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MicroRNA-targeting in spermatogenesis: Over-expressions of microRNA-23a/b-3p and its affected targeting of the genes *ODF2* and *UBQLN3* in spermatozoa of patients with oligoasthenozoospermia

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Abstract

Background: Male infertility is a multifactorial syndrome with diverse phenotypic representations. MicroRNAs (miRNAs) are small, non-coding RNAs that are involved in the post-transcriptional regulation of gene expression. Altered abundance levels of *ODF2* and *UBQLN3* have been reported in patients with different spermatogenic impairments. However, the transcriptional regulation of these two genes by miR-23a/b-3p is still unclear.

Objectives: To investigate experimentally whether miR-23a/b-3p targets the genes *ODF2* and *UBQLN3* and whether this targeting impacts abundance levels of *ODF2* and *UBQLN3* in patients with oligoasthenozoospermia.

Materials and methods: A total of 92 men attending a fertility clinic were included in the study, including 46 oligoasthenozoospermic men and 46 age-matched normozoospermic volunteers who served as controls. Reverse transcription-quantitative PCR (RT-qPCR), Western blot, and dual-luciferase (Firefly-Renilla) assays were used to validate the miRNAs and their target genes.

Results: RT-qPCR revealed that miR-23a/b-3p was more abundant and *ODF2* and *UBQLN3* targets were less abundant in men with impaired spermatogenesis. Besides, Western blot shows that ODF2 and UBQLN3 protein levels were reduced in men with impaired spermatogenesis. In silico prediction and dual-luciferase assays revealed that potential links exist between the higher abundance level of miR-23a/b-3p and the lower abundance level of *ODF2* and *UBQLN3* targets. Mutations in the miR-23a/b-3p-binding site within the 3´UTRs (3´untranslated regions) of *ODF2* and *UBQLN3* genes resulted in abrogated responsiveness to miR-23a/b-3p. Correlation analysis showed that sperm count, motility, and morphology were negatively correlated with

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miR-23a/b-3p and positively correlated with the lower abundance level of *UBQLN3*, while *ODF* lower abundance level was positively correlated with sperm motility.

Conclusion: Findings indicate that the higher abundance level of miR-23a/b-3p and the lower abundance level of *ODF2* and *UBQLN3* targets are associated with oligoasthenozoospermia and male subfertility.

KEYWORDS
male subfertility, Oligoasthenozoospermia, ODF2, UBQLN3, and miR-23a/b-3p

1 | INTRODUCTION

Male infertility is a multifactorial pathological condition with diverse phenotypic representations and affects up to 50% of couples' infertility and accounts for about 20% of total infertility.[1] The etiology of male infertility is idiopathic in approximately 40% of the cases, even after extensive endocrine, genetic, biochemical, and physical examinations.[2] Recent transcriptome analysis shows that hundreds of genes are expressed exclusively or predominantly in male germ cells.[3,4] Many of these germ cell-specific genes are thought to play a crucial role during spermatogenesis and/or sperm function. However, the cellular and biological functions of the majority of these genes are still unclear. Gene expression during spermatogenesis is highly dynamic and strictly regulated post-transcriptionally via several regulators including microRNAs (miRNAs).[5] MiRNAs are small, non-coding RNAs that are involved in the post-transcriptional regulation of gene expression by inhibiting translation or degrading messenger RNA (mRNA).[6] To date, 2300 true and validated human mature miRNAs have been reported.[7] These miRNAs are involved in many, if not all, cellular and biological processes investigated, including in the spermatogenesis process and during the early stages of embryonic development.[5,8-10] Of these miRNAs, miR-23a and miR-23b demonstrated a dysregulated abundance level in spermatozoa, seminal plasma-derived exosomes, and testicular tissue of subfertile men compared to fertile men.[11-15] Recently, increased amounts of miR-23a/b-3p and reduced amounts of testis-specific transcripts were experimentally validated in the spermatozoa and testicular tissues of oligoasthenozoospermic men as compared to that in normozoospermic men.[11] Despite significant advances made in miRNA profiling to unravel the regulatory mechanisms underlying complex multifactorial diseases, the exact molecular functions of miRNAs have only identified for a very limited number of miRNAs involved in fertility disorders. Identifying the functional mRNA targets of a particular miRNA can offer a potential therapeutic target for male infertility or male contraception. According to Human Protein Atlas (www.proteinatlas.org), 2237 testis-specific genes are showing an elevated expression in testes compared to other tissue types. Of these testis-specific genes, two genes, the outer dense fiber of sperm tail 2 (*ODF2*) and the Ubiquilin 3 (*UBQLN3*) were recently identified to be associated with spermatogenesis disorders and male infertility.[16-21] Besides, these genes have binding sites for miR-23a-3p and miR-23b-3p within their 3′UTRs. *ODF2* is expressed in the testes, preferentially in round spermatids.[22] It encodes a cytoskeleton protein that is essential in sperm cell formation,[23] and in protecting the sperm tail from shear forces that are triggered during epididymal transportation and ejaculation.[24] *ODF2* also supports the movement of the sperm tail through the axon by transmitting power in the flagellum.[20] The testis-specific gene *UBQLN3* is a ubiquitin-like protein expressed exclusively in the testes and in elongated spermatids. It is involved in protein processing in the endoplasmic reticulum pathway.[25-27] Although *UBQLN3* is highly expressed in the post-meiotic stage of spermatogenesis, and a reduced level was observed in men with non-obstructive azoospermia (NOA),[19] Yuan et al, found that *Ubqln3* is not necessary in the early stages of embryonic development and spermatogenesis.[26,27] In this study, we aim to investigate experimentally whether miR-23a/b-3p targets the genes *ODF2* and *UBQLN3* and whether this targeting impacts expression levels of *ODF2* and *UBQLN3* in men with oligoasthenozoospermia.

2 | MATERIALS AND METHODS

2.1 | Collection and preparation of human sperm samples

A total of 92 men were included in the study (mean age ±SD, 26 ± 4.2 years; range, 18–35), including 46 oligoasthenozoospermic men who attended the IVF center for infertility treatment. Another 46 age-matched normozoospermic volunteers served as controls. The semen samples were newly collected to accomplish the objective of this study and have not been included in previous studies. Semen samples were collected after at least three days of sexual abstinence and allowed to liquefy completely for ~30 min at 37°C. Basic semen parameters including sperm count (10^6/mL), motility (% motile), and morphology (%) were evaluated according to the World Health Organization (WHO) 2010 guidelines. Sperm parameters were considered normal when the semen volume was ≥1.5 mL, sperm count was ≥15 × 10^6/mL, total motility was ≥40%, and normal sperm morphology was ≥4%. Semen samples from all the participants were further purified using Puresperm 45%–90% discontinuous density gradients (Nidacon International) as previously described [14,28] and finally transferred to −80°C for long-term storage until total RNA, including miRNAs, and protein isolation.

2.2 | Sperm RNA isolation

For RNA including miRNA isolation, sperm samples stored at −80°C were thawed on ice and washed three times with phosphate-buffered saline (PBS). Spermatozoa were mixed with 700 µL QIAzol Lysis Reagent (Qiagen), supplemented with dithiothreitol (DTT) (80 mM; Sigma-Aldrich), and allowed to completely lyse for 2 h at room temperature (RT). Subsequently, the procedure was completed according to the manufacturer's instructions for Qiagen miRNeasy Mini Kit using QIAcube™ Robotic Workstation. The quantity and quality of RNA were assessed using the NanoDrop spectrophotometer (Thermo Fisher Scientific). A DNase I treatment step was included during the isolation procedure to remove any contaminating DNA using the RNase-free DNase Set (Qiagen).

2.3 | Reverse transcription and quantitative real-time PCR

Complementary DNA (cDNA) was generated in 20 µL reactions by reverse transcription (RT) of 100 ng RNA using the *mi*Script RT II Kit (Qiagen) according to the manufacturer's instructions. The 5x *mi*Script HiFlex buffer was used to prepare cDNA from total RNA including miRNAs and mRNAs. The resulting cDNA was then diluted to 0.5 and 2.5 ng/µL for the determination of miRNA and mRNA abundance levels, respectively. The cDNA was then amplified by the *mi*Script SYBR Green PCR Master Mix (Qiagen), for quantification of miR-23a-3p and miR-23b-3p and their functionally validated target genes, that is, *ODF2* and *UBQLN3*. All RT-qPCR experiments were performed using the Liquid Handling Robot QIAgility™ (Qiagen) before performing RT-qPCR using the StepOnePlus™ Real-Time PCR system (Applied Biosystems). As reference endogenous controls, the *GAPDH* was used for mRNA normalization, and the combination of RNU6B, miR-100-5p, and miR-30a-5p for miRNA normalization as previously recommended [11,14,15,28,29] because of their minimum variance between samples as observed by the DataAssist™ Software (Applied Biosystems). Moreover, no template controls (NTC) and RT negative controls were included. A miRNA RT control (miRTC) was performed to assess the performance of the RT reactions. The *mi*Script and exon-spanning QuantiTect primer assays (Qiagen) were purchased from Qiagen.

2.4 | Prediction of miR-23a/b-3p target genes

We performed an in silico analysis to identify the predicted target genes for miR-23a/b-3p, that is, hsa-miR-23a (MIMAT0000078) and hsa-miR-23b (MIMAT0000418) using miRWalk 2.0.[30] By including only the potential target genes which have been detected by at least 4 out of the 12 algorithms integrated in miRWalk, ~13,000 potential target genes were predicted. The cross-match between the predicted genes and the testis-specific genes that showed a higher abundance level in testes compared to other tissue types according to Human Protein Atlas (2237 genes) yielded 1097 targets that were further investigated for a functional role in spermatogenesis and/or sperm function. The *ODF2* and *UBQLN3* target genes were selected for further validation experiments. These two genes have binding sites for miR-23a-3p and miR-23b-3p in their 3´UTR and are also known to be involved in spermatogenesis and/or sperm function.

2.5 | Cell line, cell culture, and plasmid constructs

The human embryonic kidney cell line (HEK-293 T) was used for the dual-luciferase (Firefly-Renilla) assays. HEK-293 T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Biochrom), 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich). HEK-293 T cells were incubated at 37°C in a humidified 5% CO_2 atmosphere and subcultured following trypsinization with a 1x solution of 0.05% trypsin-EDTA (Thermo Fisher Scientific). The over-expression of miR-23a-3p and miR-23b-3p was confirmed by Northern blot in HEK-293 T cells as previously observed. [11] Both the reporter plasmid (pMIR-RNL-TK) and the expression plasmid (pSG5) harbor the SV40 promotor. The pSG5-miR-23a and pSG5-miR-23b were constructed by cloning the miR-23a-3p/5p and miR-23b-3p/5p precursors downstream to the T7 viral promoter in the pSG5 expression vector (Agilent Technologies). The 3´UTRs of *ODF2* and *UBQLN3* potential target genes were cloned in the pMIR-RNL-TK dual-luciferase reporter vector (Thermo Fisher Scientific), downstream to the luciferase gene as previously described.[11] These constructs served to determine the ability of miR-23a-3p and miR-23b-3p to suppress the luciferase expression when bound to their corresponding site in the 3´UTR. Plasmids containing the wild-type 3′UTR are designated with the suffix -WT, and those containing the 3′UTR with mutated miR-23 binding sites are designated the suffix -Mut. Specific primer sequences and restriction sites are listed in Table S1 and the size, reference, and location of the cloned fragments are listed in Table S2.

2.6 | Transfection and dual-luciferase activity assay

For dual-luciferase assays, 1×10^5 HEK-293 T cells were seeded in 24-well culture plates. After 24 h, cells in each well were simultaneously transfected with 0.2 µg of the corresponding reporter gene construct (pMIR-*ODF2* or pMIR-*UBQLN3)* and 0.8 µg of the expression vector (pSG5-miR-23a or pSG5-miR-23b). Transfections were performed using a 2 µL PolyFect transfection reagent (Qiagen) according to the manufacturer's recommendations. Empty pMIR-RNLTK and empty pSG5 vectors were included in every single treatment as controls. Two days post-transfection, cells were harvested and assayed using the dual-luciferase assay system (Promega) as recommended by the manufacturer. Each treatment was performed in duplicate, in three independent

TABLE 1 Semen characteristics

Parameters	Oligoasthenozoospermic men (*n* = 46)	Normozoospermic men (*n* = 46)	*p*-value
Count (10^6/mL)	9.40 ± 2.32	69.09 ± 15.02	<0.0001
Motility (% motile)	20.21 ± 4.84	59.33 ± 11.52	<0.0001
Morphology (%)	14.98 ± 3.94	22.26 ± 3.72	<0.0001

Note: Unpaired two-tailed t-test. Data were presented as mean ±standard deviation. $P < 0.05$ was considered statistically significant.

experiments. The results were expressed as mean relative luciferase activity ±SE (Firefly luciferase light units/Renilla luciferase light units). The experiments were carried out in seven and four independent biological replicates for the wild and mutant types, respectively, each in duplicates.

2.7 | Protein lysate preparation and western blot

Frozen human sperm samples were thawed on ice and washed four times with phosphate-buffered saline (PBS, containing 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8.1 mM Na2HPO4, and pH 7.4) by centrifugation at 10,000 × *g* at 4°C for 5 min. The pellet was resuspended in RIPA buffer containing (150 mM NaCl, 50 mM Tris, 5 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 0.5% Na-deoxycholate, and pH 7.4) supplemented with complete protease inhibitor cocktail and PhosSTOP Phosphatase Inhibitor Cocktail Tablets (Roche). The resulting sperm suspension was sonicated on ice for 2 s, incubated at 4°C for 30 min under rotation, and then centrifuged at 16,900 × *g* at 4°C for 15 min. The supernatant was collected, and the concentration of solubilized protein was determined using the bicinchoninic acid assay (BCA assay) (Thermo Fisher Scientific) according to the manufacturer's protocol. Proteins were denatured in SDS-denaturing buffer containing (4% (w/v) SDS, 60 mM Tris, 0.005% (w/v) bromophenol blue, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, and pH 6.8 at 60°C for 1 h. Western blot was performed as previously described. [31] Proteins (50 µg per lane) were separated by SDS-PAGE 4%–20% Mini-Protean TGX gels (Bio-Rad Laboratories) and transferred to a Nitrocellulose membrane (0.2 µm; Bio-Rad Laboratories). The unoccupied membrane binding sites were blocked by incubation in TBS buffer (containing in mM 150 NaCl, 10 Tris-HCl, and pH 8.0) supplemented with 5% (w/v) dry milk powder at 21°C for 1 h. The membrane was probed with rabbit polyclonal antibodies against ODF2 (1:1000; Bethyl Laboratories, Cat. Nr. A303-546A), Ubiquilin 3 (1:1000; Thermo Fisher Scientific, Cat. Nr. PA5-12080) or β-Actin (1:1000, Abcam, Cambridge, UK, Cat Nr. ab8227) overnight at 4°C under gentle shaking. After washing with TBS, the membrane was incubated with the respective secondary antibodies conjugated to the horseradish peroxidase to detect primary antibodies after the addition of the Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, Waltham, MA, USA) using the LAS-3000 analyzer (Fujifilm). Thereafter, membranes were stripped of bound antibodies and reprobed with anti-β-Actin (1:1000, Abcam, Cat Nr. ab8227) as a loading control.

2.8 | Statistical analysis

GraphPad Prism Software version 7 (GraphPad Software) was used for statistical analysis. Data were presented as mean ±SD or SEM as indicated in each paragraph. A priori power analysis with α and β error probability of 0.05 was applied to determine the sample size for each group, showing that ≥27 samples per group are required. Unpaired two-tailed t-test was used to evaluate the differences in miRNA and mRNA abundance levels between subfertile and controls. The relative quantitative method of $2^{-\Delta\Delta Ct}$ was used to measure the dynamic change of miRNA and mRNA abundance levels[32] Spearman correlation analysis was performed in GraphPad Prism to find the association between basic semen parameters and the abundance levels of *ODF2*, *UBQLN3*, miR-23a-3p, and miR-23b-3p. $p < 0.05$ was considered statistically significant. Western blot images were saved as TIFF files and transferred to Coral DRAW X7 version 17.0 for clarity and labeling.

3 | RESULTS

3.1 | Basic parameters of oligoasthenozoospermic men and normozoospermic controls

The characteristics of the spermiogram of the oligoasthenozoospermic (*n* = 46) and age-matched normozoospermic volunteers (*n* = 46) are shown in Table 1. Subfertile men with oligoasthenozoospermia were significantly different compared to normozoospermic men in terms of sperm count, motility, and morphology (p <0.05). Other parameters, such as age, volume, pH, and viscosity, were not significantly different.

3.2 | Abundance level of miR-23a-3p and miR-23b-3p in the spermatozoa

The abundance level of miR-23a-3p and miR-23b-3p was determined in 43 men with oligoasthenozoospermia compared with 43 normozoospermic volunteers using RT-qPCR analysis. As shown in Figure 1A, the abundance levels of miR-23a-3p and miR-23b-3p were significantly higher in oligoasthenozoospermic men compared to that in age-matched normozoospermic men (2.06 and 1.91 folds respectively, $p < 0.0001$).

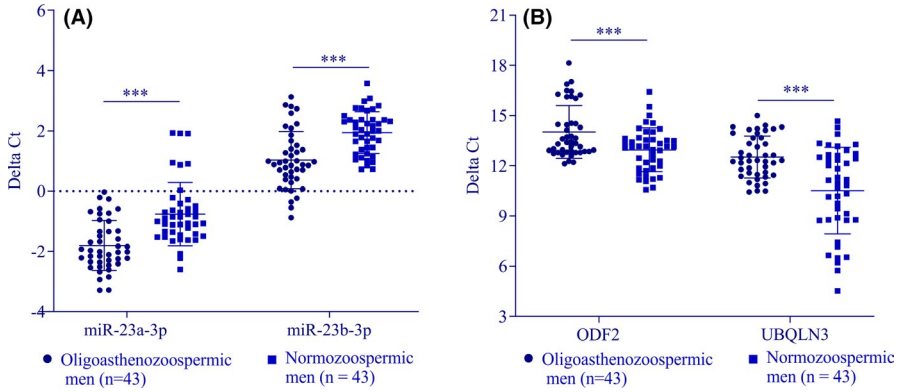

FIGURE 1 Abundance levels of (A) miR-23a/b-3p and (B) *ODF2* and *UBQLN3* in the spermatozoa from oligoasthenozoospermic men (n = 43) and age-matched normozoospermic men (n = 43) as determined by RT-qPCR. Data are presented as the mean ΔCt of oligoasthenozoospermic and normozoospermic men (lower ΔCt, higher abundance level). The p-value was calculated using unpaired two-tailed Student's t-test and $p < 0.05$ was considered statistically significant (*$p < 0.05$; **$p < 0.001$; and ***$p < 0.0001$)

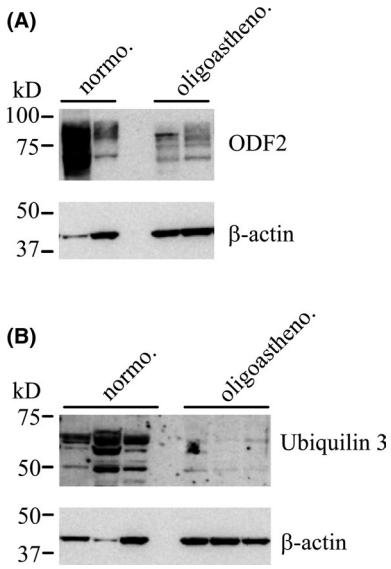

FIGURE 2 Western blot of protein lysates (50 μg per lane) isolated from spermatozoa of normozoospermic (normo.) or oligoasthenozoospermic (oligoasthen.) donors. Western blots were probed with antibodies against the (A) outer dense fiber of sperm tail 2 (ODF2) (95.4 kDa), (B) Ubiquilin 3 (70.8 kDa), and the β-actin (41.7 kDa) as a loading control

3.3 | Abundance level of ODF2 and UBQLN3 in the spermatozoa

The abundance levels of *ODF2* and *UBQLN3* genes were analyzed by RT-qPCR using the same sperm samples from men with oligoasthenozoospermia and age-matched controls. Compared with normozoospermic men, significantly reduced abundance levels of *ODF2* and *UBQLN3* were observed in oligoasthenozoospermic men compared with that in age-matched normozoospermic men (2.09- and 3.83-fold, respectively, $p < 0.0001$) (Figure 1B). In line with the RT-qPCR gene expression analysis, we performed Western blot experiments to look for the ODF2 and Ubiquilin 3 protein levels in sperm samples from normozoospermic and oligoasthenozoospermic men. In agreement with the RT-qPCR data, sperm samples from oligoasthenozoospermic men show a reduced level of ODF2 and Ubiquilin 3 protein levels compared with the control normozoospermic men (Figure 2A and B).

3.4 | Correlation of miR-23a/b-3p, ODF2, and UBQLN3 abundance levels (ΔCt) to semen parameters

To study whether there were correlations between the miR-23a/b-3p, *ODF2*, and *UBQLN3* and the basic semen parameters, Spearman correlation analyses between the abundance levels (ΔCt) of miR-23a/b-3p, *ODF2*, and *UBQLN3* and basic semen parameters were tested. As summarized in Table S3, the higher the abundance levels of miR-23a-3p and miR-23b-3p (i.e., lower ΔCt), the lower the sperm count, motility, and the number of cells exhibiting normal morphology ($p < 0.0001$). Furthermore, we analyzed the correlation between the abundance level of *ODF2* and *UBQLN3* and basic sperm parameters. The results significantly demonstrated that the *ODF2* abundance level was positively correlated with sperm motility ($p = 0.011$), while *UBQLN3* was correlated positively with sperm count, motility, and the number of cells exhibiting normal morphology ($p < 0.001$) (Table S3).

3.5 | Target gene validation by dual-luciferase assay

The binding of miR-23a-3p and miR-23b-3p on the 3′UTR of *ODF2* and *UBQLN3* target genes was validated using the dual-luciferase assay. Figure 3A and B show the location of the predicted binding sites of miR-23a-3p and miR-23b-3p in the 3′UTRs of *ODF2* and *UBQLN3*. Co-transfection of HEK-293 T cells with pMIR-ODF2-3′UTR-WT or pMIR-UBQLN3-3′UTR-WT plasmids together with plasmids expressing the corresponding miRNA leads to a reduction

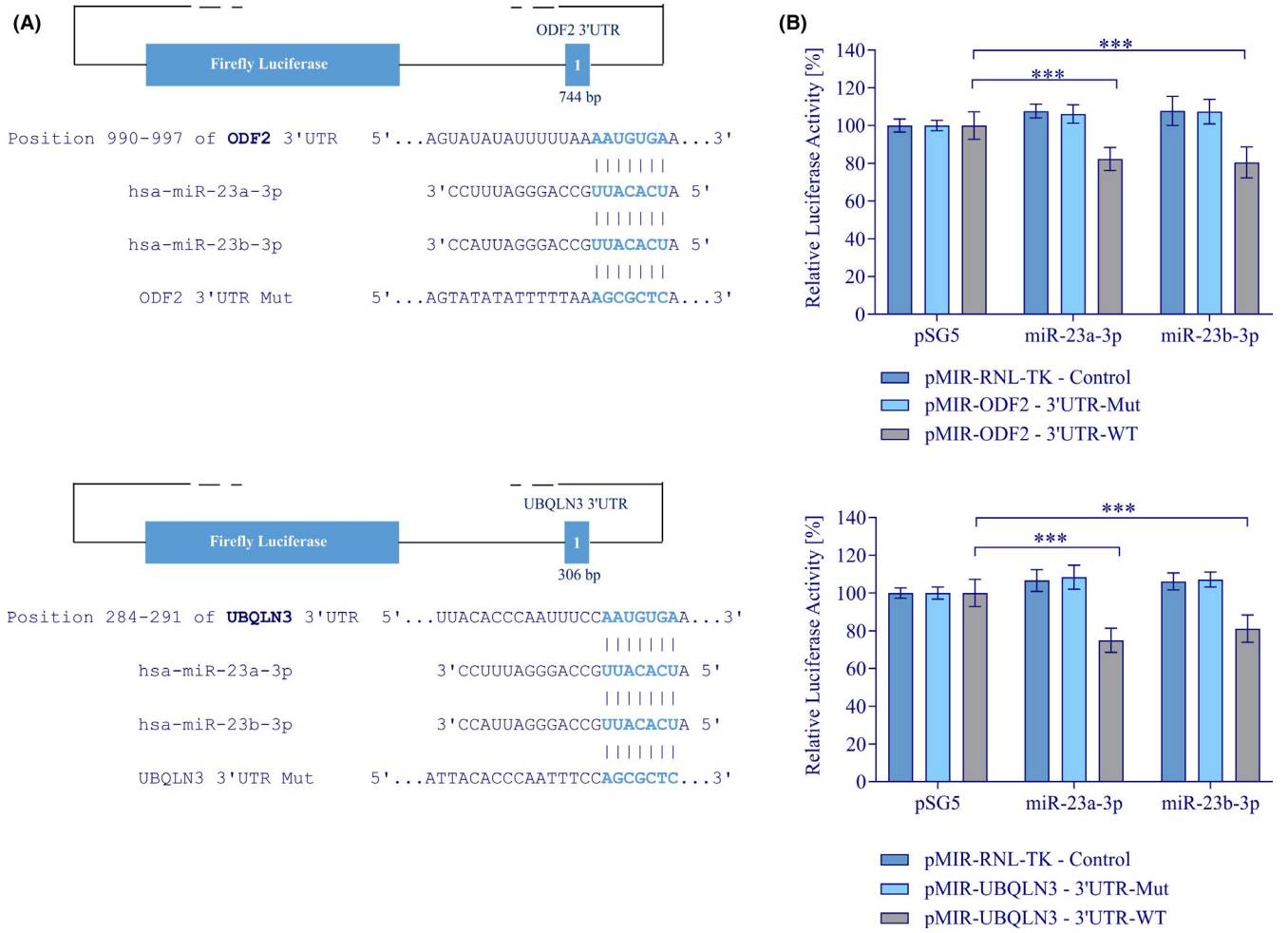

FIGURE 3 Schematic diagram of the reporter gene vectors including miR-23a/b-3p-binding sites and dual-luciferase reporter gene of the 3′UTRs of *ODF2* and *UBQLN3*. (A) ODF2-3′UTR and (B) UBQLN3-3´UTR, the location of the predicted binding sites of miR-23a-3p and miR-23b-3p in the 3´UTRs of ODF2 and UBQLN3, the sequences of the binding sites of miR-23a-3p and miR-23b-3p, and the mutated binding sites (underlined bold letters) are shown. (C) ODF2-3´UTR and (D) UBQLN3-3´UTR, HEK 293 T cells were transfected with empty vectors, reporter gene constructs, and miRNA-expression plasmids in the indicated combinations. The luciferase activity of the control vector experiment was set to 100%. The results represent the mean of seven and four independent biological replicates for the wild and mutant types, respectively, each in duplicates. Data are presented as mean ±standard deviation, and the *p*-value was calculated using unpaired two-tailed Student's t-test (*$p < 0.05$, **$p < 0.001$, and ***$p < 0.0001$)

of the luciferase activity (Figure 3C and D). A significant reduction in luciferase activity was observed when pMIR-ODF2-3′UTR-WT with plasmid bearing miR-23a-3p-binding site (reductions 22.93%, $p < 0.0001$) and miR-23b-3p (27.27%, $p < 0.0001$) (Figure 3C). Similarly, the co-transfection of miR-23a-3p and miR-23b-3p with the pMIR-UBQLN3-3′UTR-WT reporter plasmid showed a reduction of the luciferase activity by 31.63% ($p < 0.0001$) and 23.02% ($p < 0.0001$), respectively (Figure 3D). In the meanwhile, no significant reduction was observed in the case of co-transfection of an empty control vector (pMIR-RNL-TK), or vectors bearing an intentionally mutated miR-23a-3p- and miR-23b-3p-binding sites (pMIR-ODF2-3′UTR-Mut and pMIR-UBQLN3-3′UTR-Mut) (Figure 3A and B). Collectively, these results provided supporting functional evidence that miR-23a-3p and miR-23b-3p bind to the 3′UTR of *ODF2* and *UBQLN3*, resulting in a reduction of the luciferase signal activity.

4 | DISCUSSION

In this study, the higher the abundance level of miR-23a/b-3p and the lower the abundance level of *ODF2* and *UBQLN3* target genes were confirmed by RT-qPCR and Western blot, respectively, in sperm samples of 46 oligoasthenozoospermic men and 46 age-matched normozoospermic men. The abundance level of miR-23a/b-3p was negatively and *UBQLN3* was positively correlated with sperm count, motility, and the number of cells exhibiting normal morphology, while ODF2 was positively correlated with sperm motility. The over-expression of miR-23a-3p and miR-23b-3p was confirmed by Northern blot in HEK-293 T cells, as we previously described,[11] indicating that these two miRNAs (i.e., miR-23a-3p and miR-23b-3p) are actual miRNAs. A significant decrease in the luciferase activity for *ODF2* and *UBQLN3* targets was observed, suggesting a post-transcriptional regulation by miR-23a/b-3p.

Studies have revealed that the abundance level of *ODF2* and *UNQNL3* targets was decreased, both at mRNA and protein levels, in sperm and testicular tissue samples by microarray, RT-qPCR, and Western blot.[16-21] Specifically, in the sperm samples of men with asthenozoospermia, the abundance level of *ODF2* was reduced compared with that in men with normozoospermia [17] and this reduction in the abundance level might result in the aberration of the outer dense fibers (ODFs) that are surrounded the axoneme of the sperm tail and preserve the elastic rigidity of sperm flagellum,[33] resulting in reduced sperm motility. Zhao et al showed that asthenozoospermia is associated with aberrant ODF structures and that a conditional knockout of *ODF2* in mice leads to asthenozoospermia phenotype.[21] Besides, he also found a higher percentage of ODF defects in the sperm samples of men with asthenozoospermia as compared to that in normozoospermic men and the percentage of defects was significantly correlated with the percentage of axoneme defects and non-motile spermatozoa, as determined by transmission electron microscopy analysis.[21] Using Western blot analysis, the expression levels of ODF major components (*ODF1*, *ODF2*, *ODF3*, and *ODF4*) were also down-regulated in asthenozoospermic men as compared to that in normozoospermic men.[21] Altogether, these findings indicate that *ODF2* plays an essential role in protecting the sperm tail from shear forces and supports the movement of the sperm tail and its loss of function may represent an initiating event underlying male factor subfertility, specifically, asthenozoospermia. In concordance with these findings, our results show that the abundance level of *ODF2* was lower in the sperm samples of oligoasthenozoospermic men, as compared to that in normozoospermic men. Moreover, the lower abundance level was positively correlated with reduced sperm motility, providing good evidence of using *ODF2* as a biomarker for sperm dysfunction, specifically, for sperm motility.

Similarly, a significantly reduced abundance level of *UBQLN3* was observed in the testicular tissue of subfertile men with various types of NOA and controls by using microarrays.[19] In that study, the reduced abundance level was positively correlated with the degree of spermatogenic impairment.[19] Although *UBQLN3* is highly expressed in the post-meiotic stage of spermatogenesis, in elongated spermatids, Yuan et al, found that Ubqln3 is unnecessary for embryonic development and spermatogenesis in mice.[26,27] However, the abundance level of *UBQLN3* in male subfertility remains highly unclear. In agreement with our results, a reduced abundance level of *UBQLN3* was observed in the sperm samples of oligoasthenozoospermic men, when compared with the controls. It is legitimate to hypothesize that the altered abundance level of the *UBQLN3* in sperm mirrors to a high extend the *UBQLN3* expression pattern in testes, where the *UBQLN3* is highly expressed, and its altered lower expression may adversely affect the spermatogenic process and subsequently may result in the development of male subfertility. Based on our literature analysis, there is no study endeavored to ascertain the expression level of *UBQLN3*, at mRNA and/or protein levels using a reliable and robust method like RT-qPCR and/or Western blot in the spermatozoa and fresh testicular tissues from men with subfertility.

In this study, a higher abundance level of miR-23a/b-3p was validated to cause a lower abundance of *ODF2* and *UBQLN3* by RT-qPCR using the same sperm samples from men with oligoasthenozoospermia and age-matched controls. Furthermore, these results were further confirmed by Western blot showing reduced ODF2 and Ubiquilin 3 protein levels in sperm samples from men with oligoasthenozoospermia. It is thus conceivable that the binding of miR-23a/b-3p on the 3´UTR of *ODF2* and *UBQLN3* lower the abundance level of these two targets so that the phenotype of subfertility is observed.

5 | CONCLUSION

Our findings confirm our observation of the inverse correlation between the higher abundance level of miR-23a/b-3p and the lower abundance level of *ODF2* and *UBQLN3*. The observed inverse correlation levels of these two genes are likely coordinated by miR-23a/b-3p and that increased abundance level of miR-23a/b-3p may have the potential in affecting spermatogenetic activity adversely, probably through influencing the sperm count, motility, and morphology. It is important to note that the major limitation in our work was the lack of fresh testicular biopsies. The shortcoming prevented us from identifying the abundance level of these two miRNAs and genes in the testes, where the spermatogenesis process takes place. Therefore, our findings need to be further confirmed in testicular tissue samples to further define and understand the relationship between the inverse correlation of miR-23a/b-3p and *ODF2* and *UBQLN3* and male subfertility development.

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CONFLICT OF INTEREST

None of the authors have potential conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

Masood Abu-Halima performed experimental work (miRNA isolation, RT-qPCR validation, and Luciferase Assays), Masood Abu-Halima, Anouar Belkacemi, and Basim M. Ayesh drafted the manuscript. Anouar Belkacemi performed Western blot analysis. Basim M. Ayesh and Lea Simone Becker helped in the RT-qPCR and Luciferase Assays experiments, respectively. Masood Abu-Halima, Amer M. Sindiani, and Mohamed Hammadeh have recruited and diagnosed the patients and controls. Masood Abu-Halima, Ulrike Fischer, Andreas Keller, and Eckart Meese have designed the study, coordinated the molecular biology experiment, and critical revision of the manuscript. Masood Abu-Halima and Andreas Keller performed the statistical analysis. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was performed in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of the

Saarland University (Ärzekammer des Saarlandes, Saarbrücken, Ha: 195/11). All participants signed informed written consent before participating in the study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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