.
Result(s): According to miRNA array, a total of 197, 68, and 46 miRNAs were found to be differentially expressed when comparing the samples from Sertoli cell only (SCO), mixed atrophy (MA), and germ cell arrest (GA) groups, respectively, with normal spermatogenesis (N). Five miRNAs have been validated using qRT-PCR, and the results were consistent with miRNA array analysis. Bioinformatics analysis showed that five microRNAs (hsa-mir-34b*, hsa-mir-34b, hsa-mir-34c-5p, hsa-mir-449a, and hsa-mir-449b*) were involved in apoptosis, cell proliferation, and differentiation. Notably, potential target genes of these five miRNAs were involved in the spermatogenesis process.
Conclusion(s): This study provides new insights into specific miRNAs that are expressed in infertile men with different histopathologic patterns, suggesting a role of miRNAs in regulating male germ and somatic cells and that their alteration is associated with reproductive abnormalities. (Fertil Steril® 2014;101:78–86. ©2014 by American Society for Reproductive Medicine.)
Key Words: MicroRNA, small noncoding RNA, testis, nonobstructive azoospermia, male infertility

Spermatogenesis is a complex and strictly regulated developmental process in which mitosis, meiosis, and differentiation interact to coordinate the development of a haploid gamete for sexual reproduction. This process involves many unique genes (1), the expressions of which are partially coordinated via microRNA (miRNA) suppression (2). MiRNAs are a family of short (20–23 nucleotides), single-stranded noncoding RNA molecules that are required for regulating post-transcriptional gene silencing through a base pair binding on their target mRNAs, thereby inducing translational inhibition or repression (3–5). They are highly conserved among species, and play a key role in diverse biologic processes, including development, cell proliferation, differentiation, and apoptosis. Accordingly, altered miRNA expression and mutation in their recognition sites are likely to contribute to human diseases, including spermatogenic failure (6, 7). In spermatogenesis, miRNAs have a significant impact on the development of spermatozoa, particularly in germ

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cells and somatic cells (2). However, it is conceivable that any deregulation in miRNA expression patterns significantly affects spermatogenesis pathways and leads to several types of reproduction abnormalities (8, 9).

Male-factor infertility is estimated to affect more than one-half of all couples' inability to conceive and it is a result of acquired and/or congenital abnormalities. Of these, men with azoospermia compose 15%–20% (10–14). Azoospermia is primarily classified into two major types, either abnormal sperm production (nonobstructive azoospermia [NOA]) or normal sperm production in the presence of obstruction (obstructive azoospermia [OA]). Genetic abnormalities contribute to a fair enough percentage of male infertility, in ~15% of cases either as chromosomal abnormalities or as genetic translations of the Yq chromosome (11, 15–20). It is thought, however, that a substantial part of the remaining unexplained cases also have a genetic etiology. The testicular histopathologic patterns in men with NOA vary from atrophic and hyalinized seminiferous tubules with only Sertoli cells and/or tubules with complete or incomplete spermatogenesis and markedly reduced spermatogenesis suggesting mixed atrophy, through germ cell arrest at a particular stage, most often at the spermatogonial or primary spermatocyte stage and in rare case at spermatid stage, to total absence of germ cells or their products, called Sertoli cell only (21, 22). Recent studies have begun to show that miRNAs apparently are less affected, well preserved in formalin-fixed paraffin-embedded (FFPE) tissue, and recovered more easily in the extraction process, and therefore they can be safely used for miRNA profiling, quantitative real-time polymerase chain reaction (qRT-PCR), and deep sequencing (23–35). In addition, different formalin fixation times do not change the stability of miRNA (35).

With considering the important role of miRNAs in spermatogenesis, the present study was aimed at identifying and characterizing the expression profile of miRNAs in different histopathologic patterns (the most common feature of male-factor infertility) and investigating their molecular role in the spermatogenesis process and male infertility. For this purpose, we applied microarray technology to detect the differently expressed miRNAs in testis by comparing the different histopathologic patterns with normal testicular patterns. The miRNA expression data allowed us to perform a bioinformatics investigation to predict putative targets for the miRNAs expressed in these patterns to gain further knowledge of the role of the miRNAs and their targets in spermatogenesis.

MATERIALS AND METHODS

Tissue Collection and Processing

Archival formalin-fixed paraffin-embedded (FFPE) tissue samples were obtained from 56 azoospermic men, aged 21–35 (mean 25.7 ± 3.6) years, who underwent testicular biopsy. All of these samples were taken initially for diagnostic investigation and/or in connection with intracytoplasmic sperm injection treatment. The diagnosis of each sample was confirmed by histologic examination with supplemental immunohistochemical staining (data not shown). Patient samples with known medical reason for their infertility including genetic abnormalities of chromosomal abnormalities and Y chromosome microdeletions were excluded from the study. Institutional Review Board approval (no. 195/11) was obtained before initiation of this study, and informed consent was provided according to the Declaration of Helsinki.

The samples were classified into four groups according to pathologic alterations of spermatogenesis: Sertoli cell only (SCO; n = 12); mixed atrophy (MA; n = 12), germ cell arrest at spermatocyte stage (GA; n = 16), and normal spermatogenesis samples with normal structure of seminiferous tubules as well as the interstitial compartment of the testis (N; n = 16). All samples were processed and analyzed in the Institute of Human Genetics of the Saarland University. Samples were analyzed according to standard operating procedures.

Isolation of Total RNA including microRNA

Twenty-micrometer sections from each sample were cut with a microtome and processed immediately. To prevent carry-over with contaminating RNA, a fresh blade was used for each sample, microtome cutting surface and forceps were cleansed with the RNaseZap solution (Ambion) to remove any potential nuclease contamination. Cut sections were placed in 1.5-mL tubes for extraction. Thereafter, total RNA, including miRNA was extracted with the use of FFPE miRNeasy kit on a QIAcube robot (Qiagen) according to the manufacturer's instructions, and to exclude DNA contamination the tissue lysate was treated with DNase before RNA isolation. The concentration and purity of RNA samples were determined with the use of a Nanodrop ND-2000 spectrophotometer (Thermo Scientific), and its quality was verified with the use of Agilent 2100 Bioanalyzer Eukaryote Total RNA Pico assay (Agilent Technologies).

MicroRNA Microarray Assay Analysis

MicroRNA expression profiles of 48 different testicular tissue samples (12 samples from each group) were established by applying the Sureprint G3 Human v16 miRNA 8×60K (release 16.0) microarrays (Agilent Technologies). These microarrays contain ~40 replicates for each probe complement to each of the 1,205 mature miRNAs of miRBase v16. These probes act in concert to measure the miRNA of interest, and the data are combined later during software analysis. All probes are randomly distributed on the array, and cross-hybridization is prevented by the addition of a G residue and a hairpin at the 5′ end of the probe. All procedures were carried out according to the manufacturer's recommendations. Briefly, 100 ng input RNA from each sample was dephosphorylated by incubation with calf intestinal phosphatase at 37°C for 30 minutes and denatured with the use of 100% dimethyl sulfoxide at 100°C for 5 minutes. Samples were labeled with pCp-Cy3 with the use of T4 ligase at 16°C incubation for 2 hours. Each labeled RNA sample was hybridized onto an individual subarray of the 8×60K format Agilent miRNA microarray slide, with each array containing probes for 1,205 human miRNAs according to miRBase v16. Hybridizations were performed in Surehyb chambers (Agilent

Technologies) for 20 hours at 55°C with 200 rpm rotation. Arrays were washed and dried according to the manufacturer's recommendations and scanned at a resolution of 3 μm double pass with the use of an Agilent G2565BA scanner. Data were acquired with the use of Agilent AGW Feature Extraction software version 10.10.11 (Agilent Technologies).

Reverse Transcription and Quantitative Real-Time qRT-PCR

Relative qRT-PCR was performed to confirm the array results on a Steponeplus Real-Time PCR System (Applied Biosystems) with the use of miScript PCR System along with the 10× miScript Primer Assays for hsa-miR-34b*, hsa-miR-34b, hsa-miR-34c-5p, hsa-miR-449a, and has-miR-10b (Qiagen). During reverse transcription of RNA with the use of Highspec Buffer, that ensures selectively conversion of small RNAs into complementary (c) DNA. The mature miRNAs are polyadenylated by RNA-specific poly(A) polymerase and reverse transcribed into cDNA using oligo-dT primers that have a 3′ degenerate anchor and a universal tag sequence on the 5′ end, allowing amplification of mature miRNA in the RT-PCR step. The combination of polyadenylation and the universal tag addition ensures that miScript Primer Assays do not detect genomic DNA, because the binding sequence for the universal primer will be introduced during the qRT-PCR. The Poly(A) polymerase is RNA specific, so no universal tag is added to DNA fragments. Each PCR reaction contained 2 μL cDNA, 10 μL 2× Quantitect Sybr Green PCR Master Mix, 2 μL 10× miScript Universal Primer, 2 μL 10× miScript Primer Assay, and RNase-free water to a total volume of 20 μL and was placed into an individual well of a 96-well plate. Reactions were run with the following thermal cycling parameters: initial activation step 95°C for 15 minutes followed by 40 cycles at 94°C for 15 seconds (denaturation), 55°C for 30 seconds (annealing), and 70°C for 30 seconds (extension). Then final dissociation curves (melting curves) were made. The RNU6B snRNA primer assay (Qiagen) was chosen as an endogenous reference for normalization.

Statistical Analysis

In this study, we used the freely available R software (36) to analyze the differences of miRNA expression among the four tested groups. The intensity values were extracted with the use of Agilent Feature Extraction image analysis software. To compute the total expression value per miRNA and sample, we summed up the gTotalProbeSignals. Quantile normalization was applied to normalize expression values across the arrays with the use of the preprocess Core package of the R programming language. After that, the expression values were log (base 2) transformed. Different levels of miRNAs were analyzed with the use of an unpaired two-tailed *t* test for miRNAs that showed >2-fold differential expression in the considered groups. The computed *P* values for the *t* test were adjusted with the use of Benjamini-Hochberg false-discovery rate (FDR) correction (37). *P* values <.05 were considered to be statistically significant. In addition, we computed area under the receiver operating characteristic curve (AUC) values for each miRNA to investigate the molecular causes and cellular effects of infertility. For validating the microarray results, we performed qRT-PCR. We used the relative quantitative method of $2^{-\Delta\Delta Cq}$ (38) to measure the dynamic change of specific selected miRNAs.

RESULTS

Total RNA Purity and Yield

Total RNA purity and yields were assessed to ensure that the material obtained was of sufficient quality and quantity to be labeled and hybridized for miRNA profiling analysis and subsequently for qRT-PCR validation analysis. After the extraction of total RNA including miRNA from the FFPE samples with the use of FFPE miRNeasy Kits on a Qiacube robot, the 260–280 nm absorbance ratio (260/280) was ≥1.80. Furthermore, the averaged RNA yield obtained from 20-μm-thick sections ranged from 1.9 μg to 2.37 μg (Supplemental Table 1; Supplemental Tables 1–7 are available online at www.fertstert.org). In addition, the total RNA quality was assayed with the use of the Agilent 2100 Bioanalyzer Eukaryote Total RNA Pico assay. The FFPE samples consistently had RNA integrity numbers (RINs) of ∼2.6, suggesting that the samples were degraded, as expected for this type of sample.

Identifying Differentially Expressed miRNAs

Using the high-throughput Sureprint G3 Human v16 miRNA microarray platform, we analyzed the level of 1,205 human miRNAs annotated in miRBase v16. In total, we screened the miRNA expression levels in FFPE testicular tissues from 48 different individuals, including SCO, MA, GA, and N subjects, 12 from each group. By applying an unpaired two-tailed *t* test, we found 197 (16.35%), 68 (5.64%), and 46 (3.82%) significant differences in miRNA levels when comparing the samples from groups SCO, MA, and GA, respectively, with group N (Supplemental Tables 2–4; $P<.05$). In detail, 66 miRNAs were down-regulated and 131 up-regulated in the SCO-N comparison, 33 miRNAs were down-regulated and 35 up-regulated in the MA-N comparison, and 19 miRNAs were down-regulated and 27 miRNAs up-regulated in the GA-N comparison. Furthermore, 23, 36, and 7 miRNAs were shared by SCO-N and MA-N, SCO-N and GA-N, and MA-N and GA-N comparisons, respectively (Supplemental Fig. 1; Supplemental Figs. 1 and 2 available online at www.fertstert.org). The greatest fold changes detected in SCO-N, MA-N, and GA-N miRNA comparisons are summarized in Table 1. Among these highest fold change miRNAs, there were only 7 miRNAs shared among the tested groups, all of them down-regulated, including hsa-miR-34b*, hsa-miR-34c-5p, hsa-miR-449a, hsa-miR-34b, hsa-miR-449b*, hsa-miR-517b, and hsa-miR-129-3p (Supplemental Table 5). Of particular note is that several members of the oncogenic miR-17-92 family have been identified only in SCO-N comparison, with high fold change for hsa-miR-18a, hsa-miR-19a, and hsa-miR-92a which are located on chromosome 13q31.3 and hsa-miR-25 which is located on chromosome 7. Furthermore, the oncogenic cluster miR-371–373 linked to testicular cancer was also identified: hsa-miR-371-5p and hsa-miR-373* were

TABLE 1

The greatest fold changes in miRNAs with the use of human testicular samples from Sertoli cell only, mixed atrophy, and germ cell arrest compared with normal control as determined by microarray (*t* test; > 2.0-fold difference and 5% false discovery rate).

miRNA	Active sequence	Chr	Start	Stop	Strand	Fold change	Corrected *P* value	AUC	Regulation
Sertoli cell only miRNAs compared with normal									
hsa-miR-34b*	CAATCAGCTAATGACACTGCCT	chr11	110888886	110888907	–	91.222	0.00236	0.896	Down
hsa-miR-34c-5p	GCAATCAGCTAACTACACTG	chr11	110889389	110889408	–	73.749	0.00035	0.951	Down
hsa-miR-449a	ACCAGCTAACAATACACTGC	chr5	54466396	54466377	+	33.977	0.00238	0.903	Down
hsa-miR-574–5p	ACACACTCACACACACAC	chr4	38869683	38869699	–	29.112	0.03456	0.750	Down
hsa-miR-15b	TGTAAACCATGATGTGCTGC	chr3	160122398	160122416	–	22.964	0.03038	0.847	Down
hsa-miR-125b	TCACAAGTTAGGGTCTC	chr11	121970500	121970484	+	15.419	0.01260	0.844	Down
hsa-miR-125a-5p	TCACAGGTTAAAGGGTCTC	chr19	52196527	52196544	–	15.012	0.00951	0.882	Down
hsa-miR-16	CGCCAATATTTACGTGCTG	chr3	160122545	160122563	–	14.960	0.02284	0.830	Down
hsa-miR-204	AGGCATAGGATGACAAAGGG	chr9	73424944	73424926	+	14.471	0.01349	0.840	Down
hsa-miR-1260	TGGTGGCAGAGGTGG	chr14	77732578	77732591	–	12.843	0.00624	0.889	Down
hsa-miR-23a	GGAAATCCCTGGCAATGT	chr19	13947465	13947449	+	12.610	0.03477	0.778	Down
hsa-miR-145	AGGGATTCCTGGGAAAAC	chr5	148810230	148810246	–	12.113	0.01057	0.875	Down
hsa-miR-1260b	ATGGTGGCAGTGGTG	chr11	96074616	96074629	–	11.231	0.00472	0.885	Down
hsa-miR-30b	TGGCGCCTGAACAG	chr5	41475752	41475764	–	10.464	0.03564	0.785	Down
hsa-miR-25	ATGGCAGTGGAGTTAGT	chr11	111383718	111383733	–	9.629	0.02201	0.830	Down
hsa-miR-1274a	TGTGGGTGTGTGCATG	chr4	38869720	38869734	–	9.503	0.00766	0.910	Down
hsa-miR-22	ACAGTTCTTCAACTGGCAG	chr17	1617270	1617253	+	8.929	0.02567	0.750	Down
hsa-miR-34b	ATGGCAGTGGAGTTAGT	chr11	111383718	111383733	–	8.868	0.00035	0.951	Down
hsa-miR-19a	TCAGTTTTGCATAGATTTGCA	chr13	92003196	92003215	–	8.406	0.03564	0.792	Down
hsa-miR-574–3p	TGTGGGTGTGTGCATG	chr4	38869720	38869734	–	8.262	0.00408	0.889	Down
hsa-miR-92a	ACAGGCCGGGACAAGT	chr13	92003621	92003636	–	8.119	0.01699	0.764	Down
hsa-miR-3925	AGGCTCCACTTTCAGT	chr6	36590245	36590231	+	15.426	0.01152	0.108	Up
hsa-miR-135a*	CGCCACGGCTCCA	chr3	52328311	52328300	+	14.493	0.01102	0.132	Up
hsa-miR-1471	ACACCTGGCTCCACA	chr2	232756973	232756960	+	13.266	0.01260	0.139	Up
hsa-miR-642b	GGGTCCCTCTCCAA	chr19	46178257	46178245	+	11.653	0.01238	0.153	Up
hsa-miR-617	GCCACCTTCAAATGGGA	chr12	81226348	81226333	+	11.480	0.01057	0.104	Up
hsa-miR-3180–3p	GGCCTCCGGAAGC	chr16	15005147	15005159	–	11.196	0.02201	0.146	Up
hsa-miR-718	CGACGCCCGGC	chrX	153285432	153285423	+	11.116	0.01172	0.139	Up
hsa-miR-3200–5p	ACCTTGTGCGCCTTC	chr22	31127564	31127577	–	11.024	0.01187	0.167	Up
hsa-miR-99b*	CGGACCCACAGAC	chr19	52195919	52195930	–	11.002	0.01738	0.167	Up
hsa-miR-3945	ATATCAACCCTCTCCTATGC	chr4	185772211	185772193	+	10.474	0.00236	0.097	Up
hsa-miR-3648	CCCTCGGCGATCC	chr21	9825868	9825879	–	9.975	0.01699	0.153	Up
hsa-miR-575	GCTCCTGTCCAACTGGCT	chr4	83674568	83674552	+	9.972	0.02925	0.167	Up
hsa-miR-936	CTGCGATTCCTCCCT	chr10	105807882	105807869	+	9.166	0.01260	0.174	Up
hsa-miR-3137	ACCCCATTGCTCCCA	chr3	194855264	194855251	+	9.060	0.02042	0.177	Up
hsa-miR-548q	CCGCCATTACTTTTGC	chr10	12767281	12767267	+	8.990	0.01152	0.122	Up
hsa-miR-4322	CCCCACGCGCTG	chr19	10341141	10341151	–	8.970	0.02297	0.160	Up
hsa-miR-1181	CGGCTCGGGTGG	chr19	10514167	10514157	+	8.944	0.01699	0.146	Up
hsa-miR-125a-3p	GGCTCCCAAGAACCTCA	chr19	52196565	52196580	–	8.705	0.01877	0.170	Up
hsa-miR-371–5p	AGTGCCCCCACAG	chr19	54290942	54290953	–	8.661	0.02196	0.167	Up
hsa-miR-373*	GGAAAGCGCCCCC	chr19	54291974	54291985	–	8.440	0.01156	0.153	Up
hsa-miR-3197	CGCCTTTCCGAGC	chr21	42539502	42539513	–	8.105	0.00872	0.125	Up
hsa-miR-3656	CCACCCCCGCAC	chr11	118889708	118889718	–	8.095	0.00624	0.160	Up
hsa-miR-3194	CAGCCCTCCTGGTG	chr20	50069471	50069459	+	8.085	0.02515	0.163	Up
Mixed atrophy miRNAs compared with normal									
hsa-miR-34c-5p	GCAATCAGCTAACTACACTG	chr11	110889389	110889408	–	70.397	0.00017	0.993	Down
hsa-miR-34b*	CAATCAGCTAATGACACTGCCT	chr11	110888886	110888907	–	58.727	0.00017	0.951	Down
hsa-miR-449a	ACCAGCTAACAATACACTGC	chr5	54466396	54466377	+	32.386	0.00017	0.965	Down
hsa-miR-509–5p	TGATTGCCACTGTCTGC	chrX	146340317	146340301	+	7.227	0.02168	0.819	Down
hsa-miR-514	TCTACTCACAGAAGTGTC	chrX	146360843	146360826	+	7.137	0.00824	0.854	Down
hsa-miR-34b	ATGGCAGTGGAGTTAGT	chr11	111383718	111383733	–	6.338	0.00017	0.938	Down
hsa-miR-517a	ACACTCTAAAGGGATGCAC	chr19	54215579	54215596	–	5.292	0.00104	0.931	Down
hsa-miR-506	TCTACTCAGAAGGGTGC	chrX	146312328	146312313	+	5.101	0.02614	0.819	Down
hsa-miR-514b-5p	ATGATTGCCTCCCTCTT	chrX	146331702	146331687	+	4.645	0.00112	0.896	Down
hsa-miR-129–3p	ATGCTTTTTGGGGTAAGGG	chr11	43603004	43603021	–	4.467	0.00041	0.941	Down
hsa-miR-127–3p	AGCCAAGCTCAGACGGAT	chr14	100419129	100419146	–	5.345	0.00044	0.069	Up
hsa-miR-410	ACAGGCCATCTGTGTTATA	chr14	101532301	101532318	–	4.141	0.00480	0.132	Up
hsa-miR-199a-5p	GAACAGGTAGTCTGAACAC	chr1	170380350	170380332	+	4.117	0.00164	0.090	Up
hsa-miR-379	CCTACGTTCCATAGTC	chr14	101488414	101488428	–	4.009	0.00205	0.139	Up
Germ cell arrest miRNAs compared with normal									
hsa-miR-449a	ACCAGCTAACAATACACTGC	chr5	54466396	54466377	+	19.444	0.03251	0.778	Down
hsa-miR-34b*	CAATCAGCTAATGACACTGCCT	chr11	110888886	110888907	–	15.798	0.01128	0.854	Down
hsa-miR-34c-5p	GCAATCAGCTAACTACACTG	chr11	110889389	110889408	–	15.423	0.01297	0.833	Down
hsa-miR-34b	ATGGCAGTGGAGTTAGT	chr11	111383718	111383733	–	6.350	0.00089	0.924	Down

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TABLE 1

Continued.

miRNA	Active sequence	Chr	Start	Stop	Strand	Fold change	Corrected *P* value	AUC	Regulation
hsa-miR-449b*	AGTGGCAGGGTAGTTG	chr5	54466549	54466535	+	3.971	0.00013	0.958	Down
hsa-miR-135a*	CGCCACGGCTCCA	chr3	52328311	52328300	+	3.398	0.02889	0.194	Up
hsa-miR-3137	ACCCCATTGCTCCCA	chr3	194855264	194855251	+	3.122	0.04644	0.240	Up
hsa-miR-99b*	CGGACCCACAGAC	chr19	52195919	52195930	–	3.070	0.02889	0.201	Up
hsa-miR-3692*	CAGTATCCACTCCTGAC	chr6	157950177	157950192	–	3.030	0.02889	0.177	Up

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differentially up-regulated in SCO-N, hsa-miR-372 differentially down-regulated in MA-N, and hsa-miR-373* differentially up-regulated in GA-N comparisons. A ~2-fold change of the expression level of the testis/gonad-specific miRNA hsa-miR-202 was detected for the SCO-N and MA-N comparisons, but a <2-fold change for GA-N. Other clusters of miRNAs were highly expressed in the MA-N comparison, e.g., the hsa-miR-1283 and hsa-miR-506 cluster, comprising eight and seven miRNAs, respectively (Supplemental Fig. 2a). Six of these miRNAs are testis-specific miRNAs (hsa-miR-506, hsa-miR-507, hsa-miR-508, hsa-miR-509, hsa-miR-513, and hsa-miR-514). In addition, one miRNA cluster (hsa-miR-1283) was found in SCO-N comparison (Supplemental Fig. 2b), and only two miRNAs were found in the same cluster in GA-N comparison (Supplemental Fig. 2c). Six miRNAs of the miR-30 family miRNAs were identified in SCO-N comparison, namely hsa-miR-30b, hsa-miR-30c, hsa-miR-30a, hsa-miR-30a*, hsa-miR-30e*, and hsa-miR-30c-1*, and one miRNA, namely hsa-miR-30d, was identified in MA-N comparison.

Through this miRNA microarray analysis, we identified also several novel miRNAs that have not been detected before in testicular tissue and may play a role during spermatogenesis steps. Some of these miRNAs showed varying levels of expression in one or all tested testicular samples (Supplemental Tables 2–4; $P<.05$). The heat map shows two main clusters, the first containing samples from each group that show a very different expression pattern than the remaining samples and may represent outliers in this study. The second main cluster subdivides into smaller clusters that show a strong coclustering of the MA samples and a weaker coclustering of the normal and GA samples (Supplemental Fig. 2d).

miRNA Annotation, Functional Classification, and Target Prediction

We used the TAM tool (39) to annotate the five miRNAs that showed high fold change and AUC values according to function association (Supplemental Table 6; $P<.05$ [adjusted for false discovery rate]). These miRNAs (hsa-mir-34b*, hsa-mir-34b, hsa-mir-34c-5p, hsa-mir-449a, and hsa-mir-449b*) were classified to belong to the apoptosis-, cell proliferation–, and cell cycle–related miRNAs. In addition, to obtain the most complete list of targets for these five miRNAs as well as to limit the number of false positives, we took advantage of the feature in the miRWalk database (www.ma.uni-heidelberg.de/apps/zmf/mirwalk/) that allows simultaneous searches of several databases (40). We interrogated the following ten databases; DIANA-mT, miRanda, miRDB, miRWalk, RNAhybrid, PICTAR4, PICTAR5, PITA, RNA22, and TargetScan. As expected, most of these miRNAs predicted targeted hundreds of targeted genes, and >30% of the targets were regulated by more than one miRNA. Besides, it is very difficult to find miRNA targets that are specific to tissues or cell lines, because these programs predict a vast number of different targets for one given miRNA. When five or more of these programs coidentified a specific transcript, the target(s) were selected for our list of potential targets (Supplemental Table 7; $P<.05$).

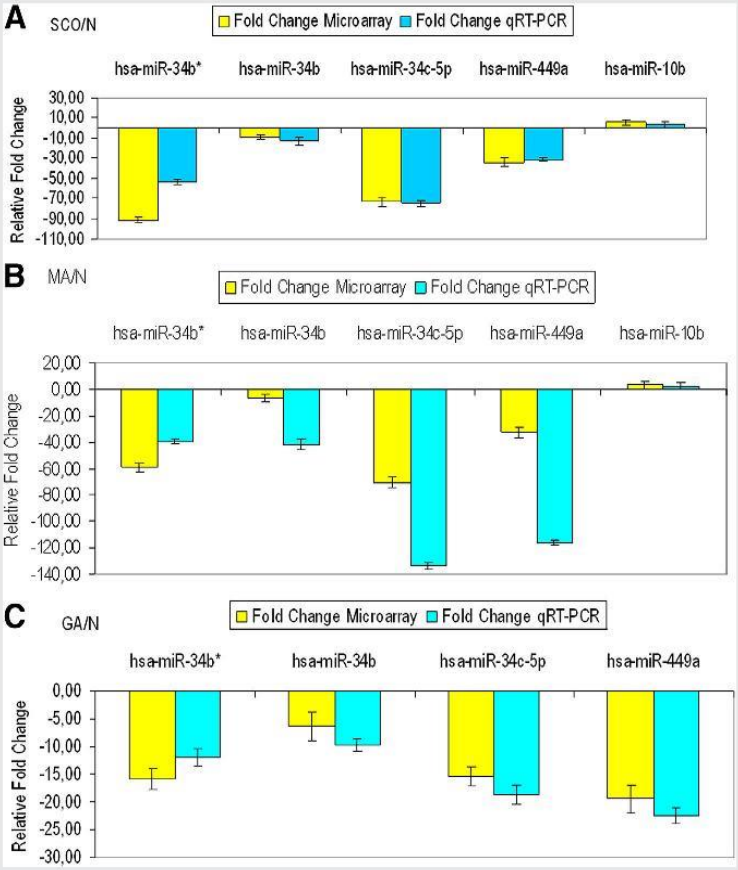
qRT-PCR Validation of the miRNA Microarray Results

For validation purposes of microarray analysis, qRT-PCR was used to confirm and validate the results of the miRNA microarray analysis with the use of SCO (n = 12); MA (n = 12), GA (n = 16), and N (n = 16). Four miRNAs, namely hsa-miR-34b*, hsa-miR-34b, hsa-miR-34c-5p, and hsa-miR-449a, were identified by the microarray as being the most down-regulated in SCO, GA, and MA men compared with N, and only one miRNA, namely hsa-miR-10b, was up-regulated in SCO and MA. A quantitative real-time PCR was performed in triplicate for these five miRNAs with the use of an ABI Steponeplus Real-Time PCR System (Applied Biosystems), and the results (Cq values) were related to RNU6B endogenous control. Melting curve analysis and agarose gel electrophoresis were used to control for the specificity of qRT-PCR products. Our results showed a high level of concordance between microarray and qRT-PCR results for the five tested miRNAs for the three comparisons SCOS-N, MA-N, GA-N (Fig. 1).

DISCUSSION

The aim of this study was to analyze the miRNA expression profile in testicular tissue of infertile men with different histopathologic spermatogenic patterns. Earlier studies have mainly focused on miRNA signatures in connection with the miRNA expression signatures in whole testicular cells or in partially purified germ cells (41–45). Despite the well known importance of miRNAs in post-transcriptional control of various developmental and cellular processes including spermatogenesis (2, 46), no studies had identified and compared the miRNA expression profiles of infertile men

FIGURE 1



Verification of miRNA level by quantitative real-time qRT-PCR of 5 differentially expressed microRNAs. (A) Sertoli cell only syndrome (SCO; n = 12); (B) mixed atrophy (MA; n = 12), (C) germ cell arrest (GA; n = 16); validated microRNAs. MicroRNAs are listed on the *x*-axis, and the *y*-axis refers to the relative expression levels ≥2.0-fold difference and 5% false-discovery rate.

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diagnosed with SCO, MA, and GA. Studies on whole testes clearly demonstrated that testicular expression of miRNA molecules changed with the stage of spermatogenesis (41, 44, 47–49). Those studies have suggested that late meiotic and haploid germ cells are the main source of miRNA production during spermatogenesis (8, 43, 50). Expression profiles of miRNAs in human seminal plasma, testicular tissue, and purified spermatozoa have been obtained by either deep sequencing or microarray strategies, and the differentially expressed miRNAs have subsequently been validated by qRT-PCR (7, 51–56). Taken together, earlier studies clearly show that miRNAs play a role in the development and/or regulation of spermatogenesis and that alteration of their expression contributes to reproduction abnormalities. In addition, deregulated miRNAs may be used as biomarkers for male infertility diagnosis.

In the present study, we identified miRNAs that are either specifically expressed in a particular histopathologic pattern compared with normal pattern or equally expressed in two or all three different histopathologic patterns. miRNAs, including miR-449 family (miR-449a, miR-449b*) and miR-34 family (miR-34b*, miR-34b, miR-34c-5p), that were differentially expressed in our study are preferentially expressed in the testis (8, 43, 47, 48, 57). We found these miRNAs to be strongly down-regulated in the SCO, MA, and GA groups compared with the normal group. These miRNAs were also reported to be down-regulated in subfertile oligozoospermic, asthenozoospermic, and oligoasthenozoospermic men (51, 56). The unique miRNA signatures in each histopathologic pattern might be responsible for observed differences in miRNA expression among SCO, MA, and GA groups compared with normal. As for the differentially expressed miR-449 family, miR-449 was significantly up-regulated in spermatogonia, spermatocytes, and spermatids (8, 57). We found this miRNA to be strongly down-regulated in SCO, and to a lesser extent in MA and GA, compared with the normal control group.

The expression of miR-449a and miR-449b is positively regulated by the E2F transcription factor 1 (E2F1), which

promotes cell cycle progression and induces apoptosis in damaged cells. Overexpression of E2F1 leads to increased apoptosis of spermatocytes (58, 59). Depletion of E2F1 causes highly reduced spermatogonial proliferation and marked testicular atrophy (60). Because miR449a/b promotes apoptosis in a p53-independent manner in tumor cell lines (46, 61–63), the deregulation of these miRNAs observed in patients with spermatogenic impairments may contribute to increased apoptosis in the gonadal tissue (7, 64, 65).

Another conserved miRNA family, miR-34, which has been demonstrated to be structurally similar to the miR-449 members, has previously been shown to be highly enriched and specifically expressed in germ cells (8, 66). miR-34b was found to be deregulated from day 7 through day 14 in mouse and in mature rhesus monkey testes and to be involved in the cellular senescence, apoptosis, and control of the cell cycle (41, 44, 67–69). miR-34b* was found to be down-regulated in patients with NOA and in subfertile men with oligoasthenozoospermia (7, 51). Likely targets of miR-34b and miR-34c include dazl (deleted in azoospermia–like) gene, which is involved germ cell differentiation in mice (46).

The alteration of miRNA expressions in testicular histopathologic patterns suggests an important role for miRNAs in male reproductive function. The high expression of both miRNA families miR-449 and miR-34 in SCO, MA, and to a lesser extent in GA is possibly due to similar expression profiles of miR-449 and miR-34b/c during testicular development and in adult spermatogenesis. Notably, both miRNA families are localized in the same spermatogenic cell types, namely spermatocytes and spermatids (8, 57). Generally, these miRNAs are found and expressed in high levels in the normal testis (62). Because total testis RNA samples may contain small amounts of the germ cell RNA, some of the miRNA signal may be derived from germ cell–expressed miRNAs. In addition, the expression levels of miRNAs are normally high in primordial germ cells (PGCs) and germ cells compared with somatic cells (41). The expression of several miRNAs seems to depend on the developmental stage of the germ cells (70, 71).

In addition, miRNA expression is completely dependent on the cell type predominantly present in the testis, i.e., Sertoli cells, spermatogonia, pachytene spermatocytes, round and elongating spermatids, and spermatozoa (8, 43, 50). miRNAs hsa-miR-1274a and hsa-miR-1274b were differentially deregulated in the SCO-N comparison, and hsa-miR-1274a in the MA-N comparison. Hsa-miR-1274a was differentially deregulated in asthenozoospermic patients compared with normozoospermic control subjects, indicating that the miR-1274 family might have a role in spermatogenesis, because it was expressed in asthenozoospermic subfertile men (51). Members of the miR-125 family (hsa-miR-125b, hsa-miR-125a-5p, hsa-miR-125a-3p, and hsa-miR-125b-1*) were also highly differentially expressed in SCO-N. hsa-miR-125a was highly expressed in PGCs, suggesting a synergistic role in developing PGCs (70). hsa-miR-125a and hsa-miR-125b were ubiquitously expressed throughout the whole lifespan of epididymis, implying a crucial role in the maintenance of basic epididymal functions (72). hsa-miR-125b was differential deregulated in androgen-dependent and -independent prostate cancer cells, suggesting that hsa-miR-125b acts as an oncogene, contributing to the pathogenesis of prostate cancer (73).

Next, we correlated our most down-regulated miRNA predicted targets to the miRNA targets that were previously reported to be significantly overexpressed in patients with NOA compared with OA. Notably, most of the correlated over-expressed target genes are related to cell growth, proliferation, and apoptosis and were highly expressed in the testicular tissues of infertile men compared with fertile men (74–79). Some of these target genes play an essential role in spermatogenesis and testicular development, such as RNA polymerase II, TATA box binding protein–associated factor (TAF5), which was overexpressed in the testis of boys with cryptorchidism and with high risk for azoospermia (79) and is a predicted target of hsa-miR-34b, hsa-miR-34c-5p, and hsa-miR-449a. In addition, Casitas B-lineage lymphoma (CBL), which is a predicted target of hsa-miR-34c-5p, was proposed to be specific biomarker of adult human spermatogonia, and was found to be overexpressed in Sertoli cells (79). Other differentially overexpressed target genes were shown to be overexpressed in NOA and involved in the regulation of spermatogenesis process. These genes included lin-28 homolog B (LIN28B), tudor domain–containing 9 (TDRD9), RNA-binding motif protein 7 (RBM7), NCK-associated protein 1 (NCKAP1), and DMRT-like family B with proline-rich C-terminal 1 (DMRTB1), all of which were predicted targets of hsa-miR-34b. Furthermore, DMRTB1 from the DMRT family, which is known to be involved in the regulation of postnatal testis differentiation, displayed an elevated expression level in NOA patients (74, 79, 80). Insulin-like growth factor–binding protein 5 (IGFBP5) gene, which was statistically highly expressed in NOA compared with OA, is a predicted target of hsa-miR-449a and hsa-miR-34c-5p (77). Furthermore, secreted frizzled-related protein 1 (SFRP1) was overexpressed in all carcinoma in situ and teratoma samples. Moreover, CCND2, which was expressed in Sertoli cells and strongly expressed in elongated spermatids and normal testis, was a predicted target of hsa-miR-34b and 449a (81, 82). Abnormal expression of these target genes may have a significant impact on male infertility. However, exact downstream effects of hsa-miR-34b, hsa-miR-34c-5p, and hsa-miR-449 need to be verified in each disease separately.

In conclusion, the present study provides insights into the roles of miRNAs involved in testicular histologic development. Specifically, this study identified several miRNA clusters and novel miRNAs specifically expressed in a particular testicular histopathology pattern or equally expressed in two or all three of the different patterns compared with normal pattern. The identified miRNAs are likely to play a role in the development and/or regulation of spermatogenesis, and alterations of their expression could directly or indirectly contribute to reproduction abnormalities.

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2.3. A Panel of Five MicroRNAs as Potential Biomarkers for the Diagnosis and Assessment of Male Infertility

Panel of five microRNAs as potential biomarkers for the diagnosis and assessment of male infertility

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Objective: To validate a set of five microRNAs (miRNAs) as specific biomarkers for the assessment of male infertility.
Design: Quantitative real-time polymerase chain reaction (qRT-PCR) validation study.
Setting: University research and clinical institutes.
Patient(s): Two hundred twenty-six men presenting at an infertility clinic.
Intervention(s): None.
Main Outcome Measure(s): Validation analysis of a set of miRNAs in human purified spermatozoa and testicular biopsies.
Result(s): Five miRNAs (hsa-miR-34b*, hsa-miR-34b, hsa-miR-34c-5p, hsa-miR-429, and hsa-miR-122) were confirmed with the use of qRT-PCR analysis in validation sets in patients with different forms of spermatogenic impairments (subfertile and nonobstructive azoospermia [NOA]) and control subjects. We found that hsa-miR-429 was significantly increased and the four other miRNAs were decreased in both tested groups compared with normal control subjects. Computing the area under the receiver operating characteristic curve (AUC) value for each of the five miRNAs, we showed that they separated the tested groups with high accuracy (range 0.777–0.988), except for hsa-miR-429 (AUC 0.475), in patient samples with NOA. Furthermore, with the use of support vector machine classification combining these five miRNAs, we found that they discriminated individuals with, respectively, subfertility and NOA from control subjects with an accuracy of 98.65% and 99.91%, a specificity of 98.44% and 99.69%, and a sensitivity of 98.83% and 100%.
Conclusion(s): Our finding suggests that these five miRNAs have potential as novel noninvasive biomarkers to diagnose patients with subfertility. Except for hsa-miR-429, the combination of these miRNAs with other conventional tests would improve the diagnostic accuracy for detecting patients with different forms of NOA. (Fertil Steril® 2014;102:989–97. ©2014 by American Society for Reproductive Medicine.)
Key Words: microRNA, spermatozoa, seminal plasma, nonobstructive azoospermia, male infertility

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Approximately 15% of couples are unable to conceive after 1 year of unprotected intercourse. A male factor is solely responsible in ~20% of infertile couples and contributory in another 30%–40% (1). Although basic male infertility evaluation requires a comprehensive history along with semen analysis, that alone has failed to accurately distinguish men with unexplained infertility, so a molecular noninvasive test can provide valuable information to the physician by further sorting males with unexplained infertility for purposes of assessment and diagnosis (2, 3). In addition, the conventional tests for assessing testicular tissue specimens with only histologic workup are not highly accurate or sensitive for detecting and classifying spermatogenesis disorders

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(4–7). An approach for improving the diagnostic value of testicular biopsies is the detection of germ and somatic cell–specific mRNA/microRNA (miRNA) expression. Thus, the quantitative determination of mRNA/miRNA expression profile in testicular tissue of patients with different forms of nonobstructive azoospermia (NOA) appears to be well suited to characterize each form and is a useful molecular diagnostic tool for supplementing histopathologic diagnostics and to determine the degree of spermatogenic impairment (4, 5). In ~60%–75% of men, no clear causal factor for unexplained infertility could be diagnosed, giving rise to the term "idiopathic male infertility." These men usually present with no earlier history associated with fertility problems and have normal findings on physical examination. Genetic abnormalities contribute to some percentage of these idiopathic cases (8, 9). A number of genome-wide expression studies have been conducted to shed more light on the issues and defects that underlie this problem based on the deregulation of a specific mRNA and/or miRNA (4, 5, 10–13). miRNAs are a class of short (20–23 nucleotides) single-stranded noncoding nucleotides and are capable of regulating gene expression through mRNA degradation or translational repression. They are involved in various biologic processes, such as proliferation, differentiation, development, and apoptosis (14–17). Recently, the presence of miRNAs in the human sperm, seminal plasma, and testicular tissue of men with subfertility and NOA has been reported (4, 10, 13, 18, 19). Interestingly, the alteration in miRNA expression patterns has been associated with different human testicular histopathologic patterns (Sertoli cell only, mixed atrophy, and germ cell arrest), spermatogenic impairments (asthenozoospermia, oligozoospermia, and oligoasthenozoospermia), and seminal plasma of patients with azoospermia (4, 10, 13, 19–21), indicating that miRNAs could be used as specific biomarkers for the assessment of human male fertility status.

In the present study we aimed to validate a set of five miRNAs, namely, hsa-miR-34b*, hsa-miR-34b, hsa-miR-34c-5p, hsa-miR-429, and hsa-miR-122, as specific biomarkers for the diagnosis of male infertility. These miRNAs were specifically chosen from our recent publications that demonstrated their deregulated expression in human purified spermatozoa and testicular tissue in men with impaired fertility potentials (4, 10).

MATERIALS AND METHODS

Study Population and Sample Collection

This study was approved by the Institutional Review Board of the Saarland University, and informed consent was obtained from each of the participants. The study samples were collected from 170 male partners of infertile couples who attended the IVF lab for infertility treatment. Out of these samples, 80 semen samples were obtained from the male partners with a spermiogram showing abnormal semen parameters (mostly oligospermia and oligoasthenospermia). In these cases, the female partner showed normal laboratory test results with good quality and quantity of oocytes. The remaining 90 semen samples were obtained from male partners with normal semen parameters. In these cases, the female partner showed abnormal laboratory test results, including poor-quality oocytes and low oocyte yield. Because the vast majority of the semen samples were from couples that underwent intracytoplasmic sperm injection, we could not utilize the penetration rate of the sperm into the oocyte for sperm characterization. However, an important criterion for sperm quality is the fertilization rate, which averaged 70%.

Semen samples were obtained by masturbation and collected into sterile containers, after 3–5 days of abstinence from sexual activity. After liquefaction, semen samples were examined for liquefaction time, volume, pH, viscosity, sperm count, motility, and morphology according to World Health Organization guidelines (22). The semen samples were then loaded onto 45%–90% discontinuous Puresperm gradients (Nidacon International) and centrifuged at 500*g* for 20 minutes at room temperature. After centrifugation, the purified spermatozoa were collected from the bottom of the tube with the use of a clean pipette and transferred to new clean-separated tubes for further analysis. The procedure for spermatozoa purification to ensure complete absence of testicular germ cells, epithelial cells, and leukocytes was described previously (10). Twenty microliters culture medium containing spermatozoa was smeared on a microscopic slide for DNA fragmentation evaluation.

Testicular biopsies were obtained from 56 men with obstructive azoospermia (OA) and NOA, and all biopsies were histologically evaluated and classified also into two main groups. OA men (n = 16), were clinically confirmed with obstruction, showed normal testicular volume, and normal structure of both the seminiferous tubules and the interstitial compartment of the testis. Furthermore, the identified late spermatids in their biopsies were the predominant feature of normal spermatogenesis. NOA men (n = 40) included 12 men with mixed atrophy as indicated by tubules with complete or incomplete spermatogenesis and by markedly reduced spermatogenesis, 16 men with germ cell arrest, most often at the spermatogonial or primary spermatocyte stage, and 12 men with total absence of germ cells or their products (Sertoli cell only). The patient samples with known medical reasons for infertility, including chromosomal abnormalities, Y chromosome microdeletions, varicocele, hydrocoele, and sexual dysfunction disorders of erection, were excluded from the study.

Isolation of Total RNA, Including miRNAs

Total RNA, including miRNAs, was isolated from spermatozoa and testicular tissue as described previously (4, 10). The concentration and purity of RNA samples were determined with the use of a Nanodrop ND-2000 spectrophotometer (Thermo Scientific), and its quality was verified with the use of Agilent 2100 Bioanalyzer Eukaryote Total RNA Pico assay (Agilent Technologies).

Generation of cDNA

Three hundred nanograms total RNA was converted to cDNA in a 20-μL reaction with the use of miScript Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. With the use of this system, miRNAs are polyadenylated by

poly(A) polymerase during the reverse transcription step. All polyadenylated miRNAs are converted to cDNA using oligo(dT) primer. The oligo(dT) primer contains a unique tag sequence on the 5′-end, which is afterward used to amplify the miRNA in the polymerase chain reaction (PCR) step.

Quantitative Real-Time PCR

For detection of miRNA, the miScript Sybr Green PCR along with miScript Primer Assays (Qiagen) were used. Genomic DNA is not detected, because the universal tag sequence is not present in genomic DNA. One microliter reverse-transcription products (4 ng cDNA) was used as templates in 20-μL reactions for quantitative real-time (qRT) PCR analysis of five selected miRNAs (hsa-miR-34b*, hsa-miR-34b, hsa-miR-34c-5p, hsa-miR-429, and has-miR-122) according to the manufacturer's instructions with the use of the following thermal cycling conditions: initial activation step 95°C for 15 minutes followed by 40 cycles at 94°C for 15 seconds (denaturation), 55°C for 30 seconds (annealing), and 70°C for 30 seconds (extension). Then final dissociation curves (melting curves) were made. All reactions were run on the Applied Biosystems Steponeplus Real-Time PCR Detection System (Applied Biosystems), and the results (Cq values) were related to RNU6B endogenous control (Qiagen).

Evaluation of DNA Fragmentation (TUNEL Assay)

The DNA fragmentation was detected with the use of TUNEL assay (In Situ Cell Death Detection Kit, Fluorescein; Roche Diagnostics). Ten microliters spermatozoa was smeared on glass slides and fixed with 4% paraformaldehyde dissolved in phosphate-buffered saline solution (pH 7.4, w/w) for 1 hour at room temperature. After being washed with distilled water (DW) for 5 minutes three times, fixed spermatozoa were permeabilized with 0.1% Triton X-100 in 0.1% sodium chloride-sodium citrate (w/w) for 30 minutes at 4°C. Slides were then washed three times with DW, air dried, and incubated with 50 μL staining solution containing terminal deoxytransferase (TdT) for 1 hour at 37°C in the dark. After three times washing with DW (5 minutes each time) and air drying, 25 μL diamidinophenylindole solution (Vector Laboratories), which is a DNA-specific stain that forms a fluorescent complex by attaching in the minor groove of A-T–rich sequences of DNA, was added to the sperm and incubated for 30 minutes at 37°C in the dark. After incubation, the slides were washed for 5 minutes three times with DW, air dried, and mounted with the use of Prolong Gold Antifade (Life Technologies). Negative control spermatozoa were treated identically except for the omission of TdT. For microscopic evaluation, the slides were evaluated with the use of fluorescent illumination in an A ×70-fluorescence microscope (Olympus) with an ×100 oil-immersion objective. For each slide, ~200 spermatozoa were evaluated and the percentage (%) of fragmented apoptotic spermatozoa was calculated.

Statistical Analysis

The expression levels of the five selected miRNAs were normalized to endogenous RNU6B. The relative miRNA quantity in the tested samples from control men versus subfertile and infertile men was calculated separately with the use of the comparative ΔCt method. The threshold cycle (Ct) reflects the cycle number at which the fluorescence curve generated within a reaction crosses the threshold in qRT-PCR. The ΔCt was calculated by subtracting the Ct values of RNU6B from the Ct values of the miRNA of interest: $\Delta Ct = (Ct_{miR\ of\ interest} - Ct_{RNU6B})$. A ΔΔCt was then calculated by subtracting the ΔCt of samples from men with known subfertility and infertility from the ΔCt of the control samples: $\Delta\Delta Ct = (\Delta Ct_{control} - Ct_{patients})$. This method determines the change in expression of a nucleic acid sequence in a test sample relative to the same sequence in a control sample. The fold-change cutoff for miRNAs was calculated by the equation $2^{-\Delta\Delta Ct}$ (23). The unpaired *t* test with Welch correction and the area under the receiver operating characteristic curve (AUC) values for each miRNA to assess its suitability as a single biomarker were computed with the use of Graphpad software, version 5.0 for Windows. Differences were considered to be significant at *P* values of $<.05$, and an AUC value close to 0 (or 1) indicates a high diagnostic value, because the expression values of one group are in this case mostly higher (or lower) than in the compared group. Classification of samples with the use of miRNA patterns was carried out with the use of support vector machines (SVM) as implemented in the R e1071 package using 20 repetitions of standard tenfold cross-validation (24).

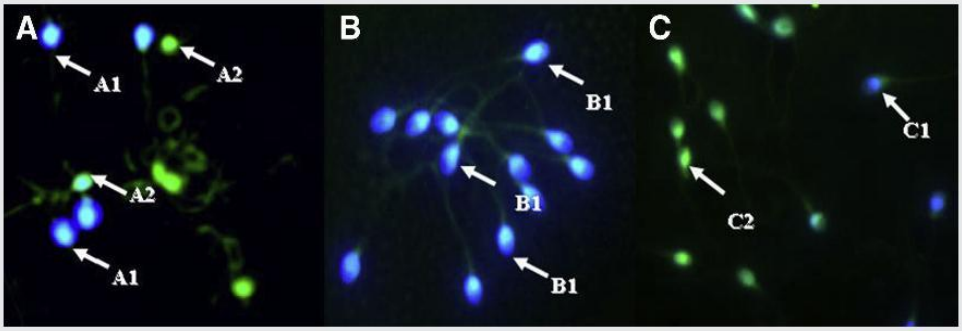
RESULTS

Characteristic Parameters of Study Subjects

The characteristics of the enrolled male partners of infertile couples and control subjects are presented in Supplemental Table 1 (available online at www.fertstert.org). The control group was significantly different from the subfertile group in sperm count, sperm motility, and sperm morphology. However, no differences were found between the two groups regarding mean age, volume, pH, and semen viscosity. Correlations between DNA fragmentation, various semen parameters, and ΔCt values of the five tested miRNAs are presented in Supplemental Table 1. The sperm apoptosis test (TUNEL) was used to determine whether the male partner of control and spermatogenic impairment couple has a high, fair or poor fertility potential by studying DNA fragmentation. The TUNEL+ spermatozoa take up more of the fluorescein stain and appeared green and the TUNEL− spermatozoa sperm take up more of the diamidinophenylindole stain and appeared blue (Fig. 1). The proportions (mean ± SEM) of TUNEL− and TUNEL+ spermatozoa were, respectively, 89.8% ± 1.15 and 10.20% ± 1.154 for the control group and 80.78% ± 1.61 and 19.22% ± 1.61 for the subfertile group ($P<.0001$).

We further analyzed the correlations between the TUNEL+ spermatozoa and various semen parameters. The results are summarized in Supplemental Table 1. We found that the amounts of TUNEL+ spermatozoa were significantly correlated with sperm count and motility: $r = -0.4889$ ($P<.0001$) and $r = -0.3366$ ($P<.0085$), respectively. In contrast, no significant correlation was observed between the amount of TUNEL+ spermatozoa and morphology: $r = -0.1701$

FIGURE 1



Representative image of sperm DNA fragmentation evaluated with the use of TUNEL assay for three different samples, showing spermatozoa stained with diamidinophenylindole (DAPI; *blue* fluorescence in the nuclear region) and spermatozoa stained with fluorescein isothiocyanate (*green* fluorescence in the nuclear region). TUNEL− spermatozoa take up the DAPI stain and appear *blue* (A1, B1, C1), wherease TUNEL+ spermatozoa appeared *bright green* (A2, C2). Magnification ×100.

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($P<.1939$). The percentage of TUNEL+ spermatozoa was significantly correlated with the ΔCt values of hsa-miR-34b* ($r = -0.3314$; $P<.0097$), hsa-miR-34b ($r = 0.3995$; $P<.0016$), hsa-miR-34c-5p ($r = 0.3087$; $P<.0164$), hsa-miR-429 ($r = -0.446$; $P<.0004$), and hsa-miR-122 ($r = 0.4159$; $P<.0009$). There were no statistically significant correlations between the ΔCt of the five tested miRNAs and morphology (%). We found moderate but significant positive correlations between the ΔCt of hsa-miR-429 and sperm count ($r = 0.657$; $P<.0001$) and motility ($r = 0.701$; $P<.0001$).

Expression Levels of the Five miRNAs in Human Purified Spermatozoa and Testicular Tissue

The expression levels of the five miRNAs were analyzed with the use of qRT-PCR in purified spermatozoa and testicular biopsies of men with subfertility and infertility compared with control men. These miRNAs were specifically chosen from our recent studies that demonstrated with the use of microarray technology their deregulated expression in men with impaired fertility potentials. With the use of the high-throughput Sureprint G3 Human v16 miRNA microarray platform, we found that hsa-miR-34b*, hsa-miR-34b, hsa-miR-34c-5p, and hsa-miR-122 were down-regulated, with AUCs of 0.98, 0.99, 0.98, and 0.99, respectively, and hsa-miR-429 was up-regulated, with an AUC of 0.96, in the spermatozoa of men with asthenozoospermia and oligoasthenozoospermia compared with normozoospermia men (10).

The deregulation expression levels for these miRNAs were further validated by qRT-PCR on an independent test set of spermatozoa samples collected for the validation purpose. Four of these miRNAs were significantly down-regulated (hsa-miR-34b*, hsa-miR-34b, hsa-miR-34c-5p, and hsa-miR-122) and one was up-regulated (hsa-miR-429; Table 1; $P<.05$

TABLE 1

Fold change of miRNAs and area under the receiver operating characteristic curve (AUC) values in human spermatozoa and testicular tissue samples of subfertile and different forms of nonobstructive azoospermia (NOA) patients compared with those from control subjects as determined by quantitative real-time polymerase chain reaction ($P<.05$; false discovery rate adjusted).

miRNA	ΔCt mean, control (n = 90)	ΔCt mean, subfertile (n = 80)	ΔΔCt	Fold change	Regulation	Corrected *P* value	AUC
Subfertility group compared with normal							
hsa-miR-34b*	8.64	10.06	−1.42	2.67	Down	2.81579E-12	0.798
hsa-miR-34b	3.23	6.18	−2.95	7.73	Down	1.34328E-32	0.944
hsa-miR-34c-5p	6.23	7.59	−1.36	2.56	Down	3.51625E-10	0.776
hsa-miR-429	6.71	2.71	4.00	16.00	Up	6.83118E-48	0.02
hsa-miR-122	4.99 (n = 16)	6.93 (n = 40)	−1.94	3.84	Down	1.69124E-11	0.777
Different forms of NOA compared with normal							
hsa-miR-34b*	9.31	12.78	−3.48	11.15	Down	2.49462E-08	0.948
hsa-miR-34b	6.61	9.06	−2.45	5.45	Down	8.17429E-06	0.852
hsa-miR-34c-5p	5.71	10.07	−4.36	20.57	Down	6.22328E-08	0.978
hsa-miR-429	14.75	14.4	0.34	0.79	Up	0.438546685	0.475
hsa-miR-122	8.8	14.37	−5.57	47.67	Down	1.42656E-09	0.988

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[false discovery rate (FDR) adjusted]). Expression values for the down-regulated miRNAs ranged from 2.56-fold for hsa-miR-34c-5p, 2.67 fold for hsa-miR-34b*, and 3.84 for hsa-miR-122 up to 7.73-fold for hsa-miR34b in spermatozoa of men with subfertility compared with control men.

For the analysis of the testicular biopsies with the use of the v16 miRNA microarray platform, we found that hsa-miR-34b*, hsa-miR-34b and hsa-miR-34c-5p were down-regulated, with AUCs of 0.89, 0.95, and 0.95, respectively, in patients with different forms of NOA compared with men with normal spermatogenesis (4). These deregulated miRNAs were also highly down-regulated in testicular biopsies of patients with different forms of NOA compared with the control men in the validation study with the use of qRT-PCR. For example, the changes ranged from 5.45-fold for hsa-miR-34b, 11.15-fold for hsa-miR-34b*, and 20.57 for hsa-miR-34c-5p up to 47.67-fold for hsa-miR-122. The hsa-miR-429 miRNA was not significantly deregulated in males with different forms of NOA compared with control men (Table 1; $P<.05$ [FDR adjusted]). Therefore, the fold change, AUC value, and deregulation direction results of the purified spermatozoa and testicular biopsies for these miRNAs according to qRT-PCR were largely concordant with the microarray data for the independent collected samples ($P<.05$ [FDR adjusted]).

Evaluation of miRNAs as New Biomarkers for Male Infertility

To investigate the characters of these five miRNAs as potential single biomarkers for the assessment of male fertility status, ROC curves were constructed on data from all 226 subjects, including 80 subfertile men compared with 90 control men and 40 patients with different forms of NOA compared with 16 control men. The receiver operating characteristic curves of hsa-miR-34b*, hsa-miR-34b, hsa-miR-34c-5p, hsa-miR-429, and hsa-miR-122 reflected strong separation between subfertile and control male groups, with AUCs of 0.798 (95% confidence interval [CI] 0.7309–0.8641), 0.944 (95% CI 0.9131–0.9713), 0.776 (95% CI 0.7052–0.8476), 0.980 (95% CI 0.9638–0.9970), and 0.777 (95% CI 0.7082–0.8460), respectively (Fig. 2A1–2A5). Similar pattern were also observed for four miRNAs (hsa-miR-34b*, hsa-miR-34b, hsa-miR-34c-5p, and hsa-miR-122) in patients with different forms of NOA compared with 16 control men, with AUCs of 0.948 (95% CI 0.8967–1.000), 0.852 (95% CI 0.7546–0.9485), 0.978 (95% CI 0.9476–1.000), and 0.988 (95% CI 0.9715–1.000), respectively. Furthermore, a very week AUC value of 0.475 (95% CI 0.3579–0.6588) was observed for hsa-miR-429 (Fig. 2B1–2B5).

Furthermore, we used support vector machines to compute the classification parameters for the combined five miRNAs for spermatozoa and testicular tissue. We found that these five miRNAs discriminated individuals with subfertility from normal control subjects with an accuracy of 98.65%, a specificity of 98.83%, and a sensitivity of 98.44%. The distinguished individuals with NOA from normal control subjects with an accuracy of 99.91%, a specificity of 99.69%, and a sensitivity of 100%. Computed means, standard deviations, and confidence intervals for the repetitions regarding specificity, sensitivity, and accuracy are presented in Table 2, together with the results for the control classifications with the randomly permuted class labels.

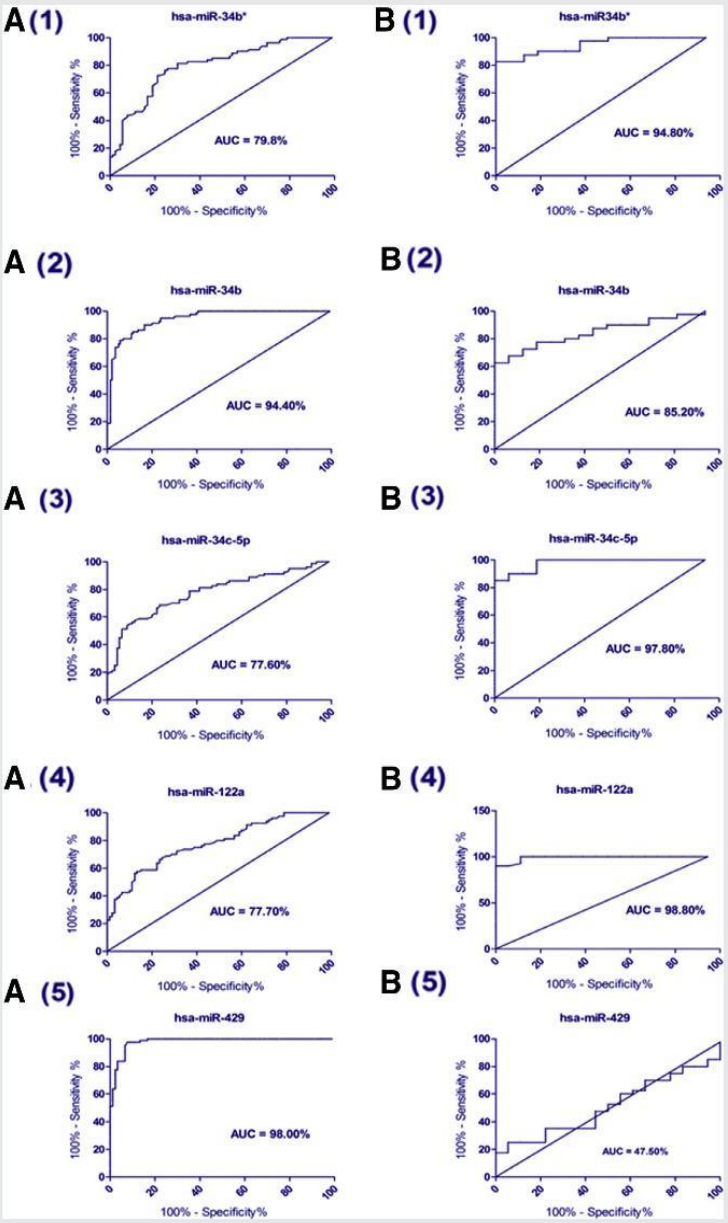
DISCUSSION

To date, there is no reliable molecular diagnostic assay to diagnose male infertility as a companion diagnosis to semen analysis. There is also a lack of markers to confirm conventional histologic diagnostics in the assessment of testicular biopsies. Alterations of miRNA expression profile in patients with different spermatogenic and histopathologic impairments have the potential to be used as new biomarkers for diagnostic purposes (4, 10, 13, 19–21). The aim of the present study was to validate a set of miRNAs toward the development of clinically practicable biomarkers for spermatogenesis and for fertility status assessment.

Besides their potential as biomarkers, the biologic role of miRNAs has increasingly been recognized in the process of spermatogenesis (25, 26). For example, many testis-specific miRNAs have been identified in male germ and Sertoli cells, and some of these miRNAs are highly expressed in the late meiotic stages of spermatogenesis (27–31). As for the biologic function of the miRNAs analyzed in this study, the miR-34 family plays an important role in the p53 tumor suppressor network, and expression of miR-34a, miR-34b, and miR-34c is robustly induced by DNA damage and oncogenic stress in a p53-dependent manner. Overall, miR-34 appears to play a vital role in apoptosis and p53-mediated cell death (29, 32, 33). Notably, miR-34c is preferentially expressed in germ cells, enhances germinal phenotypes, and is implicated in the control of the cell cycle and apoptosis (28,33–37). MiR-34b is highly expressed in adult testis compared with prepubertal testis, indicating its potential role in the differentiation of male germ cells (37–39). Similarly, miR-122a is predominately expressed in post-meiotic germ cells and suppresses the transcription of transition protein 2, a testis-specific protein involved in chromatin remodeling during spermatogenesis (31). Recently it has been shown that hsa-miR-34b*, hsa-miR-34b, hsa-miR-34c-5p, hsa-miR-429, and hsa-miR-122 are down-regulated in patients with NOA (13, 19, 20). These data indicate that the identified miRNAs are not only important as potential biomarkers but are likely to play a crucial role in processes related to spermatogenesis. A better knowledge of the biologic role of the identified miRNAs would in turn contribute to increase their value as biomarkers.

Whereas four of the five miRNAs analyzed in this study were significantly down-regulated in spermatozoa of patients with subfertility and in testicular tissue of patients with different forms of NOA, hsa-miR-429 was highly up-regulated in those spermatozoa and to lesser extent in different forms of NOA, compared with normal control subjects. Recently it was shown that hsa-miR-429 was differentially expressed in different cell types of testicular tissues with low expression in Sertoli cell–only, mixed atrophy, and germ cell arrest biopsies (4). Based on its expression pattern, it is legitimate to speculate that has-miR-429 is strongly expressed during late stages of spermatogenesis with the highest expression in spermatozoa (10), relatively high in seminal plasma (13) and lower expression in testicular

FIGURE 2



Receiver operating characteristic (ROC) curves of five miRNAs in (A1–A5) spermatozoa and (B1–B5) testicular tissues between spermatogenic impairment and different testicular histopathologic pattern groups (n = 80 and n = 40, respectively) and control subjects (n = 90 and n = 16, respectively). *P*<.05; false discovery rate adjusted.

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tissue (4), which includes somatic and/or germ cell types at different stages of maturation and accordingly different levels of testicular-expressed miRNA (40, 41). Therefore, the spermatozoa and seminal plasma hsa-miR-429 miRNA can be used to monitor the progression of spermatogenesis.

Several studies have reported increased levels of sperm DNA fragmentation in infertile men with abnormal sperm parameters (sperm count, motility, and morphology) (42–51). Moreover, men with normal basic sperm parameters may have significant levels of DNA fragmentation (52). Other

TABLE 2

Summary of the support vector machine classifications containing the means, standard deviations, and 95% confidence intervals (CIs) of the accuracy, specificity, and sensitivity running 20 repetitions of tenfold cross-validations with linear kernel.

	Classification			Permutation test		
Comparison	Accuracy	Specificity	Sensitivity	Accuracy	Specificity	Sensitivity
Subfertile (n = 80) vs. control (n = 90)	98.65 ± 0.27%	98.83 ± 0.25%	98.44 ± 0.56%	50.5 ± 4.65%	49.5 ± 5.83%	51.7 ± 6.93%
	95% CI 98.53%–98.77%	95% CI 98.73%–98.94%	95% CI 98.19%–98.68%	95% CI 48.5%–52.6%	95% CI 47.0%–52.1%	95% CI 48.7%–54.7%
NOA (n = 40) vs. control (n = 16)	99.91 ± 0.4%	99.69 ± 1.4%	100 ± 0.00%	51.6 ± 10.96%	50.0 ± 12.98%	52.3 ± 12.11%
	95% CI 99.74%–100.00%	95% CI 99.07%–100.00%	95% CI 100.00%–100.00%	95% CI 46.8%–56.40%	95% CI 44.3%–55.7%	95% CI 46.9%–57.6%

Abu-Halima. miRNA biomarkers for male infertility diagnosis. Fertil Steril 2014.

investigators however have concluded that DNA fragmentation in spermatozoa is associated with reduced rate of fertility (43, 48, 49, 53). We found a weak but significant correlation between DNA fragmentation and the five miRNAs. These weak correlations can be explained by two hypothesis: 1) The processing of spermatozoa with sperm preparation techniques such as sperm density gradient and/or sperm swim-up separately or together yields a population of spermatozoa with lower DNA fragmentation or a population with high level of genetically competent spermatozoa (54–61); or 2) the level of apoptosis during the spermatogenesis process, especially in spermatogonial and primary spermatocytes, is high compared with that in mature spermatozoa (53, 62–64). Our results lay the ground for future prospective studies with extended sample cohorts.

For the interpretation of our data, it is important to bear in mind, that we used a limited number of samples and that the control group was recruited from men of couples with inability to achieve pregnancy. In these cases, the female partner showed abnormal laboratory test results and the male partner had normal semen parameters. Although the infertility of these couples is most likely attributed to the female partner, ideally the control group would consist of men with proven fertility. In the clinical sitting, we had, however, problems in recruiting men with proven fertility.

In summary, this study has validated five miRNAs (hsa-miR-34b*, hsa-miR-34b, hsa-miR-34c-5p, hsa-miR-429, and hsa-miR-122) with altered expression in spermatogenic and histopathologic impairment patients. These five miRNAs have the potential be used as biomarkers to diagnose males with subfertility.

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4. Discussion and Conclusion

In this thesis, we investigated the differentially expressed miRNAs between asthenozoospermic and oligoasthenozoospermic subfertile as well as we identified the miRNA expression profiles of infertile males diagnosed with SCO, MA, and GA compared with control males by microarray analysis and qRT-PCR. Following the miRNA microarray-screening phase, we tested five candidate miRNAs (hsa-miR-34b*, hsa-miR-34b, hsa-miR-34c-5p, hsa-miR-429, and hsa-miR-122) in two independent validation phases in patients and controls to assess their use as specific biomarkers to diagnose male infertility. Four of these five miRNAs were significantly down-regulated in spermatozoa of patients with subfertility (in oligozoospermia, asthenozoospermia and oligoasthenozoospermia) and in testicular tissue of patients with different forms of NOA (in SCO, MA and GA), hsa-miR-429 was highly up-regulated in spermatozoa and to lesser extent in different forms of NOA, compared with normal control males. The combination of the expression levels of these five miRNAs, yielded high diagnostic accuracy, sensitivity and specificity, which might be used together as a novel biomarker to diagnose male infertility. The deregulated miRNAs in each patient group and their functional role in spermatogenesis and male infertility have been discussed in details in each article's discussion. The results presented in this thesis showed that miR-34 family ranked in the top expressed miRNAs in the spermatozoa and testicular tissue and found to be strongly down regulated in the SCO, MA, and GA males compared with the normal control males. These miRNAs were also reported to be down regulated in subfertile oligozoospermic, asthenozoospermic, and oligoasthenozoospermic males, indicating their potential roles in the later period of the sperm development. Generally, miR-34 and miR449 families are found and expressed in high levels in the testis (Bou *et al.,* 2010; Bouhallier *et al.,* 2010; Lize *et al.,* 2010). The high down regulated level in patients with different forms of NOA and to lesser extent in spermatozoa of patients with spermatogenic impairments is due to the expression level these two families are expressed in normal testis. Deletion of miR34b/c and miR-449 loci resulted in oligoasthenoteratozoospermia in mice, and the miR-34bc/449-deficiency impairs both meiosis and the final stages of spermatozoa maturation (Comazzetto *et al.,* 2014). The miR-200 family, containing miR-200a, miR-200b, miR-200c, miR-141 and miR-429, is organized into two groups based on a single nucleotide difference in their seed sequence (group A: miR-141 and miR-200a are located on chromosome 2; group B:

miR-200b, miR-200c and miR-429 are located on chromosome 1). The over expression of these five miRNAs have been observed in spermatozoa of patient with asthenozoospermia and oligoasthenozoospermia as well as in testes of patient with different forms of NOA compared with control. MiR-429, and miR-141 were significantly increased in seminal plasma of patients with NOA compared with fertile controls (Wu *et al.*, 2013). Wang *et al.* shown that hsa-miR-122 is down-regulated in patients with NOA and indicated that this miRNA marker might have confirmative molecular diagnostic value for male infertility (Wang *et al.,* 2011). These data indicate that the identified miRNAs are not only important as potential biomarkers but are likely to play a crucial role in processes related to spermatogenesis. A better knowledge of the biologic role of the identified miRNAs would in turn contribute to increase their value as biomarkers. Furthermore, understanding the complex relationship between miRNA and target gene interaction that are related to spermatogenesis is an important component of miRNA functional analysis. Hence, in this regard, the assessment of miRNA may have future diagnostic and prognostic value and shed more light on the molecular mechanisms of male infertility.

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6. Appendices

Article I: Supplementary Tables and Figures

Supplemental Table 1

Patient and semen characteristics

Characteristics	N (n=9)	A (n=9)	OA (n=9)	*P*-value A/N	*P*-value OA/N	*P*-value A/OA
Age	32.78 ± 5.63	34.67±7.87	31.33±3.94	0.567	0.538	0.279
Volume (ml)	2.589±0.804	2.467±0.456	2.611±0.782	0.698	0.953	0.640
pH	8.689±0.242	8.422±0.466	8.756±0.388	0.153	0.669	0.119
Count (mill/ml)	90.11±15.94	76.78±20.10	7.244±3.729	0.139	0.001	0.001
Motility (% motile)	55.00 ±16.39	25.89±5.56	22.78±5.07	0.001	0.001	0.233
Sperm vitality (Eosin) (%)	47.00±15.93	36.78±16.13	22.56±6.89	0.195	0.001	0.034
Membrane integrity (HOS)(%)	65.56±11.02	68.78±9.90	57.22±7.95	0.523	0.086	0.015
Morphology (%)	43.22±15.61	25.78±11.67	27.00±9.38	0.017	0.019	0.810

Data presented as Mean ± SD; P < 0.050 was considered significant and P< 0.001 was considered highly significant.

Supplemental Table 2

Expression of all miRNAs in spermatozoa samples in sub-fertile oligoasthenozoospermic males compared to that from sub-fertile asthenozoospermic males determined by miRNA-microarray.

MiRNA	Log Median Oligoastheno-zoospermia	Log Median Asthenozoo-spermia	Log Difference	Fold Change	P-value	Corrected *P*-value	AUC
hsa-miR-34c-5p	1,635	3,035	-1,400	0,379	0,055	0,2004977	0,815
hsa-miR-4323	4,047	2,753	1,293	2,451	0,105	0,2004977	0,333
hsa-miR-132	1,243	2,429	-1,186	0,439	0,067	0,2004977	0,765
hsa-miR-3148	2,989	3,999	-1,010	0,496	0,083	0,2004977	0,704
hsa-miR-93	2,582	4,258	-1,676	0,313	0,034	0,2004977	0,778
hsa-miR-767-3p	3,325	2,118	1,207	2,309	0,071	0,2004977	0,247
hsa-miR-4310	4,504	3,067	1,437	2,708	0,107	0,2004977	0,259
hsa-miR-25	3,453	4,989	-1,536	0,345	0,059	0,2004977	0,778
hsa-miR-4312	4,174	2,719	1,455	2,741	0,047	0,2004977	0,222
hsa-miR-193a-5p	4,370	3,359	1,011	2,015	0,009	0,2004977	0,117
hsa-miR-1825	5,011	3,621	1,390	2,621	0,098	0,2004977	0,315
hsa-miR-498	5,621	4,444	1,176	2,260	0,046	0,2004977	0,198
hsa-miR-34b*	2,876	5,146	-2,270	0,207	0,043	0,2004977	0,790
hsa-miR-1234	5,976	4,793	1,183	2,270	0,114	0,2004977	0,284
hsa-miR-1231	-1,852	-3,322	1,470	2,770	0,056	0,2004977	0,259
hsa-miR-1238	5,565	4,023	1,541	2,911	0,103	0,2004977	0,272
hsa-miR-1225-3p	5,483	4,062	1,421	2,677	0,122	0,2004977	0,309
hsa-miR-33b*	4,688	3,378	1,310	2,480	0,057	0,2004977	0,259
hsa-let-7b*	4,644	3,100	1,544	2,916	0,045	0,2004977	0,235
hsa-miR-378c	1,099	2,104	-1,006	0,498	0,090	0,2004977	0,716

hsa-miR-3613-3p	4,153	2,726	1,427	2,690	0,051	0,2004977	0,247
hsa-miR-425	2,465	3,809	-1,343	0,394	0,025	0,2004977	0,802
hsa-miR-940	7,725	6,256	1,469	2,768	0,100	0,2004977	0,259
hsa-miR-20a	3,898	5,565	-1,666	0,315	0,011	0,2004977	0,840
hsa-miR-20b	4,246	5,744	-1,498	0,354	0,122	0,2004977	0,728
hsa-miR-933	3,841	2,502	1,339	2,530	0,067	0,2004977	0,272
hsa-miR-1470	3,701	2,633	1,068	2,097	0,067	0,2004977	0,272
hsa-miR-1227	3,231	2,123	1,108	2,156	0,043	0,2004977	0,259
hsa-miR-595	3,412	4,459	-1,047	0,484	0,119	0,2004977	0,716
hsa-miR-572	8,073	6,667	1,407	2,651	0,110	0,2004977	0,272
hsa-miR-183	2,016	3,439	-1,423	0,373	0,060	0,2004977	0,753
hsa-miR-509-5p	1,281	2,426	-1,144	0,452	0,098	0,2004977	0,679
hsa-miR-10a	3,630	6,225	-2,595	0,165	0,082	0,2004977	0,778
hsa-miR-107	4,292	5,822	-1,530	0,346	0,096	0,2004977	0,716
hsa-miR-3940	3,227	2,006	1,220	2,330	0,071	0,2004977	0,272
hsa-miR-483-3p	4,258	2,582	1,676	3,196	0,053	0,2004977	0,259
hsa-miR-1267	3,378	2,279	1,099	2,142	0,051	0,2004977	0,222
hsa-miR-30a*	2,013	3,439	-1,426	0,372	0,029	0,2004977	0,790
hsa-miR-152	0,692	1,827	-1,135	0,455	0,109	0,2004977	0,679
hsa-miR-3180-5p	4,385	3,310	1,075	2,107	0,117	0,2004977	0,296
hsa-miR-15b	3,744	4,926	-1,182	0,441	0,028	0,2004977	0,840
hsa-miR-671-5p	7,725	6,470	1,255	2,387	0,022	0,2004977	0,167
hsa-miR-151-5p	3,548	5,103	-1,556	0,340	0,010	0,2004977	0,877
hsa-miR-584	4,477	2,758	1,719	3,291	0,031	0,2004977	0,210
hsa-miR-16	4,926	6,152	-1,226	0,427	0,030	0,2004977	0,802
hsa-miR-563	4,275	2,649	1,626	3,086	0,024	0,2004977	0,173
hsa-miR-30e*	1,586	2,912	-1,326	0,399	0,077	0,2004977	0,741
hsa-miR-1287	4,607	3,412	1,195	2,290	0,077	0,2004977	0,247
hsa-miR-1281	5,524	4,032	1,492	2,813	0,095	0,2004977	0,321
hsa-miR-100	3,848	5,146	-1,298	0,407	0,087	0,2004977	0,741
hsa-miR-3191	-0,425	0,945	-1,370	0,387	0,128	0,2021499	0,753
hsa-miR-191*	5,132	3,665	1,467	2,764	0,128	0,2021499	0,296
hsa-miR-10b	4,424	7,024	-2,600	0,165	0,140	0,2159264	0,704
hsa-miR-1228	6,735	5,588	1,147	2,214	0,147	0,2238894	0,309
hsa-miR-23c	4,543	3,150	1,392	2,625	0,152	0,2245976	0,321
hsa-miR-103	4,042	5,067	-1,025	0,491	0,153	0,2245976	0,759
hsa-miR-1237	4,755	3,712	1,043	2,060	0,162	0,2279894	0,296
hsa-miR-766	4,817	3,463	1,353	2,555	0,164	0,2279894	0,333
hsa-miR-1539	4,711	3,302	1,409	2,655	0,163	0,2279894	0,321
hsa-miR-4313	5,483	3,822	1,660	3,161	0,176	0,2409391	0,321
hsa-miR-195	3,026	4,300	-1,274	0,413	0,184	0,2473266	0,716
hsa-miR-3676	5,037	3,961	1,076	2,108	0,193	0,2557082	0,321
hsa-miR-425*	4,736	3,369	1,368	2,580	0,214	0,2758563	0,327
hsa-miR-3937	7,024	5,744	1,280	2,429	0,215	0,2758563	0,346
hsa-let-7f-1*	4,493	3,100	1,393	2,626	0,219	0,2765928	0,340
hsa-miR-129-3p	3,645	2,633	1,012	2,016	0,223	0,2776159	0,364
hsa-miR-129*	3,638	2,535	1,103	2,148	0,231	0,2830398	0,407

hsa-miR-24	3,325	4,688	-1,363	0,389	0,248	0,2974876	0,654
hsa-miR-2116*	4,144	3,082	1,061	2,087	0,250	0,2974876	0,321
hsa-miR-30b	5,684	6,788	-1,104	0,465	0,281	0,3159681	0,722
hsa-miR-130b	1,685	2,890	-1,205	0,434	0,276	0,3159681	0,630
hsa-miR-17	4,292	5,588	-1,296	0,407	0,281	0,3159681	0,704
hsa-let-7d	3,763	4,868	-1,105	0,465	0,273	0,3159681	0,642
hsa-miR-30c	3,724	4,817	-1,093	0,469	0,353	0,3821164	0,667
hsa-miR-342-3p	3,574	4,736	-1,163	0,447	0,349	0,3821164	0,667
hsa-miR-4286	9,540	10,601	-1,062	0,479	0,354	0,3821164	0,642
hsa-miR-23a	4,385	5,744	-1,359	0,390	0,391	0,4163074	0,605
hsa-miR-3610	6,820	5,766	1,054	2,077	0,453	0,4764699	0,358
hsa-miR-887	3,733	4,868	-1,134	0,456	0,529	0,5492681	0,556
hsa-miR-99b	3,302	2,157	1,145	2,212	0,635	0,6510861	0,444
hsa-miR-891a	3,016	1,146	1,870	3,656	0,755	0,7646101	0,506
hsa-miR-205	3,898	2,477	1,421	2,679	0,882	0,8823472	0,494

Supplemental Table 3

Expression of all miRNAs in spermatozoa samples in sub-fertile oligoasthenozoospermic males compared to that from fertile normozoospermic males determined by miRNA-microarray.

MiRNA	Log Median Oligoastheno-zoospermia	Log Median Normozoo -spermia	Log Difference	Fold Change	P-value	Corrected *P*-value	AUC
hsa-miR-122	2,180	5,808	-3,628	0,081	5,63E-07	0,000	1,000
hsa-miR-34b	1,742	5,976	-4,233	0,053	4,34E-07	0,000	1,000
hsa-miR-449b	1,773	3,054	-1,282	0,411	4,37E-06	0,000	0,988
hsa-miR-34b*	2,876	7,976	-5,100	0,029	3,81E-06	0,000	0,988
hsa-miR-15b	3,744	8,073	-4,329	0,050	3,47E-06	0,000	1,000
hsa-miR-29b	5,103	3,261	1,842	3,585	8,81E-06	0,000	0,012
hsa-miR-34c-5p	1,635	5,961	-4,326	0,050	1,90E-05	0,000	0,988
hsa-miR-449a	1,853	5,808	-3,955	0,064	1,72E-05	0,000	0,975
hsa-miR-1973	3,834	7,484	-3,650	0,080	3,65E-05	0,001	1,000
hsa-miR-2115*	1,184	2,413	-1,230	0,426	6,58E-05	0,001	0,975
hsa-miR-320e	6,310	4,188	2,122	4,353	8,68E-05	0,001	0,049
hsa-miR-141	6,638	3,600	3,038	8,214	0,0001823	0,002	0,074
hsa-miR-29b-2*	1,161	2,258	-1,097	0,467	0,000218	0,002	0,969
hsa-miR-16	4,926	8,346	-3,420	0,093	0,000229	0,002	0,938
hsa-miR-150	1,079	2,253	-1,174	0,443	0,000306	0,003	0,938
hsa-miR-19a	2,563	5,864	-3,301	0,101	0,000367	0,003	0,926
hsa-miR-320d	6,945	5,637	1,309	2,478	0,0004325	0,003	0,037
hsa-miR-122*	1,374	2,444	-1,070	0,476	0,0004935	0,004	0,963
hsa-miR-98	2,697	1,657	1,039	2,055	0,0005092	0,004	0,056
hsa-miR-429	4,246	1,645	2,601	6,068	0,0005854	0,004	0,099
hsa-miR-200a	5,229	2,665	2,564	5,913	0,0007061	0,004	0,074
hsa-miR-15a	4,444	6,538	-2,094	0,234	0,0006979	0,004	0,914
hsa-miR-363	5,915	3,477	2,439	5,421	0,0009972	0,005	0,086

hsa-miR-499-5p	1,382	3,875	-2,493	0,178	0,0010208	0,005	0,938
hsa-miR-132	1,243	3,690	-2,447	0,183	0,0009208	0,005	0,926
hsa-miR-520b	5,067	3,600	1,467	2,765	0,0009888	0,005	0,074
hsa-miR-520e	4,424	3,337	1,087	2,125	0,0009161	0,005	0,037
hsa-miR-873	1,120	2,353	-1,233	0,425	0,0010346	0,005	0,975
hsa-miR-625	2,306	4,300	-1,994	0,251	0,0010413	0,005	0,926
hsa-miR-30d	5,822	4,258	1,564	2,956	0,0011937	0,005	0,074
hsa-miR-9*	1,013	3,231	-2,218	0,215	0,0012584	0,005	0,877
hsa-miR-193b	4,493	1,596	2,897	7,448	0,0014907	0,006	0,123
hsa-miR-769-3p	3,936	2,658	1,279	2,426	0,0015634	0,006	0,049
hsa-miR-29c	6,902	4,755	2,148	4,431	0,0017725	0,007	0,099
hsa-miR-518e	1,150	3,405	-2,255	0,210	0,0021861	0,008	0,889
hsa-miR-30e	3,867	2,149	1,718	3,289	0,0021352	0,008	0,099
hsa-miR-3154	3,386	5,423	-2,037	0,244	0,0020975	0,008	0,914
hsa-miR-595	3,412	5,864	-2,452	0,183	0,0020362	0,008	0,901
hsa-miR-25	3,453	5,353	-1,900	0,268	0,0023184	0,008	0,895
hsa-miR-520h	1,739	2,817	-1,078	0,474	0,0027226	0,009	0,877
hsa-miR-4299	6,603	9,353	-2,750	0,149	0,0029969	0,009	0,914
hsa-let-7c	7,484	5,700	1,784	3,443	0,0029922	0,009	0,093
hsa-miR-200b	6,288	4,188	2,100	4,287	0,0032306	0,010	0,086
hsa-miR-99a	6,096	3,610	2,486	5,602	0,0032203	0,010	0,136
hsa-miR-375	6,501	4,607	1,893	3,715	0,0034005	0,010	0,099
hsa-miR-520a-5p	1,517	2,676	-1,159	0,448	0,0036914	0,011	0,889
hsa-miR-206	2,477	3,906	-1,429	0,371	0,0039186	0,011	0,852
hsa-miR-335	0,928	2,857	-1,929	0,263	0,0040328	0,011	0,864
hsa-miR-29a	6,357	3,655	2,702	6,507	0,0048819	0,013	0,123
hsa-miR-1306	3,310	5,103	-1,794	0,288	0,0051344	0,013	0,840
hsa-miR-374b	2,932	1,574	1,358	2,564	0,0050842	0,013	0,123
hsa-miR-148a	6,096	4,424	1,672	3,186	0,0055848	0,014	0,123
hsa-miR-99b	3,302	1,180	2,122	4,353	0,0057486	0,014	0,099
hsa-miR-127-3p	2,234	3,690	-1,456	0,365	0,0061676	0,015	0,852
hsa-miR-200c	6,357	3,655	2,702	6,507	0,0062925	0,015	0,111
hsa-miR-24	3,325	2,097	1,228	2,342	0,0064826	0,015	0,123
hsa-miR-4284	5,524	6,603	-1,079	0,473	0,0065698	0,015	0,883
hsa-miR-193a-3p	2,531	0,956	1,575	2,980	0,00672	0,015	0,148
hsa-miR-762	9,353	8,346	1,006	2,009	0,0071409	0,016	0,136
hsa-miR-374a	3,165	1,581	1,584	2,997	0,0073896	0,016	0,148
hsa-miR-331-3p	3,848	2,811	1,037	2,052	0,0079306	0,017	0,136
hsa-miR-425	2,465	3,875	-1,410	0,376	0,0081501	0,017	0,852
hsa-miR-125a-5p	3,350	2,016	1,333	2,520	0,0087815	0,018	0,148
hsa-let-7b	8,803	7,334	1,469	2,767	0,0090765	0,019	0,111
hsa-miR-192	2,329	3,565	-1,235	0,425	0,0093802	0,019	0,864
hsa-miR-20a	3,898	6,096	-2,197	0,218	0,0097533	0,019	0,840
hsa-miR-513a-5p	5,588	8,346	-2,758	0,148	0,012212	0,024	0,815
hsa-miR-27b	3,424	2,071	1,353	2,555	0,0136464	0,026	0,173
hsa-miR-590-5p	2,247	3,453	-1,206	0,433	0,0137301	0,026	0,864
hsa-miR-193a-5p	4,370	3,095	1,274	2,419	0,015046	0,028	0,142

hsa-miR-3609	1,661	3,150	-1,489	0,356	0,0177154	0,033	0,778
hsa-miR-26a	5,524	2,786	2,738	6,672	0,0178752	0,033	0,111
hsa-miR-373*	2,786	1,705	1,081	2,115	0,0189862	0,034	0,179
hsa-miR-205	3,898	1,934	1,965	3,903	0,0198332	0,035	0,235
hsa-miR-205*	1,817	4,162	-2,345	0,197	0,020119	0,035	0,765
hsa-miR-361-5p	2,817	1,493	1,323	2,502	0,0205336	0,035	0,173
hsa-let-7d	3,763	2,395	1,368	2,581	0,0207973	0,035	0,185
hsa-miR-3149	4,332	5,838	-1,505	0,352	0,0211157	0,035	0,765
hsa-miR-1228*	2,250	3,638	-1,388	0,382	0,0240493	0,040	0,802
hsa-miR-3679-5p	9,080	10,351	-1,272	0,414	0,0264287	0,043	0,790
hsa-miR-574-5p	7,334	8,713	-1,379	0,384	0,0291809	0,047	0,815
hsa-miR-891a	3,016	0,931	2,085	4,243	0,0302582	0,048	0,222
hsa-miR-23b	4,408	3,142	1,266	2,406	0,0304573	0,048	0,222
hsa-miR-187*	4,444	2,683	1,761	3,389	0,0309642	0,048	0,160
hsa-miR-4298	7,725	9,540	-1,815	0,284	0,0319954	0,049	0,790
hsa-miR-139-3p	4,370	3,030	1,340	2,531	0,0325726	0,050	0,185
hsa-miR-320b	5,941	4,661	1,280	2,429	0,0336794	0,051	0,210
hsa-miR-629*	4,385	3,100	1,284	2,436	0,0346252	0,051	0,198
hsa-miR-92a	5,766	3,302	2,464	5,517	0,0348344	0,051	0,160
hsa-miR-1180	2,903	4,755	-1,852	0,277	0,0362923	0,053	0,765
hsa-miR-151-5p	3,548	4,989	-1,441	0,368	0,0375297	0,054	0,815
hsa-miR-22	6,394	4,627	1,768	3,405	0,0394891	0,056	0,198
hsa-miR-1972	1,995	3,359	-1,364	0,389	0,0428626	0,060	0,765
hsa-miR-27a	2,768	1,338	1,430	2,695	0,0459805	0,064	0,235
hsa-miR-3161	3,886	2,804	1,082	2,117	0,0468246	0,065	0,198
hsa-miR-1261	1,752	3,251	-1,500	0,354	0,0503679	0,069	0,728
hsa-miR-3136	1,399	2,413	-1,014	0,495	0,0511404	0,069	0,735
hsa-miR-1280	7,484	6,256	1,228	2,342	0,0517367	0,069	0,228
hsa-miR-888	3,621	2,053	1,568	2,964	0,0537153	0,071	0,247
hsa-miR-26b	5,961	4,354	1,607	3,045	0,0633231	0,083	0,265
hsa-miR-423-5p	5,146	3,733	1,413	2,662	0,0672296	0,086	0,259
hsa-miR-720	11,975	9,455	2,520	5,736	0,0667064	0,086	0,247
hsa-miR-670	2,779	5,203	-2,424	0,186	0,0692607	0,088	0,704
hsa-miR-3148	2,989	5,229	-2,240	0,212	0,0710246	0,089	0,691
hsa-miR-21	8,147	7,138	1,009	2,012	0,0728486	0,090	0,247
hsa-miR-2278	3,142	4,678	-1,536	0,345	0,0730078	0,090	0,679
hsa-miR-497	2,753	1,698	1,055	2,078	0,0820214	0,100	0,272
hsa-miR-574-3p	4,678	3,296	1,382	2,607	0,0884003	0,107	0,247
hsa-miR-32*	4,105	5,575	-1,470	0,361	0,0965571	0,116	0,716
hsa-miR-1915*	2,140	1,140	1,001	2,001	0,1092322	0,130	0,333
hsa-miR-382	1,992	3,314	-1,322	0,400	0,1163594	0,137	0,704
hsa-miR-146b-5p	1,682	2,688	-1,006	0,498	0,1241099	0,145	0,679
hsa-miR-539	2,306	3,396	-1,090	0,470	0,1262747	0,146	0,667
hsa-miR-3692*	3,848	5,621	-1,773	0,293	0,1300607	0,149	0,765
hsa-miR-30a	4,590	2,152	2,437	5,416	0,1343708	0,153	0,284
hsa-miR-3689b*	1,847	3,350	-1,502	0,353	0,1361307	0,154	0,679
hsa-miR-3682	4,408	5,547	-1,139	0,454	0,1398576	0,155	0,778

hsa-miR-10a	3,630	5,294	-1,665	0,315	0,1394732	0,155	0,778
hsa-miR-1274b	9,080	7,670	1,410	2,657	0,1502259	0,165	0,265
hsa-miR-513b	3,424	5,766	-2,342	0,197	0,1778344	0,193	0,704
hsa-miR-101	2,775	1,504	1,271	2,414	0,1786664	0,193	0,309
hsa-miR-4286	9,540	8,435	1,104	2,150	0,1837389	0,197	0,284
hsa-let-7f	7,294	6,096	1,199	2,295	0,1989691	0,212	0,352
hsa-miR-513c	3,118	4,688	-1,570	0,337	0,2020102	0,213	0,679
hsa-miR-1274a	5,664	3,775	1,888	3,702	0,2472939	0,259	0,272
hsa-miR-30b	5,684	4,444	1,239	2,361	0,2700134	0,280	0,296
hsa-miR-3127	5,011	6,357	-1,346	0,393	0,2718685	0,280	0,605
hsa-miR-378c	1,099	2,132	-1,034	0,488	0,3341931	0,342	0,630
hsa-miR-3680*	3,337	5,091	-1,754	0,296	0,5404948	0,549	0,605
hsa-miR-3152	2,085	3,585	-1,501	0,353	0,69884	0,704	0,630
hsa-miR-887	3,733	4,856	-1,123	0,459	0,8552888	0,855	0,481

Supplemental Table 4

Expression of all miRNAs in spermatozoa samples in sub-fertile asthenozoospermic males compared to that from fertile normozoospermic males determined by miRNA-microarray.

MiRNA	Log Median Asthenozoo-spermia	Log Median Normozoo-spermia	Log Difference	Fold Change	P-value	Corrected *P*-value	AUC
hsa-miR-24	4,688	2,097	2,591	6,025	6,04E-05	0,0051946	0,037
hsa-miR-29b	5,146	3,261	1,885	3,693	5,40E-05	0,0051946	0,037
hsa-miR-27b	3,986	2,071	1,915	3,771	0,0001289	0,0073902	0,062
hsa-miR-429	4,493	1,645	2,848	7,202	0,0010792	0,0167719	0,049
hsa-miR-363	6,538	3,477	3,062	8,349	0,0023955	0,0167719	0,136
hsa-miR-27a	3,600	1,338	2,262	4,795	0,0010311	0,0167719	0,099
hsa-miR-30e	4,607	2,149	2,458	5,495	0,0009361	0,0167719	0,111
hsa-miR-148a	6,902	4,424	2,479	5,574	0,0019879	0,0167719	0,099
hsa-miR-4298	7,294	9,540	-2,245	0,211	0,0025259	0,0167719	0,889
hsa-miR-23b	5,048	3,142	1,906	3,748	0,0013232	0,0167719	0,086
hsa-miR-3154	3,424	5,423	-1,999	0,250	0,0023908	0,0167719	0,889
hsa-miR-99a	5,664	3,610	2,054	4,153	0,0024545	0,0167719	0,086
hsa-miR-29c	7,055	4,755	2,300	4,926	0,0005573	0,0167719	0,099
hsa-miR-29a	6,394	3,655	2,739	6,676	0,0015416	0,0167719	0,111
hsa-miR-939	7,434	8,864	-1,430	0,371	0,0018175	0,0167719	0,901
hsa-miR-185	1,628	0,374	1,254	2,385	0,0014884	0,0167719	0,074
hsa-miR-98	2,981	1,657	1,323	2,503	0,0024044	0,0167719	0,099
hsa-miR-141	6,574	3,600	2,974	7,858	0,0014185	0,0167719	0,086
hsa-miR-3679-5p	8,670	10,351	-1,682	0,312	0,0022037	0,0167719	0,895
hsa-miR-671-5p	6,470	8,035	-1,566	0,338	0,0008934	0,0167719	0,938
hsa-miR-3917	4,856	6,501	-1,644	0,320	0,0013789	0,0167719	0,914
hsa-miR-331-3p	4,424	2,811	1,613	3,059	0,0004591	0,0167719	0,086
hsa-miR-210	3,337	2,136	1,201	2,298	0,0008695	0,0167719	0,111
hsa-miR-26b	6,866	4,354	2,511	5,702	0,0025353	0,0167719	0,111
hsa-miR-26a	5,838	2,786	3,052	8,292	0,0024228	0,0167719	0,099

hsa-miR-146a	2,218	1,203	1,015	2,021	0,0024076	0,0167719	0,099
hsa-miR-30d	5,838	4,258	1,580	2,989	0,0027994	0,0172766	0,173
hsa-miR-200a	5,700	2,665	3,035	8,197	0,0028125	0,0172766	0,111
hsa-miR-374b	2,817	1,574	1,243	2,366	0,0031842	0,0185075	0,086
hsa-miR-374a	3,110	1,581	1,529	2,886	0,0032281	0,0185075	0,148
hsa-miR-20b	5,744	3,857	1,888	3,700	0,0033637	0,018663	0,123
hsa-miR-324-5p	2,287	1,177	1,110	2,159	0,0035174	0,0189059	0,123
hsa-let-7d	4,868	2,395	2,473	5,551	0,0039026	0,0197428	0,136
hsa-miR-103	5,067	3,182	1,885	3,693	0,0038006	0,0197428	0,099
hsa-miR-3682	4,202	5,547	-1,345	0,394	0,0042061	0,0206702	0,889
hsa-miR-638	10,351	11,488	-1,136	0,455	0,0052412	0,0217367	0,840
hsa-miR-193b	4,408	1,596	2,812	7,023	0,005298	0,0217367	0,136
hsa-miR-4290	2,337	3,518	-1,182	0,441	0,0047668	0,0217367	0,827
hsa-miR-193a-3p	2,097	0,956	1,141	2,206	0,004802	0,0217367	0,123
hsa-miR-1825	3,621	5,621	-2,000	0,250	0,0050622	0,0217367	0,833
hsa-miR-3613-3p	2,726	4,459	-1,733	0,301	0,0053078	0,0217367	0,889
hsa-miR-497	3,150	1,698	1,453	2,737	0,0048303	0,0217367	0,123
hsa-miR-361-3p	3,001	1,797	1,204	2,303	0,0054892	0,0219566	0,173
hsa-miR-1281	4,032	5,864	-1,832	0,281	0,0057222	0,0223685	0,889
hsa-miR-572	6,667	8,580	-1,913	0,265	0,0059189	0,0226234	0,852
hsa-miR-17	5,588	3,325	2,263	4,800	0,0066445	0,0243224	0,148
hsa-let-7e	3,936	2,825	1,112	2,161	0,0066462	0,0243224	0,148
hsa-miR-520b	4,926	3,600	1,326	2,508	0,0075495	0,0270524	0,160
hsa-miR-122	2,732	5,808	-3,076	0,119	0,0086261	0,0300192	0,796
hsa-miR-575	7,243	8,346	-1,104	0,465	0,0087265	0,0300192	0,944
hsa-miR-1973	3,848	7,484	-3,636	0,080	0,0091152	0,0305037	0,765
hsa-miR-361-5p	2,876	1,493	1,382	2,607	0,009222	0,0305037	0,099
hsa-miR-125a-5p	3,518	2,016	1,502	2,833	0,0094322	0,0306101	0,160
hsa-miR-1470	2,633	3,945	-1,312	0,403	0,0099302	0,0316294	0,827
hsa-miR-33b*	3,378	4,755	-1,377	0,385	0,0103545	0,0323814	0,864
hsa-miR-130b	2,890	1,298	1,591	3,013	0,0124863	0,0383508	0,148
hsa-miR-1231	-3,322	-1,370	-1,952	0,259	0,0127837	0,0385753	0,815
hsa-miR-34b	2,371	5,976	-3,605	0,082	0,0134204	0,0397984	0,778
hsa-miR-22	7,138	4,627	2,512	5,702	0,0139627	0,0403475	0,130
hsa-miR-1274a	6,394	3,775	2,619	6,143	0,0142894	0,0403475	0,210
hsa-miR-92a	5,547	3,302	2,245	4,741	0,0143093	0,0403475	0,173
hsa-miR-550a	3,261	4,424	-1,162	0,447	0,015713	0,0435909	0,821
hsa-miR-2861	10,877	11,975	-1,099	0,467	0,0166684	0,0441072	0,815
hsa-miR-320e	5,725	4,188	1,537	2,903	0,0164108	0,0441072	0,173
hsa-miR-151-3p	2,712	1,412	1,300	2,462	0,0165834	0,0441072	0,173
hsa-miR-4313	3,822	5,838	-2,016	0,247	0,0176046	0,0458787	0,815
hsa-miR-4324	2,545	1,456	1,089	2,127	0,0182291	0,046201	0,228
hsa-miR-4286	10,601	8,435	2,166	4,487	0,0182655	0,046201	0,210
hsa-miR-3614-5p	3,047	4,105	-1,058	0,480	0,0193093	0,0481334	0,840
hsa-miR-4312	2,719	3,834	-1,115	0,462	0,0218523	0,0494556	0,827
hsa-miR-30a	5,294	2,152	3,142	8,828	0,0202984	0,0494556	0,235
hsa-miR-200b	6,524	4,188	2,336	5,049	0,02214	0,0494556	0,210

hsa-let-7f	8,073	6,096	1,978	3,939	0,0221373	0,0494556	0,253
hsa-miR-127-3p	2,371	3,690	-1,319	0,401	0,0217332	0,0494556	0,802
hsa-miR-3159	1,351	0,262	1,089	2,127	0,0208105	0,0494556	0,148
hsa-miR-1299	4,047	5,838	-1,791	0,289	0,0217923	0,0494556	0,802
hsa-miR-320b	5,700	4,661	1,039	2,055	0,0212756	0,0494556	0,160
hsa-miR-425*	3,369	5,067	-1,698	0,308	0,0229474	0,0506019	0,815
hsa-miR-1225-3p	4,062	5,506	-1,444	0,368	0,0234782	0,0511171	0,815
hsa-miR-140-3p	2,507	1,000	1,506	2,841	0,0240864	0,0513871	0,198
hsa-miR-766	3,463	4,856	-1,393	0,381	0,0241997	0,0513871	0,765
hsa-miR-21	8,488	7,138	1,350	2,549	0,0254388	0,0514761	0,099
hsa-miR-1307	2,253	1,205	1,048	2,068	0,0252807	0,0514761	0,173
hsa-miR-375	6,074	4,607	1,467	2,765	0,024933	0,0514761	0,222
hsa-miR-107	5,822	4,531	1,291	2,447	0,0246636	0,0514761	0,123
hsa-miR-30e*	2,912	1,821	1,091	2,130	0,0264203	0,0528406	0,198
hsa-miR-30b	6,788	4,444	2,343	5,075	0,0279601	0,0528573	0,160
hsa-miR-1238	4,023	5,588	-1,565	0,338	0,0279652	0,0528573	0,790
hsa-miR-23a	5,744	4,023	1,721	3,297	0,0278731	0,0528573	0,173
hsa-miR-183	3,439	2,301	1,138	2,201	0,0275142	0,0528573	0,247
hsa-miR-101	3,188	1,504	1,684	3,212	0,0275105	0,0528573	0,210
hsa-miR-200c	6,310	3,655	2,655	6,297	0,0285801	0,0534323	0,241
hsa-miR-483-3p	2,582	3,972	-1,390	0,381	0,0302726	0,0559881	0,815
hsa-miR-888	3,638	2,053	1,585	2,999	0,0313011	0,0570309	0,210
hsa-miR-940	6,256	7,670	-1,414	0,375	0,0314996	0,0570309	0,778
hsa-miR-1234	4,793	5,915	-1,122	0,459	0,0331195	0,0593391	0,778
hsa-miR-3680*	3,314	5,091	-1,777	0,292	0,0344895	0,0611566	0,778
hsa-miR-205*	2,189	4,162	-1,973	0,255	0,0365462	0,0641422	0,778
hsa-miR-563	2,649	3,857	-1,207	0,433	0,0379147	0,065872	0,790
hsa-miR-601	5,838	6,945	-1,108	0,464	0,0389163	0,066936	0,778
hsa-miR-595	4,459	5,864	-1,405	0,378	0,0399503	0,0678707	0,765
hsa-miR-3132	3,219	2,180	1,039	2,054	0,0406435	0,0678707	0,185
hsa-let-7g	6,207	5,067	1,141	2,205	0,0402829	0,0678707	0,210
hsa-miR-891b	3,675	2,143	1,532	2,892	0,0411686	0,0680865	0,259
hsa-miR-3180-5p	3,310	4,354	-1,045	0,485	0,0425892	0,0695657	0,765
hsa-miR-3911	6,419	7,535	-1,116	0,461	0,0428719	0,0695657	0,765
hsa-let-7b*	3,100	4,292	-1,192	0,438	0,0442462	0,0711248	0,765
hsa-miR-191*	3,665	5,111	-1,446	0,367	0,0462479	0,0736541	0,765
hsa-miR-1267	2,279	3,282	-1,003	0,499	0,0467064	0,0737018	0,784
hsa-miR-15b	4,926	8,073	-3,147	0,113	0,0479276	0,0749413	0,778
hsa-miR-449a	2,568	5,808	-3,239	0,106	0,04965	0,0761279	0,765
hsa-miR-4257	4,504	5,547	-1,043	0,485	0,0500143	0,0761279	0,765
hsa-miR-4271	6,288	7,334	-1,046	0,484	0,0492381	0,0761279	0,728
hsa-miR-3675-3p	3,075	4,105	-1,030	0,490	0,0507207	0,076526	0,778
hsa-miR-4299	7,294	9,353	-2,059	0,240	0,0522195	0,0780259	0,753
hsa-let-7a	8,670	7,243	1,427	2,689	0,0526221	0,0780259	0,272
hsa-miR-1274b	9,741	7,670	2,071	4,202	0,0588145	0,0864624	0,222
hsa-miR-3190	4,081	5,146	-1,065	0,478	0,0617685	0,0899119	0,778
hsa-miR-29b-1*	2,297	3,494	-1,197	0,436	0,0622065	0,0899119	0,802

hsa-miR-187*	3,744	2,683	1,060	2,086	0,0639547	0,0916684	0,247
hsa-let-7c	7,484	5,700	1,784	3,443	0,0644949	0,0916788	0,296
hsa-miR-148b	3,016	1,692	1,324	2,504	0,0673325	0,0933967	0,272
hsa-miR-1539	3,302	4,424	-1,122	0,460	0,0671972	0,0933967	0,753
hsa-miR-1280	7,535	6,256	1,279	2,427	0,066317	0,0933967	0,272
hsa-miR-1306	3,645	5,103	-1,459	0,364	0,0686811	0,0938056	0,704
hsa-miR-23c	3,150	4,590	-1,439	0,369	0,0692635	0,0938056	0,753
hsa-miR-4284	5,203	6,603	-1,400	0,379	0,0692454	0,0938056	0,728
hsa-miR-513a-5p	6,074	8,346	-2,272	0,207	0,0701307	0,0941491	0,747
hsa-miR-584	2,758	4,032	-1,274	0,413	0,0706118	0,0941491	0,753
hsa-miR-1246	7,535	8,713	-1,178	0,442	0,0751596	0,0994419	0,747
hsa-miR-3149	4,408	5,838	-1,429	0,371	0,0764647	0,1003964	0,728
hsa-miR-4310	3,067	4,408	-1,342	0,395	0,0836487	0,1089968	0,753
hsa-miR-933	2,502	3,936	-1,434	0,370	0,0883563	0,1142653	0,741
hsa-miR-3692*	3,477	5,621	-2,144	0,226	0,0927452	0,1190461	0,728
hsa-miR-3152	2,026	3,585	-1,559	0,339	0,0994653	0,1261673	0,741
hsa-miR-129-3p	2,633	3,675	-1,042	0,486	0,0997602	0,1261673	0,772
hsa-miR-3713	4,627	3,557	1,070	2,099	0,1104948	0,1387234	0,272
hsa-miR-720	11,975	9,455	2,520	5,736	0,1139404	0,1420127	0,253
hsa-miR-10b	7,024	5,111	1,913	3,766	0,1193537	0,1476894	0,278
hsa-miR-373*	2,779	1,705	1,074	2,105	0,1284973	0,1578681	0,272
hsa-miR-765	6,026	7,090	-1,063	0,478	0,1367043	0,1667599	0,691
hsa-miR-30c	4,817	3,369	1,448	2,728	0,1409226	0,170695	0,259
hsa-miR-34c-5p	3,035	5,961	-2,926	0,132	0,1462327	0,1758883	0,753
hsa-miR-1273c	2,857	1,500	1,358	2,563	0,1487991	0,1777322	0,296
hsa-miR-3127	5,336	6,357	-1,022	0,493	0,1503839	0,1783864	0,673
hsa-miR-15a	4,841	6,538	-1,698	0,308	0,1545356	0,1820556	0,753
hsa-miR-19a	3,540	5,864	-2,324	0,200	0,1644224	0,1912923	0,716
hsa-miR-34b*	5,146	7,976	-2,830	0,141	0,1646003	0,1912923	0,753
hsa-miR-1180	3,274	4,755	-1,481	0,358	0,1779761	0,2054489	0,704
hsa-miR-106b	4,543	3,439	1,104	2,149	0,1888392	0,2165356	0,296
hsa-miR-3609	1,884	3,150	-1,267	0,416	0,1904712	0,2169605	0,691
hsa-miR-539	2,374	3,396	-1,022	0,493	0,1979388	0,2239833	0,642
hsa-miR-2278	3,405	4,678	-1,272	0,414	0,2041544	0,2285201	0,605
hsa-miR-32*	4,332	5,575	-1,243	0,423	0,2046052	0,2285201	0,685
hsa-miR-518e	1,574	3,405	-1,831	0,281	0,2073587	0,2301013	0,691
hsa-miR-132	2,429	3,690	-1,261	0,417	0,2264112	0,2496329	0,716
hsa-miR-625	2,512	4,300	-1,789	0,289	0,2385821	0,2613766	0,679
hsa-miR-449b	1,952	3,054	-1,102	0,466	0,2534075	0,2758613	0,741
hsa-miR-2115*	1,389	2,413	-1,024	0,492	0,2666106	0,2884089	0,691
hsa-miR-382	2,189	3,314	-1,125	0,459	0,2792122	0,3001531	0,642
hsa-miR-2116*	3,082	4,153	-1,071	0,476	0,2822014	0,3014822	0,679
hsa-let-7f-1*	3,100	4,300	-1,200	0,435	0,2914626	0,3094542	0,617
hsa-miR-365	5,483	4,424	1,059	2,083	0,2997759	0,316328	0,370
hsa-miR-670	3,484	5,203	-1,719	0,304	0,3049532	0,319829	0,605
hsa-miR-3689b*	2,128	3,350	-1,222	0,429	0,3126744	0,3259393	0,630
hsa-miR-9*	1,374	3,231	-1,857	0,276	0,3428401	0,3552319	0,630

hsa-miR-3148	3,999	5,229	-1,229	0,427	0,3628078	0,3736703	0,593
hsa-miR-499-5p	2,123	3,875	-1,752	0,297	0,3701491	0,3789622	0,691
hsa-miR-16	6,152	8,346	-2,194	0,218	0,3769466	0,383638	0,704
hsa-miR-1260	8,670	7,243	1,427	2,689	0,4055539	0,4103251	0,284
hsa-miR-335	1,546	2,857	-1,312	0,403	0,4461552	0,4487643	0,667
hsa-miR-513b	4,188	5,766	-1,578	0,335	0,6196648	0,6196648	0,605

Supplemental Table 5

Expression of miRNAs in spermatozoa samples in sub-fertile asthenozoospermic males compared to that from fertile normozoospermic males determined by miRNA-microarray(t-test - >2.0-fold difference and 5% false-discovery rate).

MiRNA	Fold Change	Regulation	P-value	Corrected *P*-value	AUC	Sequence	Chr
hsa-let-7d	5.55123	Up	0.00390	0.01974	0.13580	AACTATGCAACCTACTACC	chr9
hsa-let-7e	2.16084	Up	0.00665	0.02432	0.14815	AACTATACAACCTCCTACC	chr19
hsa-let-7f	3.93872	Up	0.02214	0.04946	0.25309	AACTATACAATCTACTACCTC	chr9
hsa-miR-103	3.69319	Up	0.00380	0.01974	0.09877	TCATAGCCCTGTACAATG	chr5
hsa-miR-125a-5p	2.83258	Up	0.00943	0.03061	0.16049	TCACAGGTTAAAGGGTCTC	chr19
hsa-miR-1274a	6.14306	Up	0.01429	0.04035	0.20988	TGGCGCCTGAACAG	chr5
hsa-miR-130b	3.01315	Up	0.01249	0.03835	0.14815	ATGCCCTTTCATCATTGC	chr22
hsa-miR-141	7.85796	Up	0.00142	0.01677	0.08642	CCATCTTTACCAGACAG	chr12
hsa-miR-146a	2.02069	Up	0.00241	0.01677	0.09877	AACCCATGGAATTCAGTTC	chr5
hsa-miR-148a	5.57358	Up	0.00199	0.01677	0.09877	ACAAAGTTCTGTAGTGCACT	chr7
hsa-miR-151-3p	2.46195	Up	0.01658	0.04411	0.17284	CCTCAAGGAGCTTCAGT	chr8
hsa-miR-17	4.80043	Up	0.00664	0.02432	0.14815	CTACCTGCACTGTAAGC	chr13
hsa-miR-185	2.38531	Up	0.00149	0.01677	0.07407	TCAGGAACTGCCTTTCT	chr22
hsa-miR-193a-3p	2.20558	Up	0.00480	0.02174	0.12346	ACTGGGACTTTGTAGGC	chr17
hsa-miR-193b	7.02336	Up	0.00530	0.02174	0.13580	AGCGGGACTTTGAGGG	chr16
hsa-miR-200a	8.19739	Up	0.00281	0.01728	0.11111	ACATCGTTACCAGACAGT	chr1
hsa-miR-200b	5.04890	Up	0.02214	0.04946	0.20988	TCATCATTACCAGGCAG	chr1
hsa-miR-20b	3.70011	Up	0.00336	0.01866	0.12346	CTACCTGCACTATGAGCAC	chrX
hsa-miR-210	2.29835	Up	0.00087	0.01677	0.11111	TCAGCCGCTGTCACAC	chr11
hsa-miR-22	5.70227	Up	0.01396	0.04035	0.12963	ACAGTTCTTCAACTGGCAG	chr17
hsa-miR-23b	3.74794	Up	0.00132	0.01677	0.08642	GGTAATCCCTGGCAATG	chr9
hsa-miR-24	6.02507	Up	0.00006040	0.00519	0.03704	CTGTTCCTGCTGAACTGA	chr9
hsa-miR-26a	8.29168	Up	0.00242	0.01677	0.09877	AGCCTATCCTGGATT	chr3
hsa-miR-26b	5.70175	Up	0.00254	0.01677	0.11111	ACCTATCCTGAATTACTTGA	chr2
hsa-miR-27a	4.79529	Up	0.00103	0.01677	0.09877	GCGGAACTTAGCCACTG	chr19
hsa-miR-27b	3.77142	Up	0.00013	0.00739	0.06173	GCAGAACTTAGCCACTGT	chr9
hsa-miR-29a	6.67581	Up	0.00154	0.01677	0.11111	TAACCGATTTCAGATGGTGC	chr7
hsa-miR-29b	3.69278	Up	0.00005	0.00519	0.03704	AACACTGATTTCAAATGGTGC	chr1
hsa-miR-29c	4.92576	Up	0.00056	0.01677	0.09877	TAACCGATTTCAAATGGTGCTA	chr1
hsa-miR-30a	8.82776	Up	0.02030	0.04946	0.23457	CTTCCAGTCGAGGATG	chr6
hsa-miR-30d	2.98905	Up	0.00280	0.01728	0.17284	CTTCCAGTCGGGGA	chr8
hsa-miR-30e	5.49486	Up	0.00094	0.01677	0.11111	CTTCCAGTCAAGGATGT	chr1
hsa-miR-3159	2.12746	Up	0.02081	0.04946	0.14815	GTGGCCGACACTTG	chr11

hsa-miR-320b	2.05499	Up	0.02128	0.04946	0.16049	TTGCCCTCTCAACCC	chr1
hsa-miR-320e	2.90264	Up	0.01641	0.04411	0.17284	CCTTCTCAACCCAGC	chr19
hsa-miR-324-5p	2.15864	Up	0.00352	0.01891	0.12346	ACACCAATGCCCTAGGG	chr17
hsa-miR-331-3p	3.05863	Up	0.00046	0.01677	0.08642	TTCTAGGATAGGCCCAGGG	chr12
hsa-miR-361-3p	2.30335	Up	0.00549	0.02196	0.17284	AAATCAGAATCACACCTGGG	chrX
hsa-miR-361-5p	2.60678	Up	0.00922	0.03050	0.09877	GTACCCCTGGAGATTC	chrX
hsa-miR-363	8.34870	Up	0.00240	0.01677	0.13580	TACAGATGGATACCGTGCA	chrX
hsa-miR-374a	2.88555	Up	0.00323	0.01851	0.14815	CACTTATCAGGTTGTATTATAA	chrX
hsa-miR-374b	2.36643	Up	0.00318	0.01851	0.08642	CACTTAGCAGGTTGTATTA	chrX
hsa-miR-4286	4.48740	Up	0.01827	0.04620	0.20988	GGTACCAGGAGTGGG	chr8
hsa-miR-429	7.20231	Up	0.00108	0.01677	0.04938	ACGGTTTTACCAGACAGTA	chr1
hsa-miR-4324	2.12704	Up	0.01823	0.04620	0.22840	TTAAGGTTAGGGTCTCAGG	chr19
hsa-miR-497	2.73710	Up	0.00483	0.02174	0.12346	ACAAACCACAGTGTGCTG	chr17
hsa-miR-520b	2.50773	Up	0.00755	0.02705	0.16049	CCCTCTAAAAGGAAGCACT	chr19
hsa-miR-92a	4.74058	Up	0.01431	0.04035	0.17284	ACAGGCCGGGACAAGT	chr13
hsa-miR-98	2.50266	Up	0.00240	0.01677	0.09877	AACAATACAACTTACTACCTC	chrX
hsa-miR-99a	4.15290	Up	0.00245	0.01677	0.08642	CACAAGATCGGATCTACGG	chr21
hsa-miR-122	8.43115	Down	0.00863	0.03002	0.79630	CAAACACCATTGTCACACT	chr18
hsa-miR-1231	3.86783	Down	0.01278	0.03858	0.81481	GCAGCTGTCCG	chr1
hsa-miR-127-3p	2.49462	Down	0.02173	0.04946	0.80247	AGCCAAGCTCAGACGGAT	chr14
hsa-miR-1281	3.55994	Down	0.00572	0.02237	0.88889	GGGAGAGGAGGAGG	chr22
hsa-miR-1299	3.46113	Down	0.02179	0.04946	0.80247	TCCCTCACACAGAATTC	chr9
hsa-miR-1470	2.48303	Down	0.00993	0.03163	0.82716	CGGGGTGCACGG	chr19
hsa-miR-1825	3.99885	Down	0.00506	0.02174	0.83333	GGAGAGGAGGGCAC	chr20
hsa-miR-1973	12.43053	Down	0.00912	0.03050	0.76543	TATGCTACCTTTGCACG	chr4
hsa-miR-2861	2.14168	Down	0.01667	0.04411	0.81481	CCGCCCACCGC	chr9
hsa-miR-3154	3.99748	Down	0.00239	0.01677	0.88889	TCTGCTCCCAACTCC	chr9
hsa-miR-33b*	2.59703	Down	0.01035	0.03238	0.86420	CAATCAGCTAATGACACTGCCT	chr11
hsa-miR-34b	12.16602	Down	0.01342	0.03980	0.77778	ATGGCAGTGGAGTTAGT	chr11
hsa-miR-3613-3p	3.32460	Down	0.00531	0.02174	0.88889	GAAGGGTTGGGCTTT	chr13
hsa-miR-3614-5p	2.08236	Down	0.01931	0.04813	0.83951	GGGCAGCCTTCAGA	chr17
hsa-miR-3679-5p	3.20819	Down	0.00220	0.01677	0.89506	TCCCCTTCCCTGCC	chr2
hsa-miR-3682	2.54043	Down	0.00421	0.02067	0.88889	CTACCTCCACCTGTATC	chr2
hsa-miR-3917	3.12616	Down	0.00138	0.01677	0.91358	CCCACCTGCTCAGT	chr1
hsa-miR-4290	2.26861	Down	0.00477	0.02174	0.82716	GAGGGAAGAAAGGAGG	chr9
hsa-miR-4298	4.74176	Down	0.00253	0.01677	0.88889	CTGCCTCCTCCTCC	chr11
hsa-miR-4312	2.16555	Down	0.02185	0.04946	0.82716	TGGGGACAGGAACAA	chr15
hsa-miR-4313	4.04353	Down	0.01760	0.04588	0.81481	GGGTTTGGGGCCA	chr15
hsa-miR-550a	2.23843	Down	0.01571	0.04359	0.82099	GGGCTCTTACTCCCT	chr7
hsa-miR-572	3.76705	Down	0.00592	0.02262	0.85185	TGGGCCACCGCCG	chr4
hsa-miR-575	2.14915	Down	0.00873	0.03002	0.94444	GCTCCTGTCCAACTGGCT	chr4
hsa-miR-638	2.19805	Down	0.00524	0.02174	0.83951	AGGCCGCCACCCGC	chr19
hsa-miR-671-5p	2.96026	Down	0.00089	0.01677	0.93827	CTCCAGCCCCT	chr7
hsa-miR-939	2.69447	Down	0.00182	0.01677	0.90123	CACCCCCAGAGCC	chr8

Supplemental Table 6

Expression of miRNAs in spermatozoa samples in sub-fertile oligoasthenozoospermic males compared to that from fertile normozoospermic males determined by miRNA-microarray (t-test - >2.0-fold difference and 5% false-discovery rate).

MiRNA	Fold Change	Regulation	P-value	Corrected *P*-value	AUC	Sequence	Chr
hsa-let-7b	2.76744	Up	0.00908	0.01858	0.1111	AACCACACAACCTACTACC	chr22
hsa-let-7c	3.44310	Up	0.00299	0.00935	0.0925	AACCATACAACCTACTACC	chr21
hsa-let-7d	2.58130	Up	0.02080	0.03538	0.1851	AACTATGCAACCTACTACC	chr9
hsa-miR-125a-5p	2.51983	Up	0.00878	0.01826	0.1481	TCACAGGTTAAAGGGTCTC	chr19
hsa-miR-139-3p	2.53093	Up	0.03257	0.04962	0.1851	ACTCCAACAGGGCCG	chr11
hsa-miR-141	8.21370	Up	0.00018	0.00199	0.0740	CCATCTTTACCAGACAG	chr12
hsa-miR-148a	3.18599	Up	0.00558	0.01407	0.1234	ACAAAGTTCTGTAGTGCACT	chr7
hsa-miR-187*	3.38918	Up	0.03096	0.04829	0.1604	GCCCGGGTCCTGT	chr18
hsa-miR-193a-3p	2.97967	Up	0.00672	0.01518	0.1481	ACTGGGACTTTGTAGGC	chr17
hsa-miR-193a-5p	2.41907	Up	0.01505	0.02816	0.1419	TCATCTCGCCCGC	chr17
hsa-miR-193b	7.44771	Up	0.00149	0.00610	0.1234	AGCGGGACTTTGAGGG	chr16
hsa-miR-200a	5.91279	Up	0.00071	0.00420	0.0740	ACATCGTTACCAGACAGT	chr1
hsa-miR-200b	4.28730	Up	0.00323	0.00962	0.0864	TCATCATTACCAGGCAG	chr1
hsa-miR-200c	6.50708	Up	0.00629	0.01499	0.1111	TCCATCATTACCCGG	chr12
hsa-miR-205	3.90320	Up	0.01983	0.03511	0.2345	CAGACTCCGGTGGAAT	chr1
hsa-miR-23b	2.40566	Up	0.03046	0.04807	0.2222	GGTAATCCCTGGCAATG	chr9
hsa-miR-24	2.34212	Up	0.00648	0.01510	0.12346	CTGTTCCTGCTGAACTGA	chr9
hsa-miR-26a	6.67219	Up	0.01788	0.03252	0.11111	AGCCTATCCTGGATT	chr3
hsa-miR-27b	2.55469	Up	0.01365	0.02607	0.1728	GCAGAACTTAGCCACTGT	chr9
hsa-miR-29a	6.50708	Up	0.00488	0.01305	0.1234	TAACCGATTTCAGATGGTGC	chr7
hsa-miR-29b	3.58511	Up	0.00001	0.00019	0.0123	AACACTGATTTCAAATGGTGC	chr1
hsa-miR-29c	4.43130	Up	0.00177	0.00683	0.09877	TAACCGATTTCAAATGGTGCTA	chr1
hsa-miR-30d	2.95591	Up	0.00119	0.00521	0.0740	CTTCCAGTCGGGGA	chr8
hsa-miR-30e	3.28943	Up	0.00214	0.00754	0.0987	CTTCCAGTCAAGGATGT	chr1
hsa-miR-320d	2.47766	Up	0.00043	0.00333	0.0370	TTGCCCTCTCAACCC	chr1
hsa-miR-320e	4.35316	Up	0.00009	0.00103	0.0493	CCTTCTCAACCCAGC	chr19
hsa-miR-331-3p	2.05185	Up	0.00793	0.01703	0.1358	TTCTAGGATAGGCCCAGGG	chr12
hsa-miR-361-5p	2.50234	Up	0.02053	0.03538	0.1728	GTACCCCTGGAGATT	chrX
hsa-miR-363	5.42084	Up	0.00100	0.00470	0.0864	TACAGATGGATACCGTGCA	chrX
hsa-miR-373*	2.11546	Up	0.01899	0.03407	0.1790	GGAAAGCGCCCCC	chr19
hsa-miR-374a	2.99710	Up	0.00739	0.01613	0.1481	CACTTATCAGGTTGTATTATAA	chrX
hsa-miR-374b	2.56413	Up	0.00508	0.01319	0.1234	CACTTAGCAGGTTGTATTA	chrX
hsa-miR-375	3.71493	Up	0.00340	0.00990	0.0987	TCACGCGAGCCGAAC	chr2
hsa-miR-429	6.06763	Up	0.00059	0.00383	0.0987	ACGGTTTTACCAGACAGTA	chr1

hsa-miR-520b	2.76481	Up	0.00099	0.00470	0.0740	CCCTCTAAAAGGAAGCACT	chr19
hsa-miR-520e	2.12475	Up	0.00092	0.00470	0.03704	CCCTCAAAAAGGAAGCACT	chr19
hsa-miR-762	2.00888	Up	0.00714	0.01586	0.1358	GCTCGGCCCCGG	chr16
hsa-miR-769-3p	2.42617	Up	0.00156	0.00621	0.0493	AACCAAGACCCCGGAG	chr19
hsa-miR-891a	4.24263	Up	0.03026	0.04807	0.2222	TCAGTGGCTCAGGT	chrX
hsa-miR-98	2.05530	Up	0.00051	0.00351	0.0555	AACAATACAACTTACTACCTC	chrX
hsa-miR-99a	5.60164	Up	0.00322	0.00962	0.1358	CACAAGATCGGATCTACGG	chr21
hsa-miR-99b	4.35320	Up	0.00575	0.01421	0.0987	CGCAAGGTCGGTTCTA	chr19
hsa-miR-122	12.35956	Down	0.00000056	0.00004	1.0000	CAAACACCATTGTCACACT	chr18
hsa-miR-122*	2.09968	Down	0.00049	0.00351	0.9696	TATTTAGTGTGATAATGGCGTT	chr18
hsa-miR-1228*	2.61694	Down	0.02405	0.03988	0.80247	CACACACCTGCC	chr12
hsa-miR-127-3p	2.74325	Down	0.00617	0.01496	0.85185	AGCCAAGCTCAGACGGAT	chr14
hsa-miR-1306	3.46708	Down	0.00513	0.01319	0.83951	CACCACCAGAGCCA	chr22
hsa-miR-132	5.45193	Down	0.00092	0.00470	0.9259	CGACCATGGCTGTAGA	chr17
hsa-miR-150	2.25692	Down	0.00031	0.00267	0.9382	CACTGGTACAAGGGTTGG	chr19
hsa-miR-15a	4.26904	Down	0.00070	0.00420	0.9135	CACAAACCATTATGTGCTGCT	chr13
hsa-miR-15b	20.10457	Down	0.00000347	0.00011	1.0000	TGTAAACCATGATGTGCTGC	chr3
hsa-miR-16	10.70660	Down	0.00023	0.00214	0.9382	CGCCAATATTTACGTGCTG	chr3
hsa-miR-192	2.35421	Down	0.00938	0.01890	0.8642	GGCTGTCAATTCATAGGTC	chr11
hsa-miR-1973	12.55374	Down	0.00003654	0.00053	1.0000	TATGCTACCTTTGCACG	chr4
hsa-miR-19a	9.85754	Down	0.00036	0.00299	0.9259	TCAGTTTTGCATAGATTTGCA	chr13
hsa-miR-205*	5.08077	Down	0.02012	0.03514	0.7654	GAACTTCACTCCACTGA	chr1
hsa-miR-206	2.69288	Down	0.00392	0.01092	0.8518	CCACACACTTCCTTAC	chr6
hsa-miR-20a	4.58603	Down	0.00975	0.01936	0.8395	CTACCTGCACTATAAGCAC	chr13
hsa-miR-2115*	2.34517	Down	0.00006579	0.00086	0.9753	CTAGCCTCCATGAATTCT	chr3
hsa-miR-25	3.73282	Down	0.00232	0.00779	0.8950	TCAGACCGAGACAAGTGC	chr7
hsa-miR-29b-2*	2.13933	Down	0.00022	0.00214	0.9691	CTAAGCCACCATGTGA	chr1
hsa-miR-3149	2.83904	Down	0.02112	0.03546	0.7654	ATACACACACATATCCATACA	chr8
hsa-miR-3154	4.10432	Down	0.00210	0.00754	0.9135	TCTGCTCCCAACTCC	chr9
hsa-miR-335	3.80914	Down	0.00403	0.01101	0.8642	ACATTTTTCGTTATTGCTC	chr7
hsa-miR-34b	18.80819	Down	0.00000043	0.00004	1.0000	ATGGCAGTGGAGTTAGT	chr11
hsa-miR-34b*	34.30493	Down	0.00000381	0.00011	0.9876	CAATCAGCTAATGACACTGCCT	chr11
hsa-miR-34c-5p	20.04993	Down	0.00001902	0.00031	0.9876	GCAATCAGCTAACTACACTG	chr11
hsa-miR-3609	2.80783	Down	0.01772	0.03252	0.7777	CAGCCAGTATTACTCATCA	chr7
hsa-miR-3679-5p	2.41444	Down	0.02643	0.04328	0.7901	TCCCCTTCCCTGCC	chr2
hsa-miR-425	2.65654	Down	0.00815	0.01722	0.8518	TCAACGGGAGTGATCGTG	chr3
hsa-miR-4284	2.11225	Down	0.00657	0.01510	0.8827	ATGGGGTGATGTGAGC	chr7
hsa-miR-4298	3.51761	Down	0.03200	0.04931	0.7901	CTGCCTCCTCCTCC	chr11
hsa-miR-4299	6.72579	Down	0.00300	0.00935	0.9135	GCCTCTCATGTCACC	chr11
hsa-miR-449a	15.50551	Down	0.00001717	0.00031	0.97531	ACCAGCTAACAATACACTGC	chr5

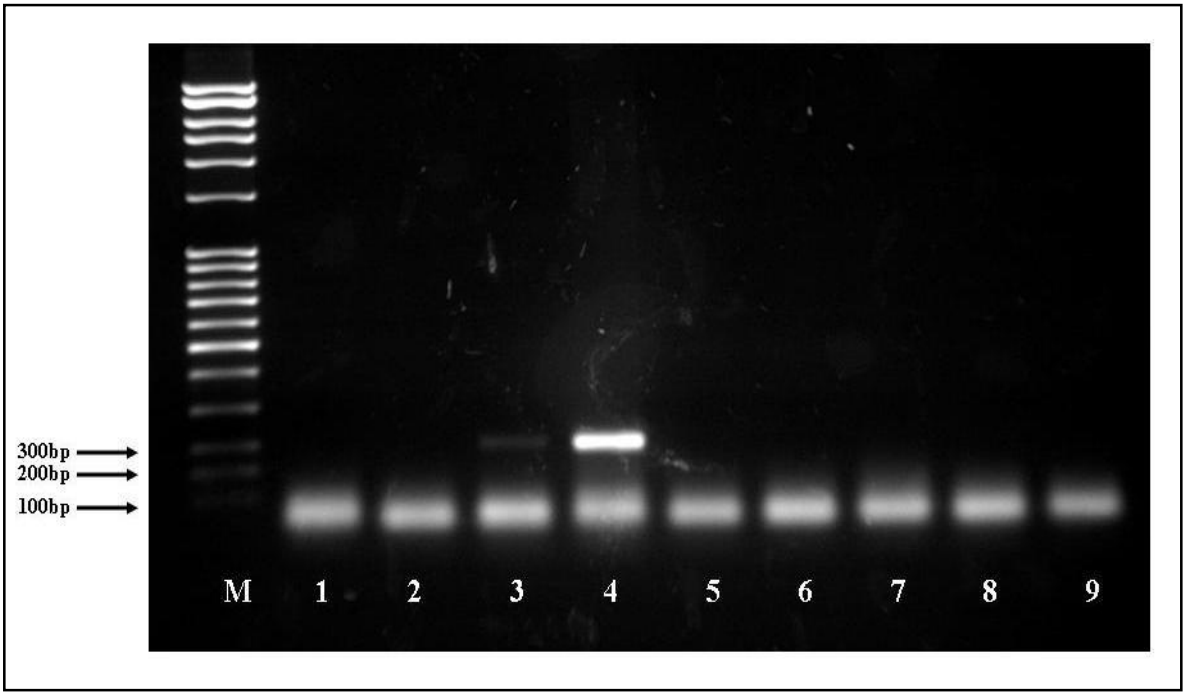
hsa-miR-449b	2.43107	Down	0.00000437	0.00011	0.98765	GCCAGCTAACAATACACTG	chr5
hsa-miR-499-5p	5.62847	Down	0.00102	0.00470	0.9382	AAACATCACTGCAAGTCTTAA	chr20
hsa-miR-513a-5p	6.76585	Down	0.01221	0.02388	0.8148	ATGACACCTCCCTGTG	chrX
hsa-miR-518e	4.77325	Down	0.00219	0.00754	0.8888	CACTCTGAAGGGAAGCGC	chr19
hsa-miR-520a-5p	2.23340	Down	0.00369	0.01051	0.8888	AGAAAGTACTTCCCTCTGG	chr19
hsa-miR-520h	2.11132	Down	0.00272	0.00892	0.8765	ACTCTAAAGGGAAGCACTTTG	chr19
hsa-miR-574-5p	2.60149	Down	0.02918	0.04719	0.8148	ACACACTCACACACACAC	chr4
hsa-miR-590-5p	2.30707	Down	0.01373	0.02607	0.8642	CTGCACTTTTATGAATAAGCTC	chr7
hsa-miR-595	5.47199	Down	0.00204	0.00754	0.9012	AGACACACCACGGCACA	chr7
hsa-miR-625	3.98266	Down	0.00104	0.00470	0.9259	GGACTATAGAACTTTCCCC	chr14
hsa-miR-873	2.35100	Down	0.00103	0.00470	0.9753	AGGAGACTCACAAGTTCCTG	chr9
hsa-miR-9*	4.65198	Down	0.00126	0.00532	0.8765	ACTTTCGGTTATCTAGCTT	chr1

Supplemental Table 7

Validation by qRT-PCR of array results for selected miRNAs analyzed in sub-fertile asthenozoospermic and oligoasthenozoospermic males as compared to fertile normozoospermic males.

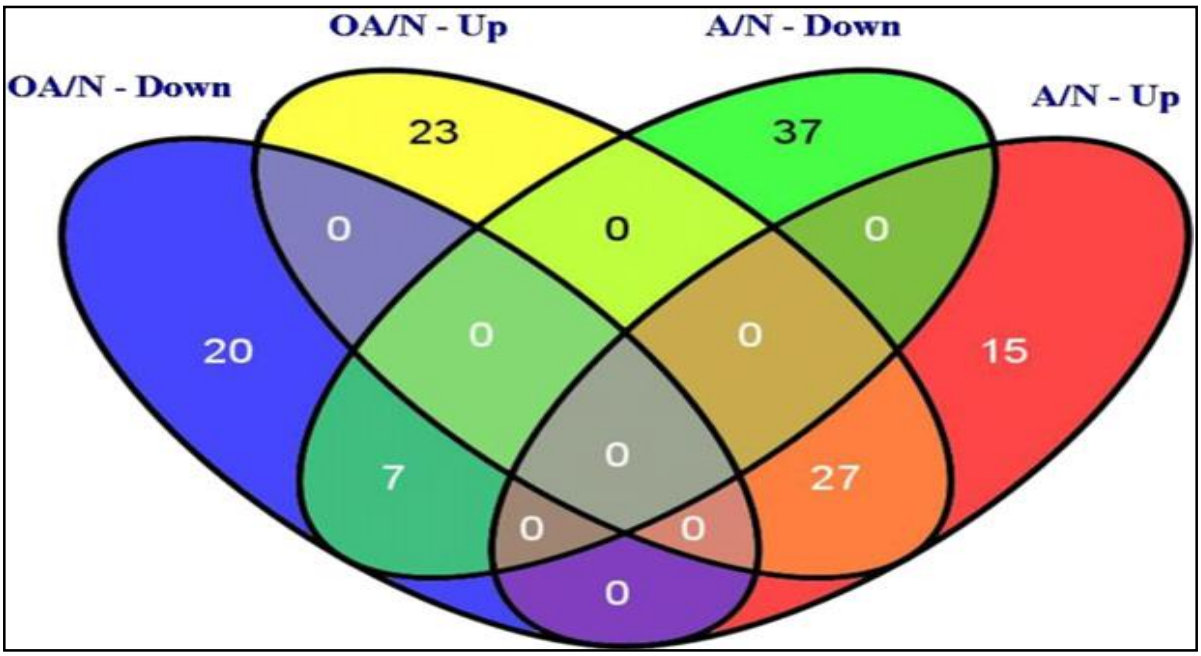
miRNA	Fold Change OA/N	Fold Change A/N	P-value OA/N	P-value A/N	P-value A/OA	Std. Error OA/N	Std. Error A/N
miR-122	- 15.15	- 3.95	0.029	0.002	0.246	± 0.34	± 0.42
miR-34c-5p	- 5.62	- 1.55	0.158	0.469	0.288	± 0.60	± 0.63
miR-34b	- 12.35	- 3.26	0.009	0.019	0.172	± 0.43	± 0.49
miR-16	- 5.78	- 1.55	0.023	0.709	0.288	± 0.65	± 0.65
miR-200a	13.40	15.67	0.001	0.012	0.884	± 0.36	±1.30
miR-141	6.39	20.65	0.008	0.019	0.329	± 0.53	±1.53

A: Asthenozoospermic males; OA; oligoasthenozoospermic males; N; normozoospermic males



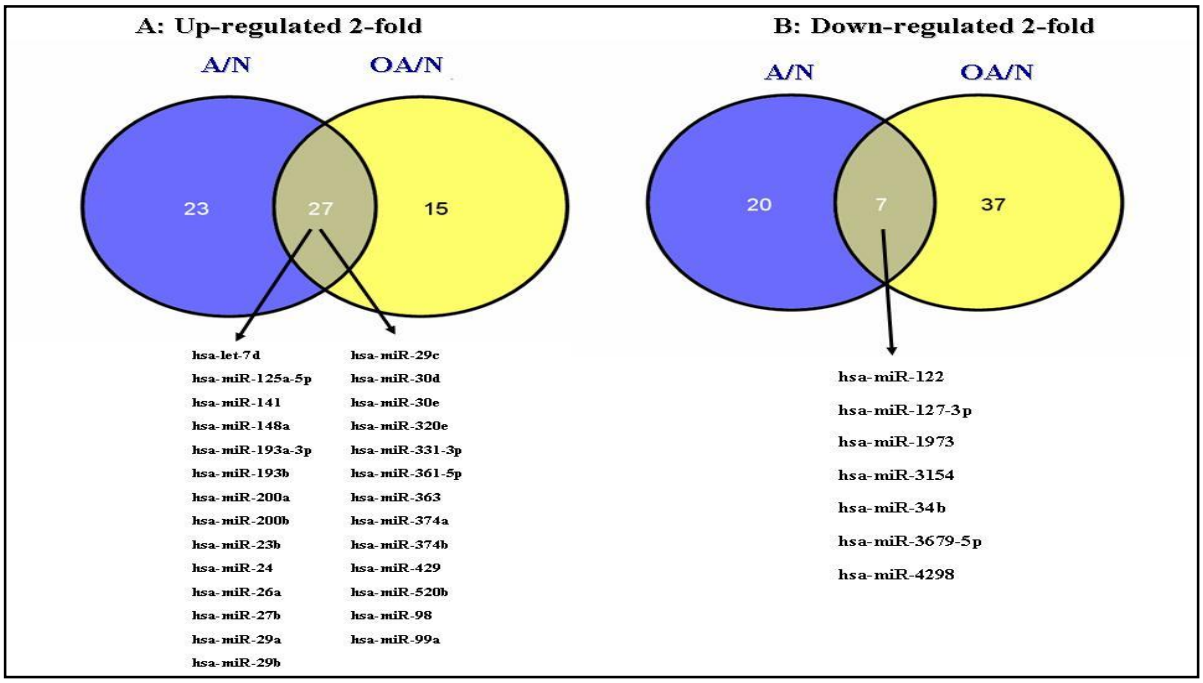
Supplemental Figure 1

Detection of Ago2/EIF2C2 (Eukaryotic Translation Initiation Factor 2C, 2): Agarose gel showing the PCR products of semen samples were prepared individually by PureSperm® density gradient purification. Spermatozoa, whole ejaculate and HeLa cell type (positive control). A representative photograph is shown for randomly samples selected from our tested groups. Highly purified spermatozoa were obtained from the PureSperm® fractions as described earlier (lane 1 and 2), whole ejaculate from the same patient sample (lane 3), HeLa cell type (lane 4), lanes 5, 6, 7, and 8 are RT- control and lane 9 PCR negative control. M corresponds to DNA ladder (100 bp).



Supplemental Figure 2

A Venn diagram showing the overlap of miRNAs that were found to be down-regulated and up-regulated in asthenozoospermic (A) and oligoasthenozoospermic (OA) compared with normozoospermic (N) sample groups. Venn diagram created with the use of Venny (http://bioinfogp.cnb.csic.es/tools/venny/index.html).



Supplemental Figure 3

Up-regulated and down-regulated miRNAs in asthenozoospermic (A) and oligoasthenozoospermic (OA) compared with normozoospermic (N) sample groups. Venn diagrams show miRNA expressions that were (A) increased 2-fold or (B) decreased 2-fold. Listed are the miRNAs that overlapped between these two different data sets. Venn diagram created with the use of Venny (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

Article II: Supplementary Tables and Figures

Supplemental Table 1

Total RNA purity and yield results from testicular tissue FFPE blocks that ranged in age from 1 to 3-year-old. The A260/280 ratio is ≥1.80, indicating that the RNA was isolated with very few contaminants.

Pathological State	Avg RNA Conc. (ng/µl)	A260/A280	Avg RNA yield (µg)
Normal Spermatogensis pattren	126.50	≥1.80	1.90
Sertoli cell only	158.78	≥1.80	2.26
Germ cell arrest	152.62	≥1.80	2.06
Mixed atrophy	114.11	≥1.80	2.37

Supplemental Table 2

Expression of all miRNAs in human testicular samples from Sertoli cell only (SCO) pattern compared to that from normal control pattern as determined by miRNA microarray (t-test ->2.0-fold difference and 5% false-discovery rate).

miRNA	Log Median SCOS	Log Median Normal	Log Difference	Fold Change	Corrected *P*-value	AUC	Regulation
hsa-miR-34b*	2,74098	9,25229	-6,51131	91,222	0,00236	0,896	Down
hsa-miR-34c-5p	2,12260	8,32715	-6,20456	73,749	0,00035	0,951	Down
hsa-miR-449a	2,20167	7,28816	-5,08649	33,977	0,00238	0,903	Down
hsa-miR-574-5p	7,98059	12,84415	-4,86356	29,112	0,03456	0,750	Down
hsa-miR-15b	4,03809	8,55942	-4,52133	22,964	0,03038	0,847	Down
hsa-miR-125b	6,37396	10,32055	-3,94659	15,419	0,01260	0,844	Down
hsa-miR-125a-5p	3,66273	7,57074	-3,90801	15,012	0,00951	0,882	Down
hsa-miR-16	5,51802	9,42105	-3,90303	14,960	0,02284	0,830	Down
hsa-miR-204	3,59361	7,44875	-3,85514	14,471	0,01349	0,840	Down
hsa-miR-1260	6,26493	9,94780	-3,68287	12,843	0,00624	0,889	Down
hsa-miR-23a	4,09031	7,74680	-3,65648	12,610	0,03477	0,778	Down
hsa-miR-145	4,72857	8,32708	-3,59851	12,113	0,01057	0,875	Down
hsa-miR-1260b	5,36078	8,85021	-3,48943	11,231	0,00472	0,885	Down
hsa-miR-30b	3,04612	6,43345	-3,38733	10,464	0,03564	0,785	Down
hsa-miR-25	2,77685	6,04422	-3,26737	9,629	0,02201	0,830	Down
hsa-miR-1274a	6,79707	10,04549	-3,24842	9,503	0,00766	0,910	Down
hsa-miR-22	5,81955	8,97805	-3,15849	8,929	0,02567	0,750	Down
hsa-miR-34b	1,81911	4,96764	-3,14853	8,868	0,00035	0,951	Down
hsa-miR-19a	2,36087	5,43230	-3,07143	8,406	0,03564	0,792	Down
hsa-miR-574-3p	3,71209	6,75852	-3,04643	8,262	0,00408	0,889	Down
hsa-miR-92a	4,53279	7,55416	-3,02137	8,119	0,01699	0,764	Down
hsa-miR-30c	2,95694	5,78689	-2,82995	7,111	0,01877	0,809	Down
hsa-miR-720	11,87932	14,67397	-2,79465	6,939	0,01348	0,792	Down
hsa-miR-342-3p	2,88557	5,49676	-2,61119	6,110	0,02201	0,792	Down
hsa-miR-1274b	10,26084	12,84415	-2,58331	5,993	0,01877	0,813	Down
hsa-miR-126	3,15328	5,72108	-2,56781	5,929	0,04306	0,757	Down
hsa-miR-517a	1,91636	4,48216	-2,56580	5,921	0,00236	0,955	Down
hsa-miR-30a	2,92674	5,46629	-2,53956	5,814	0,03948	0,813	Down
hsa-miR-425	2,26858	4,63808	-2,36950	5,168	0,04520	0,767	Down
hsa-miR-4286	9,15497	11,37001	-2,21504	4,643	0,01614	0,819	Down

hsa-miR-99b	3,13764	5,33162	-2,19398	4,576	0,01057	0,840	Down
hsa-miR-193b	3,59290	5,61606	-2,02316	4,065	0,01720	0,781	Down
hsa-miR-449b*	2,03255	4,02565	-1,99310	3,981	0,00020	0,986	Down
hsa-miR-197	4,51035	6,48984	-1,97949	3,944	0,00238	0,896	Down
hsa-miR-132	2,14879	4,10065	-1,95186	3,869	0,03598	0,785	Down
hsa-miR-517b	2,39763	4,32263	-1,92501	3,797	0,00035	0,979	Down
hsa-miR-129-3p	4,43485	6,27999	-1,84514	3,593	0,01779	0,826	Down
hsa-miR-769-5p	2,49977	4,27380	-1,77403	3,420	0,00639	0,875	Down
hsa-miR-518e	1,77446	3,53257	-1,75811	3,383	0,00121	0,993	Down
hsa-miR-320d	5,56555	7,18763	-1,62208	3,078	0,02382	0,778	Down
hsa-miR-30a*	2,25567	3,79371	-1,53803	2,904	0,02022	0,813	Down
hsa-miR-127-3p	3,31795	4,81794	-1,49999	2,828	0,04706	0,740	Down
hsa-miR-31	1,46923	2,94413	-1,47490	2,780	0,03905	0,792	Down
hsa-miR-299-5p	2,66305	4,13392	-1,47087	2,772	0,02072	0,795	Down
hsa-miR-199b-5p	2,81336	4,21617	-1,40281	2,644	0,02698	0,771	Down
hsa-miR-328	4,11851	5,49343	-1,37491	2,594	0,00192	0,944	Down
hsa-miR-3653	1,75126	3,10353	-1,35226	2,553	0,01057	0,854	Down
hsa-miR-3935	4,35857	5,68462	-1,32605	2,507	0,00238	0,917	Down
hsa-miR-30e*	1,89174	3,21208	-1,32034	2,497	0,04523	0,743	Down
hsa-miR-654-3p	2,39280	3,70521	-1,31241	2,484	0,02515	0,771	Down
hsa-miR-320e	5,35788	6,64996	-1,29208	2,449	0,02284	0,840	Down
hsa-miR-323b-3p	-1,03589	0,25298	-1,28887	2,443	0,01057	0,892	Down
hsa-miR-18a	1,45785	2,73183	-1,27398	2,418	0,02989	0,792	Down
hsa-miR-4324	3,17674	4,42273	-1,24599	2,372	0,04969	0,760	Down
hsa-miR-361-3p	2,38595	3,53191	-1,14596	2,213	0,02831	0,757	Down
hsa-miR-520g	2,56893	3,71275	-1,14382	2,210	0,00035	0,972	Down
hsa-miR-625	2,91882	4,05628	-1,13746	2,200	0,01877	0,785	Down
hsa-miR-362-3p	2,01349	3,13616	-1,12268	2,178	0,04969	0,750	Down
hsa-miR-520h	2,01179	3,13296	-1,12117	2,175	0,00136	0,931	Down
hsa-miR-224	1,61945	2,69910	-1,07965	2,114	0,00648	0,889	Down
hsa-miR-520a-5p	1,94685	3,01399	-1,06714	2,095	0,01057	0,861	Down
hsa-miR-31*	1,49001	2,54873	-1,05872	2,083	0,03477	0,833	Down
hsa-miR-512-3p	3,23256	4,28790	-1,05534	2,078	0,00035	0,965	Down
hsa-miR-1280	6,76974	7,81754	-1,04780	2,067	0,03097	0,799	Down
hsa-miR-202	2,36951	3,41698	-1,04747	2,067	0,03948	0,792	Down
hsa-miR-3907	3,65900	4,67030	-1,01130	2,016	0,00532	0,903	Down
hsa-miR-3925	6,27999	2,33269	3,94730	15,426	0,01152	0,108	Up
hsa-miR-135a*	7,99700	4,13973	3,85726	14,493	0,01102	0,132	Up
hsa-miR-1471	7,78820	4,05856	3,72965	13,266	0,01260	0,139	Up
hsa-miR-642b	9,85155	6,30893	3,54262	11,653	0,01238	0,153	Up
hsa-miR-617	6,44400	2,92295	3,52105	11,480	0,01057	0,104	Up
hsa-miR-3180-3p	6,08822	2,60334	3,48488	11,196	0,02201	0,146	Up
hsa-miR-718	7,70308	4,22854	3,47454	11,116	0,01172	0,139	Up
hsa-miR-3200-5p	5,17987	1,71731	3,46256	11,024	0,01187	0,167	Up
hsa-miR-99b*	7,00923	3,54960	3,45963	11,002	0,01738	0,167	Up
hsa-miR-3945	5,79886	2,41010	3,38877	10,474	0,00236	0,097	Up
hsa-miR-3648	9,49295	6,17458	3,31837	9,975	0,01699	0,153	Up
hsa-miR-575	8,95755	5,63974	3,31782	9,972	0,02925	0,167	Up
hsa-miR-936	5,78743	2,59120	3,19623	9,166	0,01260	0,174	Up
hsa-miR-3137	7,69974	4,52015	3,17959	9,060	0,02042	0,177	Up
hsa-miR-548q	7,28741	4,11907	3,16835	8,990	0,01152	0,122	Up
hsa-miR-4322	7,94324	4,77813	3,16511	8,970	0,02297	0,160	Up
hsa-miR-1181	8,45779	5,29679	3,16100	8,944	0,01699	0,146	Up
hsa-miR-125a-3p	8,34204	5,22014	3,12190	8,705	0,01877	0,170	Up
hsa-miR-371-5p	8,62077	5,50624	3,11453	8,661	0,02196	0,167	Up
hsa-miR-373*	6,57022	3,49290	3,07731	8,440	0,01156	0,153	Up

hsa-miR-3197	5,67681	2,65795	3,01886	8,105	0,00872	0,125	Up
hsa-miR-3656	14,67397	11,65687	3,01710	8,095	0,00624	0,160	Up
hsa-miR-3194	8,07028	5,05509	3,01519	8,085	0,02515	0,163	Up
hsa-miR-1469	6,66906	3,71806	2,95100	7,733	0,01463	0,181	Up
hsa-miR-3621	5,70917	2,75907	2,95011	7,728	0,02317	0,177	Up
hsa-miR-298	5,19216	2,27434	2,91782	7,557	0,00408	0,090	Up
hsa-miR-877	6,23720	3,33892	2,89829	7,455	0,02063	0,153	Up
hsa-miR-1254	4,77385	1,93794	2,83591	7,140	0,01057	0,132	Up
hsa-miR-3610	7,71796	4,93265	2,78530	6,894	0,03948	0,264	Up
hsa-miR-520b	6,30937	3,56081	2,74856	6,720	0,01057	0,101	Up
hsa-miR-762	10,44226	7,69513	2,74713	6,714	0,00951	0,139	Up
hsa-miR-198	6,03504	3,31934	2,71569	6,569	0,00855	0,132	Up
hsa-miR-887	5,58675	2,87648	2,71026	6,544	0,01720	0,181	Up
hsa-miR-516a-5p	4,94361	2,23894	2,70467	6,519	0,01877	0,181	Up
hsa-miR-4257	8,20009	5,58210	2,61799	6,139	0,04530	0,208	Up
hsa-miR-520e	5,96452	3,39030	2,57423	5,956	0,00951	0,108	Up
hsa-miR-4314	5,58900	3,02327	2,56573	5,921	0,03948	0,208	Up
hsa-miR-422a	4,50477	1,95230	2,55247	5,866	0,04995	0,257	Up
hsa-miR-3682	7,03034	4,50034	2,52999	5,776	0,03948	0,233	Up
hsa-miR-3131	5,79459	3,30316	2,49143	5,623	0,02201	0,188	Up
hsa-miR-4253	6,71967	4,24185	2,47782	5,571	0,01699	0,160	Up
hsa-miR-378	5,69262	3,23216	2,46047	5,504	0,01180	0,167	Up
hsa-miR-10b	5,70217	3,24257	2,45960	5,501	0,00238	0,101	Up
hsa-miR-1275	9,55984	7,13202	2,42781	5,381	0,01461	0,174	Up
hsa-miR-3622a-5p	4,57717	2,15289	2,42428	5,368	0,01653	0,160	Up
hsa-miR-1224-5p	9,90547	7,48487	2,42059	5,354	0,01699	0,177	Up
hsa-miR-3188	8,12904	5,71706	2,41198	5,322	0,04513	0,229	Up
hsa-miR-874	7,72651	5,36990	2,35660	5,122	0,00236	0,076	Up
hsa-miR-3663-3p	8,24271	5,91406	2,32865	5,023	0,03948	0,226	Up
hsa-miR-155	5,89370	3,56790	2,32580	5,013	0,01257	0,135	Up
hsa-miR-3678-3p	4,05548	1,75349	2,30199	4,931	0,02015	0,167	Up
hsa-miR-1273e	5,84049	3,54279	2,29770	4,917	0,03542	0,184	Up
hsa-miR-1208	5,00545	2,71834	2,28711	4,881	0,02538	0,208	Up
hsa-miR-630	7,36886	5,10188	2,26698	4,813	0,04969	0,257	Up
hsa-miR-1249	8,91437	6,67250	2,24186	4,730	0,01057	0,146	Up
hsa-miR-134	9,28717	7,05199	2,23517	4,708	0,02063	0,205	Up
hsa-miR-769-3p	5,10214	2,91975	2,18239	4,539	0,00776	0,104	Up
hsa-miR-3692*	4,48895	2,31025	2,17870	4,527	0,00985	0,153	Up
hsa-miR-345	5,05798	2,89121	2,16677	4,490	0,01463	0,146	Up
hsa-miR-622	5,77953	3,61988	2,15964	4,468	0,03373	0,194	Up
hsa-miR-4294	5,08628	2,93500	2,15128	4,442	0,02284	0,201	Up
hsa-miR-3937	7,57074	5,43301	2,13773	4,401	0,04306	0,247	Up
hsa-miR-564	6,49929	4,37647	2,12281	4,355	0,01187	0,153	Up
hsa-miR-3666	4,35327	2,24249	2,11078	4,319	0,02163	0,174	Up
hsa-miR-188-5p	9,52378	7,41671	2,10706	4,308	0,03979	0,236	Up
hsa-miR-663b	4,30170	2,21337	2,08833	4,253	0,03373	0,194	Up
hsa-miR-3154	5,08879	3,01649	2,07230	4,206	0,02436	0,201	Up
hsa-miR-614	4,71430	2,65599	2,05831	4,165	0,00106	0,056	Up
hsa-miR-490-5p	4,53256	2,47946	2,05309	4,150	0,03613	0,215	Up
hsa-miR-149*	5,76244	3,72350	2,03894	4,109	0,02667	0,188	Up
hsa-miR-150*	8,83531	6,81381	2,02151	4,060	0,03200	0,215	Up
hsa-miR-296-5p	7,35687	5,36166	1,99521	3,987	0,01699	0,194	Up
hsa-miR-3202	4,86046	2,87070	1,98976	3,972	0,01463	0,181	Up
hsa-miR-3201	0,96763	-0,91691	1,88454	3,692	0,01102	0,097	Up
hsa-miR-3616-3p	4,39234	2,50869	1,88365	3,690	0,00060	0,056	Up
hsa-miR-3646	6,86179	4,99708	1,86470	3,642	0,02515	0,260	Up

hsa-miR-3161	4,52738	2,67847	1,84891	3,602	0,04588	0,250	Up
hsa-miR-3122	3,22045	1,41826	1,80219	3,487	0,01102	0,181	Up
hsa-miR-3163	3,68459	1,88995	1,79463	3,469	0,01598	0,181	Up
hsa-miR-639	3,79435	2,00504	1,78931	3,456	0,02941	0,299	Up
hsa-miR-1273c	4,12445	2,35757	1,76688	3,403	0,02607	0,201	Up
hsa-miR-187*	5,06146	3,30951	1,75195	3,368	0,02382	0,201	Up
hsa-miR-183*	2,78763	1,05549	1,73214	3,322	0,01720	0,250	Up
hsa-miR-3605-5p	4,16926	2,47112	1,69814	3,245	0,04513	0,278	Up
hsa-miR-3622b-5p	4,93666	3,24169	1,69497	3,238	0,02989	0,229	Up
hsa-miR-1266	3,07699	1,38920	1,68779	3,222	0,01238	0,160	Up
hsa-miR-663	9,14394	7,51898	1,62495	3,084	0,03477	0,174	Up
hsa-miR-3196	10,71165	9,09567	1,61598	3,065	0,01238	0,194	Up
hsa-miR-3176	4,00416	2,38879	1,61537	3,064	0,03979	0,250	Up
hsa-miR-4274	6,59514	5,06240	1,53274	2,893	0,03905	0,257	Up
hsa-miR-628-3p	3,02320	1,50264	1,52056	2,869	0,01057	0,153	Up
hsa-miR-3177	3,58333	2,07827	1,50506	2,838	0,02607	0,236	Up
hsa-miR-1290	5,25280	3,74975	1,50305	2,834	0,03477	0,212	Up
hsa-miR-1228	8,13162	6,64121	1,49042	2,810	0,02868	0,194	Up
hsa-miR-3162	11,03711	9,55951	1,47760	2,785	0,02831	0,205	Up
hsa-miR-648	3,82405	2,35452	1,46952	2,769	0,04066	0,292	Up
hsa-miR-498	5,95893	4,53638	1,42255	2,681	0,03948	0,278	Up
hsa-miR-602	6,67574	5,31850	1,35724	2,562	0,03979	0,233	Up
hsa-miR-654-5p	4,18880	2,83494	1,35386	2,556	0,01877	0,146	Up
hsa-miR-423-5p	5,92799	4,58923	1,33876	2,529	0,02989	0,250	Up
hsa-miR-494	12,28141	10,96426	1,31715	2,492	0,02941	0,205	Up
hsa-miR-200b*	3,10547	1,80311	1,30237	2,466	0,03131	0,264	Up
hsa-miR-1234	7,10015	5,79888	1,30126	2,464	0,03979	0,194	Up
hsa-miR-1292	2,65010	1,36042	1,28968	2,445	0,03979	0,313	Up
hsa-miR-431	3,69312	2,41124	1,28188	2,432	0,02727	0,222	Up
hsa-miR-1243	3,29161	2,02231	1,26929	2,410	0,01102	0,115	Up
hsa-miR-452	2,58741	1,33474	1,25267	2,383	0,01749	0,198	Up
hsa-miR-492	3,20369	1,95903	1,24467	2,370	0,01001	0,156	Up
hsa-miR-296-3p	2,73223	1,48772	1,24450	2,369	0,04513	0,306	Up
hsa-miR-370	4,40878	3,17863	1,23015	2,346	0,03589	0,201	Up
hsa-miR-3174	4,28941	3,07548	1,21393	2,320	0,01463	0,188	Up
hsa-miR-30c-1*	4,06984	2,88957	1,18027	2,266	0,00624	0,097	Up
hsa-miR-1225-5p	12,20632	11,03711	1,16921	2,249	0,01544	0,177	Up
hsa-miR-4251	2,44437	1,29555	1,14882	2,217	0,03979	0,285	Up
hsa-miR-193b*	4,19187	3,04559	1,14628	2,213	0,03979	0,250	Up
hsa-miR-339-3p	3,27586	2,13036	1,14551	2,212	0,00855	0,139	Up
hsa-miR-3121	3,02622	1,88347	1,14275	2,208	0,04306	0,250	Up
hsa-miR-3065-3p	2,84015	1,73399	1,10616	2,153	0,01996	0,208	Up
hsa-miR-665	4,81032	3,70664	1,10368	2,149	0,04995	0,229	Up
hsa-miR-3180	2,01142	0,91899	1,09243	2,132	0,02727	0,229	Up
hsa-miR-10a*	2,47133	1,38289	1,08844	2,126	0,01057	0,153	Up
hsa-miR-518a-5p	3,10708	2,02350	1,08359	2,119	0,03711	0,319	Up
hsa-miR-939	9,90547	8,84668	1,05878	2,083	0,02929	0,208	Up
hsa-miR-4289	2,88668	1,82903	1,05765	2,082	0,01706	0,215	Up
hsa-miR-2114*	2,83548	1,77842	1,05706	2,081	0,01699	0,170	Up
hsa-miR-125b-1*	2,91136	1,85911	1,05225	2,074	0,04183	0,257	Up
hsa-miR-3144-5p	2,01993	0,97723	1,04269	2,060	0,00035	0,035	Up
hsa-miR-4266	2,47581	1,43342	1,04239	2,060	0,04306	0,302	Up
hsa-miR-675	2,15057	1,10838	1,04219	2,059	0,02711	0,271	Up
hsa-miR-4280	3,07328	2,06311	1,01017	2,014	0,03070	0,271	Up
hsa-miR-4291	3,28057	2,27825	1,00233	2,003	0,01902	0,181	Up

Supplemental Table 3

Expression of all miRNAs in human testicular samples from Mixed atrophy (MA) pattern compared to that from normal control pattern as determined by miRNA microarray (t-test ->2.0-fold difference and 5% false-discovery rate).

miRNA	Log Median MA	Log Median Normal	Log Difference	Fold Change	Corrected *P*-value	AUC	Regulation
hsa-miR-34c-5p	2,18971	8,32715	-6,13744	70,397	0,00017	0,993	Down
hsa-miR-34b*	3,37634	9,25229	-5,87595	58,727	0,00017	0,951	Down
hsa-miR-449a	2,27085	7,28816	-5,01730	32,386	0,00017	0,965	Down
hsa-miR-509-5p	5,17599	8,02939	-2,85341	7,227	0,02168	0,819	Down
hsa-miR-514	5,46021	8,29550	-2,83529	7,137	0,00824	0,854	Down
hsa-miR-34b	2,30352	4,96764	-2,66412	6,338	0,00017	0,938	Down
hsa-miR-517a	2,07824	4,48216	-2,40391	5,292	0,00104	0,931	Down
hsa-miR-506	5,32592	7,67676	-2,35083	5,101	0,02614	0,819	Down
hsa-miR-514b-5p	7,68965	9,90547	-2,21581	4,645	0,00112	0,896	Down
hsa-miR-129-3p	4,12069	6,27999	-2,15930	4,467	0,00041	0,941	Down
hsa-miR-507	3,29963	5,28817	-1,98854	3,968	0,00061	0,924	Down
hsa-miR-3935	3,76601	5,68462	-1,91862	3,781	0,00018	0,986	Down
hsa-miR-508-3p	3,81292	5,65385	-1,84093	3,582	0,02349	0,806	Down
hsa-miR-517b	2,50564	4,32263	-1,81699	3,523	0,00017	0,986	Down
hsa-miR-1274a	8,24862	10,04549	-1,79687	3,475	0,01060	0,833	Down
hsa-miR-34c-3p	1,82458	3,61852	-1,79394	3,468	0,00139	0,899	Down
hsa-miR-129-5p	3,43574	5,17273	-1,73699	3,333	0,00060	0,917	Down
hsa-miR-516b	2,12507	3,74536	-1,62029	3,074	0,00315	0,861	Down
hsa-miR-518e	1,91245	3,53257	-1,62012	3,074	0,00056	0,993	Down
hsa-miR-513c	6,38629	7,99700	-1,61071	3,054	0,04237	0,826	Down
hsa-miR-4298	6,78864	8,37403	-1,58539	3,001	0,03919	0,760	Down
hsa-miR-449b*	2,50873	4,02565	-1,51692	2,862	0,00029	0,944	Down
hsa-miR-513a-5p	8,15408	9,67029	-1,51620	2,860	0,01976	0,885	Down
hsa-miR-31	1,56409	2,94413	-1,38004	2,603	0,00917	0,819	Down
hsa-miR-520a-5p	1,69183	3,01399	-1,32216	2,500	0,00164	0,955	Down
hsa-miR-202	7,38637	8,68049	-1,29412	2,452	0,00059	0,917	Down
hsa-miR-551b	2,32492	3,48463	-1,15972	2,234	0,00101	0,979	Down
hsa-miR-520c-3p	2,78355	3,90100	-1,11745	2,170	0,00105	0,983	Down
hsa-miR-520g	2,59614	3,71275	-1,11661	2,168	0,00017	0,958	Down
hsa-miR-625	2,97423	4,05628	-1,08205	2,117	0,00517	0,816	Down
hsa-miR-372	1,53761	2,59132	-1,05371	2,076	0,01474	0,792	Down
hsa-miR-135b	2,66979	3,71556	-1,04577	2,064	0,00089	0,944	Down
hsa-miR-515-3p	2,59132	3,60622	-1,01489	2,021	0,00061	0,944	Down
hsa-miR-127-3p	7,23618	4,81794	2,41823	5,345	0,00044	0,069	Up
hsa-miR-410	4,58165	2,53161	2,05004	4,141	0,00480	0,132	Up
hsa-miR-199a-5p	8,17161	6,13013	2,04148	4,117	0,00164	0,090	Up
hsa-miR-379	4,57142	2,56835	2,00306	4,009	0,00205	0,139	Up
hsa-miR-432	5,45499	3,54679	1,90820	3,753	0,00017	0,069	Up
hsa-miR-193b	7,47677	5,61606	1,86071	3,632	0,02349	0,188	Up
hsa-miR-376c	6,51329	4,70860	1,80469	3,494	0,01060	0,215	Up

hsa-miR-199a-3p	9,31776	7,53632	1,78144	3,438	0,03913	0,219	Up
hsa-miR-377	5,89731	4,12604	1,77127	3,414	0,01194	0,194	Up
hsa-miR-376a	5,71968	4,01767	1,70201	3,254	0,01363	0,236	Up
hsa-miR-10b	9,25588	7,61461	1,64127	3,119	0,01736	0,132	Up
hsa-miR-409-3p	6,13750	4,50098	1,63652	3,109	0,00186	0,111	Up
hsa-miR-381	5,08579	3,46596	1,61984	3,073	0,00483	0,174	Up
hsa-miR-140-3p	5,81955	4,26749	1,55207	2,932	0,00738	0,156	Up
hsa-miR-485-5p	3,50329	2,03017	1,47312	2,776	0,00104	0,111	Up
hsa-miR-214	8,29516	6,82730	1,46787	2,766	0,02107	0,201	Up
hsa-miR-455-3p	3,96294	2,49888	1,46406	2,759	0,04672	0,247	Up
hsa-miR-193a-5p	4,80549	3,38490	1,42059	2,677	0,01060	0,153	Up
hsa-miR-382	3,91064	2,58547	1,32516	2,506	0,00148	0,125	Up
hsa-miR-370	4,50285	3,17863	1,32422	2,504	0,00766	0,139	Up
hsa-miR-150	5,71968	4,45696	1,26272	2,399	0,02836	0,233	Up
hsa-let-7i	8,40663	7,14717	1,25947	2,394	0,03112	0,226	Up
hsa-miR-299-3p	3,87441	2,62298	1,25143	2,381	0,01285	0,160	Up
hsa-miR-320b	8,53842	7,29608	1,24234	2,366	0,00273	0,111	Up
hsa-miR-99a	10,08805	8,85482	1,23323	2,351	0,01959	0,167	Up
hsa-miR-320e	7,85845	6,64996	1,20850	2,311	0,00186	0,139	Up
hsa-miR-433	3,15303	1,95803	1,19500	2,289	0,00315	0,125	Up
hsa-miR-320d	8,37403	7,18763	1,18640	2,276	0,00357	0,135	Up
hsa-miR-320c	7,87945	6,72176	1,15770	2,231	0,00186	0,097	Up
hsa-miR-193b*	4,13534	3,04559	1,08975	2,128	0,00104	0,056	Up
hsa-miR-654-5p	3,91301	2,83494	1,07806	2,111	0,00059	0,063	Up
hsa-miR-495	3,72391	2,65758	1,06633	2,094	0,03407	0,250	Up
hsa-miR-30d	7,22913	6,16317	1,06597	2,094	0,03066	0,201	Up
hsa-miR-100	9,00581	7,97598	1,02983	2,042	0,04601	0,222	Up
hsa-miR-26a	8,55942	7,54770	1,01172	2,016	0,02763	0,153	Up

Supplemental Table 4

Expression of all miRNAs in human testicular samples from Germ cell arrest (GA) pattern compared to that from normal control pattern as determined by miRNA microarray (t-test ->2.0-fold difference and 5% false-discovery rate).

miRNA	Log Median GA	Log Median Normal	Log Difference	Fold Change	Corrected *P*-value	AUC	Regulation
hsa-miR-449a	3,00689	7,28816	-4,28126	19,444	0,03251	0,778	Down
hsa-miR-34b*	5,27058	9,25229	-3,98171	15,798	0,01128	0,854	Down
hsa-miR-34c-5p	4,38014	8,32715	-3,94701	15,423	0,01297	0,833	Down
hsa-miR-34b	2,30079	4,96764	-2,66685	6,350	0,00089	0,924	Down
hsa-miR-449b*	2,03603	4,02565	-1,98962	3,971	0,00013	0,958	Down
hsa-miR-1260	7,97376	9,94780	-1,97403	3,929	0,00195	0,938	Down
hsa-miR-328	3,77796	5,49343	-1,71547	3,284	0,00007	1,000	Down
hsa-miR-574-3p	5,09241	6,75852	-1,66611	3,174	0,01597	0,802	Down
hsa-miR-1909*	2,88845	4,52738	-1,63893	3,114	0,01376	0,840	Down
hsa-miR-1260b	7,35994	8,85021	-1,49027	2,809	0,01012	0,858	Down
hsa-miR-1910	2,36061	3,70191	-1,34130	2,534	0,00935	0,868	Down
hsa-miR-517b	3,02566	4,32263	-1,29697	2,457	0,02470	0,792	Down
hsa-miR-129-3p	5,00429	6,27999	-1,27570	2,421	0,02560	0,802	Down
hsa-miR-197	5,30579	6,48984	-1,18405	2,272	0,00722	0,854	Down
hsa-miR-885-5p	2,49829	3,60813	-1,10984	2,158	0,00195	0,917	Down

hsa-let-7d*	2,59304	3,65830	-1,06526	2,093	0,00015	0,958	Down
hsa-miR-615-3p	2,51517	3,54452	-1,02935	2,041	0,04456	0,771	Down
hsa-miR-1280	6,79761	7,81754	-1,01993	2,028	0,02889	0,788	Down
hsa-miR-941	-1,03589	-0,03261	-1,00328	2,005	0,03564	0,733	Down
hsa-miR-135a*	5,90426	4,13973	1,76452	3,398	0,02889	0,194	Up
hsa-miR-3137	6,16274	4,52015	1,64259	3,122	0,04644	0,240	Up
hsa-miR-99b*	5,16783	3,54960	1,61823	3,070	0,02889	0,201	Up
hsa-miR-3692*	3,90966	2,31025	1,59941	3,030	0,02889	0,177	Up
hsa-miR-548q	5,61154	4,11907	1,49247	2,814	0,00787	0,111	Up
hsa-miR-614	4,12227	2,65599	1,46628	2,763	0,00015	0,056	Up
hsa-miR-3925	3,75343	2,33269	1,42074	2,677	0,04644	0,174	Up
hsa-miR-3945	3,78792	2,41010	1,37782	2,599	0,01975	0,181	Up
hsa-miR-1254	3,23114	1,93794	1,29321	2,451	0,01597	0,160	Up
hsa-miR-373*	4,78151	3,49290	1,28861	2,443	0,02638	0,153	Up
hsa-miR-3197	3,86742	2,65795	1,20947	2,313	0,03210	0,201	Up
hsa-miR-138-2*	3,23103	2,02511	1,20592	2,307	0,00935	0,181	Up
hsa-miR-3678-3p	2,92749	1,75349	1,17400	2,256	0,03056	0,160	Up
hsa-miR-198	4,48951	3,31934	1,17017	2,250	0,02602	0,184	Up
hsa-miR-1224-5p	8,63969	7,48487	1,15481	2,227	0,03210	0,229	Up
hsa-miR-4291	3,42744	2,27825	1,14920	2,218	0,00928	0,153	Up
hsa-miR-1268	11,19453	10,05659	1,13794	2,201	0,01376	0,149	Up
hsa-miR-1273c	3,46441	2,35757	1,10685	2,154	0,03210	0,194	Up
hsa-miR-3660	4,02565	2,93807	1,08758	2,125	0,00184	0,069	Up
hsa-miR-874	6,44601	5,36990	1,07611	2,108	0,00722	0,108	Up
hsa-miR-1291	3,09559	2,03217	1,06342	2,090	0,01591	0,194	Up
hsa-miR-1208	3,75750	2,71834	1,03916	2,055	0,01382	0,156	Up
hsa-miR-3622a-5p	3,18481	2,15289	1,03193	2,045	0,02889	0,215	Up
hsa-miR-2114*	2,80978	1,77842	1,03136	2,044	0,00184	0,083	Up
hsa-miR-3666	3,27192	2,24249	1,02943	2,041	0,03251	0,181	Up
hsa-miR-516a-5p	3,26381	2,23894	1,02488	2,035	0,03210	0,163	Up
hsa-miR-1469	4,74132	3,71806	1,02326	2,033	0,03210	0,215	Up

Supplemental Table 5

The shared miRNAs in testicular tissue samples from Sertoli-cell-only syndrome, mixed atrophy, germ cell arrest patients compared with those from normal spermatogenesis control males as determined by microarray (t test:>2.0-fold difference and 5% false-discovery rate).

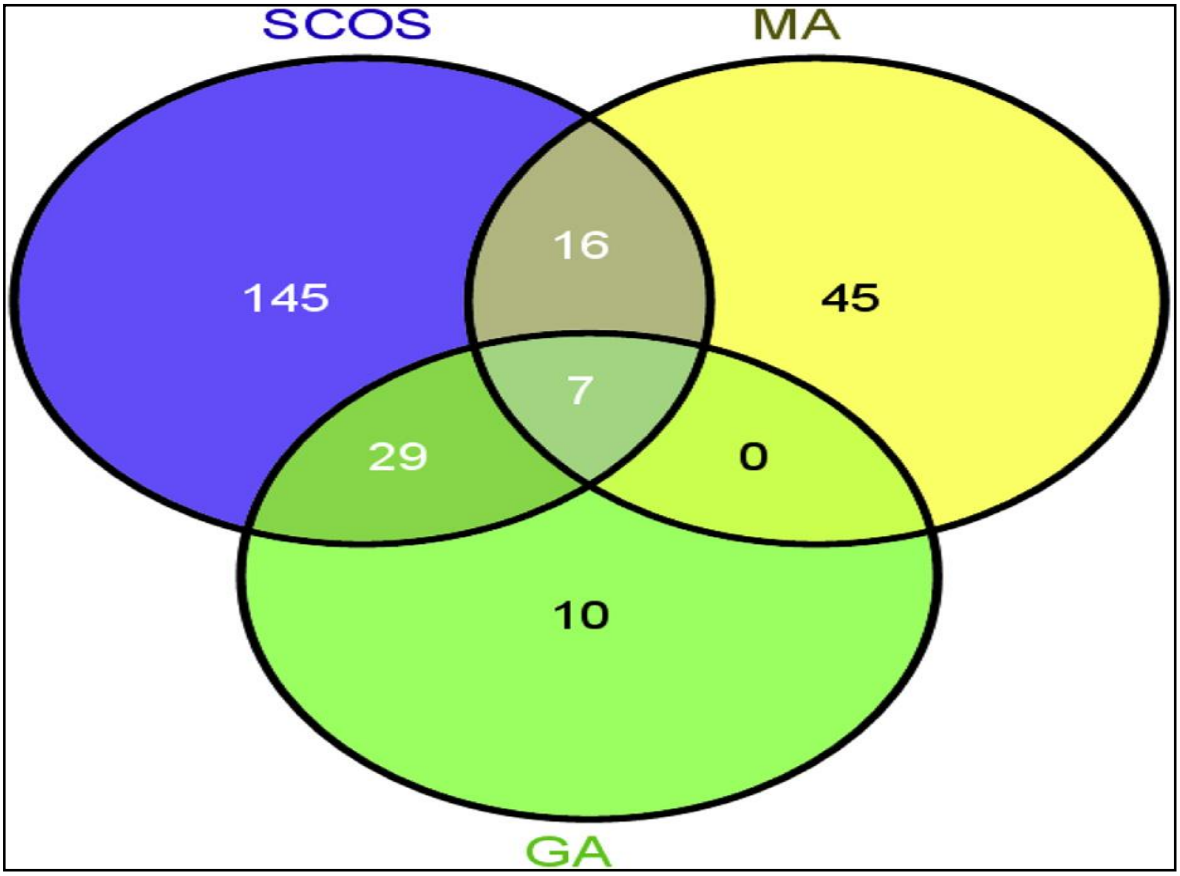
	Sertoli-cell-only			**Mixed atrophy**			**Germ cell arrest**		
miRNA	**Fold change**	**Corrected *P*-value**	**AUC**	**Fold change**	**Corrected *P*-value**	**AUC**	**Fold change**	**Corrected *P*-value**	**AUC**
hsa-miR-34b*	91,222	0,00236	0,896	58,727	0,00017	0,951	15,798	0,01128	0,854
hsa-miR-34c-5p	73,749	0,00035	0,951	70,397	0,00017	0,993	15,423	0,01297	0,833
hsa-miR-449a	33,977	0,00238	0,903	32,386	0,00017	0,965	19,444	0,03251	0,778
hsa-miR-34b	8,868	0,00035	0,951	6,338	0,00017	0,938	6,350	0,00089	0,924
hsa-miR-449b*	3,981	0,00020	0,986	2,862	0,00029	0,944	3,971	0,00013	0,958
hsa-miR-517b	3,797	0,00035	0,979	3,523	0,00017	0,986	2,457	0,02470	0,792
hsa-miR-129-3p	3,593	0,01779	0,826	4,467	0,00041	0,941	2,421	0,02560	0,802

Supplemental Table 6

Significant miRNA categories of down-regulated miRNAs in testicular tissue samples from Sertoli-cell-only syndrome, mixed atrophy, germ cell arrest patients compared with those from normal spermatogenesis obtained by TAM.

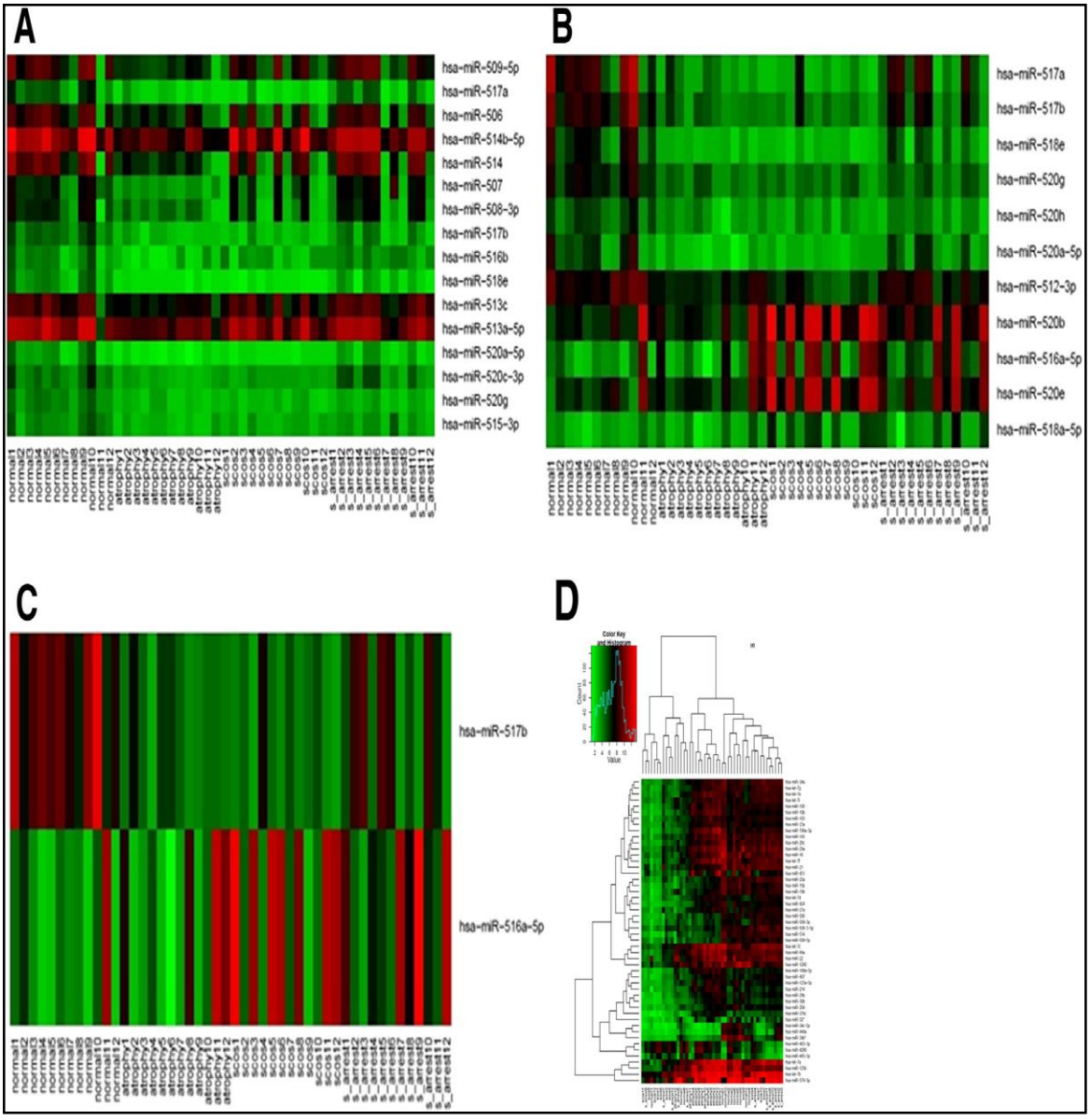
Category	Fold	P-value	FDR †
Cluster			
hsa-mir-34b cluster	97.75	7.87E-05	0.0143
hsa-mir-449a cluster	65.17	2.35E-04	0.0177
Family			
mir-34 family	65.1667	2.35E-04	0.0264
mir-449 family	65.1667	2.35E-04	0.0211
Function			
Apoptosis	9.775	9.53E-05	0.0176
Cell cycle related	6.5167	5.08E-04	0.0326
Cell proliferation	15.0385	1.56E-05	7.00E-03

†FDR < 0.05. FDR, false discovery rate.



Supplemental Figure 1

Venn diagram showing the overlap of miRNAs that were found to be down-regulated and up-regulated in Sertoli cell only syndrome (SCOS), mixed atrophy (MA), and germ cell arrest (GA) groups compared with that from normal control group (N). Diagram created with the use of Venny (http://bioinfogp.cnb.csic.es/tools/venny/index.html).



Supplemental Figure 2

Visualization of the microRNA microarray data of differentially expressed microRNAs between the samples. (A) Heat map of microRNAs correlating with mixed atrophic cell content (MA) vs. normal. (B) Heat map of microRNAs correlating with Sertoli cell only cell content (SCO) vs. normal. (C) Heat map of microRNAs correlating with germ cell arrest cell content (GA) vs. normal. (D) Clustering of 50 microRNAs with the highest variances; SCOS, MA, GA, and normal samples. Complete linkage hierarchic clustering was performed with the euclidian distance measure. The colors in the heatmap represent the normalized values, with lower values being colored in shades of green and higher values in shades of red.

Article III: Supplementary Tables and Figures

Supplemental Table 1

Semen parameters in patients and controls used in the present study.

Characteristic	Fertile Control (n=90)	Sub-Fertile (n=80)	P-value
Spermiogram			
Count (10^6/mL)	59.7 ± 2.91	17.57 ± 2.3	< 0.0001
Motility (% motile)	59.59 ± 1.21	29.31 ± 1.3	< 0.0001
Morphology (%)	33.6 ± 2.25	21.53 ± 1.62	< 0.0001
DNA Fragmentation			
TUNEL (-)	89.8% ± 1.15	80.78 % ± 1.61	< 0.0001
TUNEL (+)	10.20% ± 1.154	19.22% ± 1.61	< 0.0001

Correlation coefficient of semen parameters and DNA fragmentation

Semen parameter	TUNEL (-)	TUNEL (+)	P-value TUNEL (-)	P-value TUNEL (+)
Count (10^6/mL)	0.4889	-0.4889	0.0001	0.0001
Motility (% motile)	0.3366	-0.3366	0.0085	0.0085
Morphology (%)	0.1701	-0.1701	0.1939	0.1939

Correlation coefficient of miRNAs ΔCt and DNA fragmentation

miRNAs	TUNEL (-)	TUNEL (+)	P-value TUNEL (-)	P-value TUNEL (+)
hsa-miR-34b*	-0.3314	0.3314	0.0097	0.0097
hsa-miR-34b	-0.3995	0.3995	0.0016	0.0016
hsa-miR-34c-5p	-0.3087	0.3087	0.0164	0.0164
hsa-miR-429	0.446	-0.446	0.0004	0.0004
hsa-miR-122	-0.4159	0.4159	0.0009	0.0009

Correlation coefficient of miRNAs ΔCt and semen parameters

miRNAs	Count (10^6/mL)	Motility (% motile)	Morphology (%)	P-value (Count)	P-value (Motility)	P-value (Morphology)
hsa-miR-34b*	-0.329	-0.456	0.067	0.01	0.0003	0.612
hsa-miR-34b	-0.628	-0.596	-0.186	< 0.0001	< 0.0001	0.156
hsa-miR-34c-5p	-0.259	-0.421	-0.007	0.0455	0.0008	0.959
hsa-miR-429	0.657	0.701	0.171	< 0.0001	< 0.0001	0.192
hsa-miR-122	-0.422	-0.43	-0.035	0.00079	0.0006	0.792

Note: P<.05 was considered to be significant; P<.0001 was considered to be highly significant

7. Acknowledgements

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