Supplemental methods

Seminal plasma small RNA purification

Semen samples were obtained by masturbation followed by 30 min of liquefaction at 37 ℃. Seminal plasma was obtained by centrifugating semen samples first at 1,500×g for 10 min, and three hundred microliters of seminal plasma were transferred to new tubes, and 1 ml of TRIzol™ LS Reagent (Thermo Fisher; #10296028) was added, followed by 200 µL of chloroform. Briefly, after mixing thoroughly, the samples were centrifuged at 10,000×g for 15 min to separate phases. Approximately 600 µL of the aqueous phase was carefully transferred into new tubes, and an equal volume of cold isopropanol was added, followed by centrifugation at 15,000×g for 30 min to pellet the total RNA. The RNA pellets were washed twice with 75% ethanol and resolved in 100 µL of hot nuclease-free water. To remove the large amount of polysaccharide coprecipitated with RNA and enrich small RNAs (sRNAs) (<200 nt), we performed sRNA purification using the mirVana™ miRNA Isolation Kit (Thermo Fisher; #AM1561). Briefly, 500 µL of lysis/binding buffer was added to 100 µL of total RNA together with 50 µL of additive. A 1/3 volume of 100% ethanol was added first to the mixture and passed through the column to remove the large RNAs. The filtrate was saved for sRNA isolation, and a 2/3 volume of 100% alcohol was added to the filtrate and passed through the second column to recover the sRNA. After 3 washes, 30 µL of sRNAs was eluted with nuclease-free water.

Multiplex RT-qPCR for microRNAs

Briefly, 1 µL of 50 nM SLP cocktail (13 SLP mixture) together with 10 mM dNTPs, 0.1 M DTT, 40 units of RNaseOUT™ (Thermo Fisher; #10777-019) and 200 units of SuperScript™ II Reverse Transcriptase (SS II RTase) (Thermo Fisher; #18064014) were used for each RT reaction. All 13 microRNAs were converted into cDNAs in one reaction. The conditions were as follows: 16 ℃ for 30 min, followed by 60 cycles of 20 ℃ for 30 s, 42 ℃ for 30 s, 50 ℃ for 1 s and termination at 85 ℃ for 5 min. For the preamplification, the total RT product (20 µL) was used as a template, together with 50 nM of forward primer (FP) cocktail (mixture of 13 microRNA-specific primers), 5 µM of universal reverse primer (URP) and 25 µL of 2× Q5® High-Fidelity Master Mix (NEB; #M0492L). The conditions for the pre-PCR were 98 ℃ for 30 s followed by 12 cycles of 98 ℃ for 10 s, 68 ℃ for 30 s, 72 ℃ for 30 s and 72 ℃ for 5 min. The cleanup treatment with ExoSAP-IT reagent was performed as follows: 10 µL of pre-PCR product together with 4 µL of ExoSAP-IT reagent (Thermo Fisher; #75001) was incubated at 37 ℃ for 30 min followed by 15 min of inactivation at 80 ℃.

One microliter of 1:200 diluted pre-PCR product was used as a qPCR template for a 20-µL reaction, together with 250 nM of FP, 250 nM of URP and 1× TB Green Premix Ex Taq II (TAKARA; #RR820A). The probe qPCR was performed using the same amount of template and primers, together with 0.2 µM of hydrolysis probe and 1× Premix Ex Taq™ (TAKARA; #RR390L). The conditions were as follows: 95 ℃ for 30 s, followed by 40 cycles at 95 ℃ for 10 s and 60 ℃ for 1 min. All the reactions were run on a LightCycler 480 System (Roche).

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Table S1 Purified microRNA characteristics

Sample No.	Sample name	Con. (ng/μL)	Amount (μg)	RNA integrity number (RIN)	28S/18S	A260/A280	A260/A230
1	P-1471	104	0.88	2.6	0.0	1.92	2.07
2	P-3073	32	0.27	1.1	0.0	1.95	2.2
3	P-3072	23	0.21	2.6	0.0	2.01	2.13
4	P-2972	19	0.15	2.5	0.0	1.95	2.12
5	P-3306	19	0.16	2.5	0.0	1.91	2.08
6	P-3204	16	0.14	2.4	0.0	2.03	2.14
7	F-361	134	1.21	2.6	0.0	1.97	2.02
8	F-188	62	0.56	2.6	0.0	1.93	2.36
9	F-024	46	0.41	2.6	0.0	1.93	2.17
10	F-3922	67	0.54	2.8	0.0	1.92	2.21
11	F-3292	21	0.18	2.3	0.0	2.02	2.35
12	F-3231	15	0.13	2.3	0.0	1.95	2.27
13	G-0544	44	0.4	2.6	0.0	1.92	2.21
14	G-1045	56	0.48	1.5	0.0	1.93	2.17
15	G-0629	51	0.46	2.6	0.0	2.01	2.25
16	G-50	36	0.32	2.6	0.0	2.03	2.23
17	G-68	34	0.29	2.6	0.0	1.97	2.36
18	G-48	26	0.23	2.8	0.0	1.97	2.33

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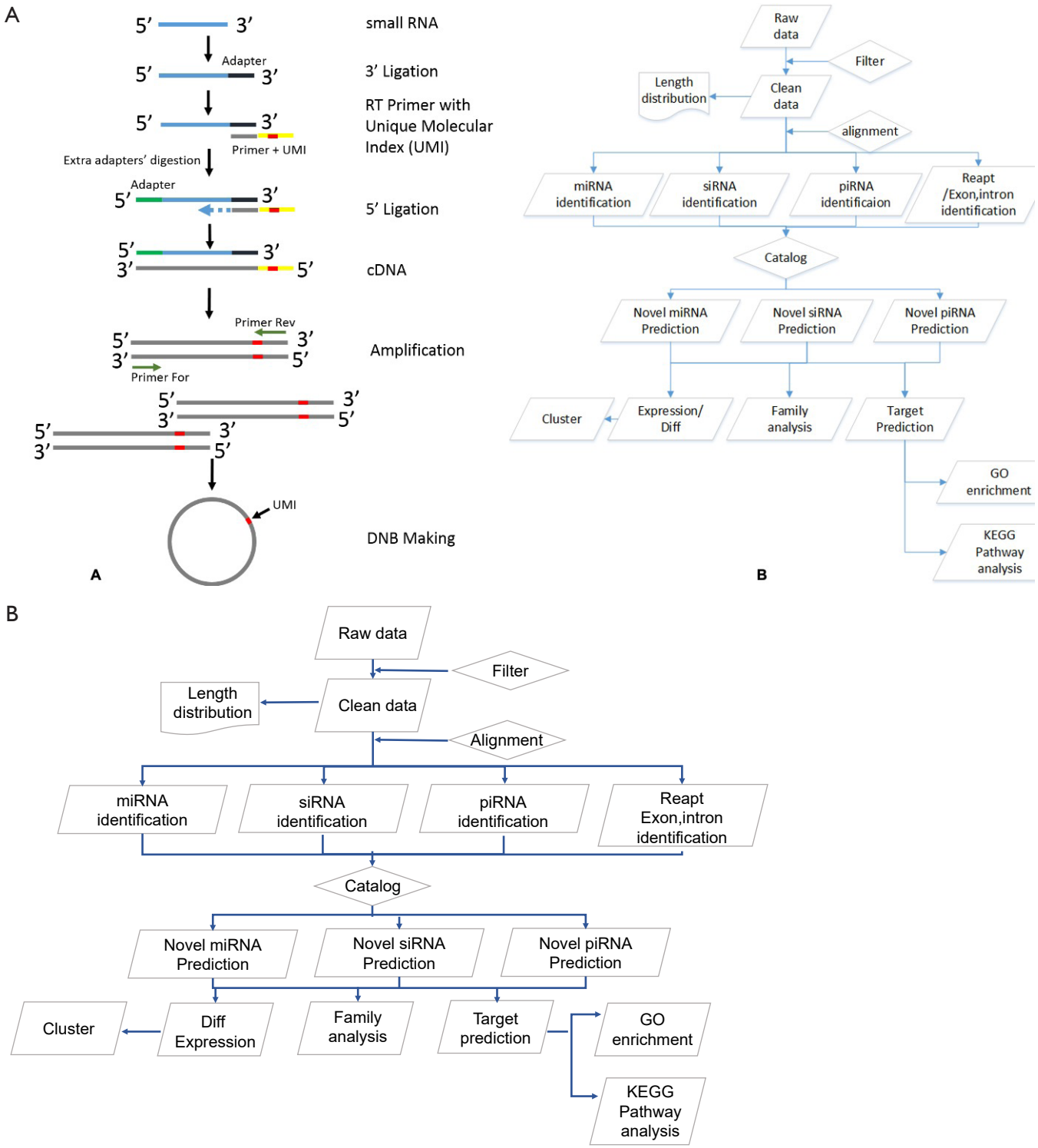

Figure S1 Absolute quantitative HTS and bioinformatic analysis. (A) The experiment pipeline steps for sRNA-seq. (B) The bioinformatics pipeline. HTS, high-throughput sRNA sequencing; Primer Rev, primer reverse; Primer For, primer forward; DNB, DNA nanoball; miRNA, microRNA; siRNA, small interfering RNA; piRNA, PIWI-interacting RNA; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

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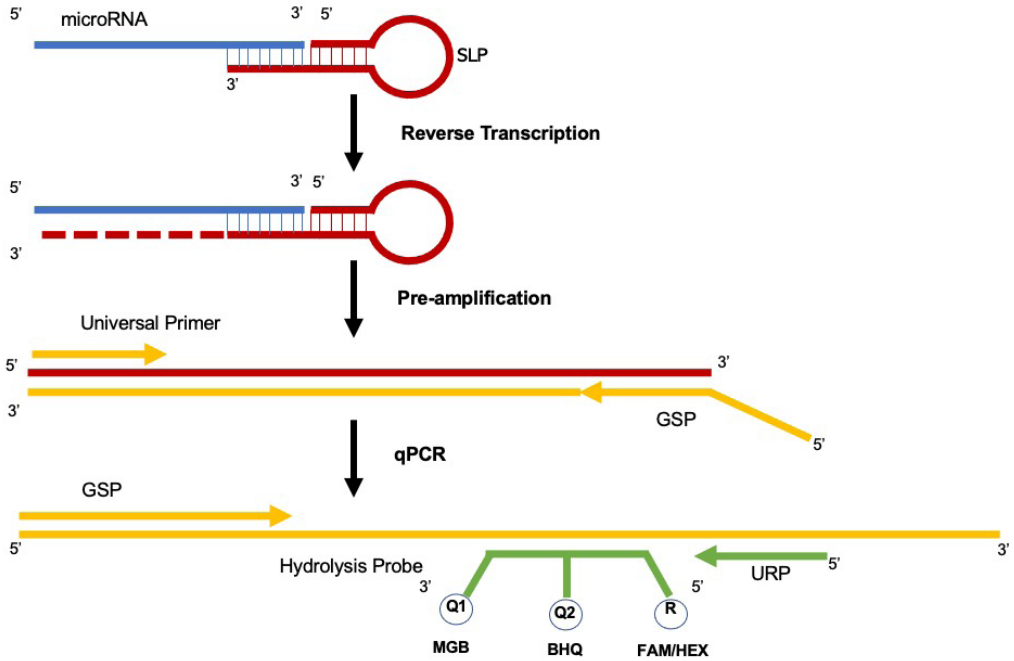

Figure S2 Schematic of stem-loop-based microRNA qRT-PCR. SLP, stem-loop primer; GSP, gene specific primer; URP, universal reverse primer.

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Table S2 Primer set

Primers	Sequence
miR-34b-3p SLR	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGATGGCAGT
miR-34c-3p SLR	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCCTGGCCG
miR-3065-3p SLP	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCTCCAACA
miR-4446-3p SLP	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACCCATGT
miR-676-3p SLP	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAACTCAAC
miR-376a-3p SLP	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACGTGGAT
miR-449b-3p SLP	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGTGGCAG
miR-202-5p SLP	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCAAAGAAG
miR-942-5p SLP	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCACATGGC
miR-518e-3p SLP	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCACTCTGA
miR-891b SLP	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCAATGAC
miR-30b-3p SLP	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGAAGTAAA
cel-miR-39-3p SLP	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCAAGCTGA
hsa-miR-34b-3p F	ACACTCCAGCTATACAATCACTAACT
hsa-miR-34c-3p F	ACACTCCAGCTATAAATCACTAACCA
hsa-miR-3065-3p F	ACACTCCAGCTATATCAGCACCAGG
hsa-miR-4446-3p F	ACACTCCAGCTATACAGGGCTGGC
hsa-miR-676-3p F	ACACTCCAGCTATACTGTCCTAAGGT
hsa-miR-376a-3p F	ACACTCCAGCTATAATCATAGAGGA
hsa-miR-449b-3p F	ACACTCCAGCTATACAGCCACAACTA
hsa-miR-202-5p F	ACACTCCAGCTATATTCCTATGCATA
hsa-miR-942-5p F	ACACTCCAGCTATATCTTCTCTGTTT
hsa-miR-518e-3p F	ACACTCCAGCTATAAAAGCGCTTCCC
hsa-miR-891b F	ACACTCCAGCTATATGCAACTTACCT
hsa-miR-30b-3p F	ACACTCCAGCTATACTGGGAGGTGGA
cel-miR-39-3p F	ACACTCCAGCTATATCACCGGGTGTA
mir-URP	CTCAACTGGTGTCGTGGAGTC
probe-34c	5‘FAM/CAGTTGAGCCTGGCCGTG/3'MGB
probe-34b	5'HEX/CAGTTGAGA/T-BHQ/GGCAGTGGAGT/3'MGB
probe-3065	5'HEX/CAGTTGAGC/T-BHQ/CCAACAATATCCTGG/3'MGB
probe-4446	5'FAM/CAGTTGAGACCCA/T-BHQ/GTCACTGCCA/3'MGB
probe-cel-39	5'FAM/CAGTTGAGCAAGCTGATTTAC/3'MGB

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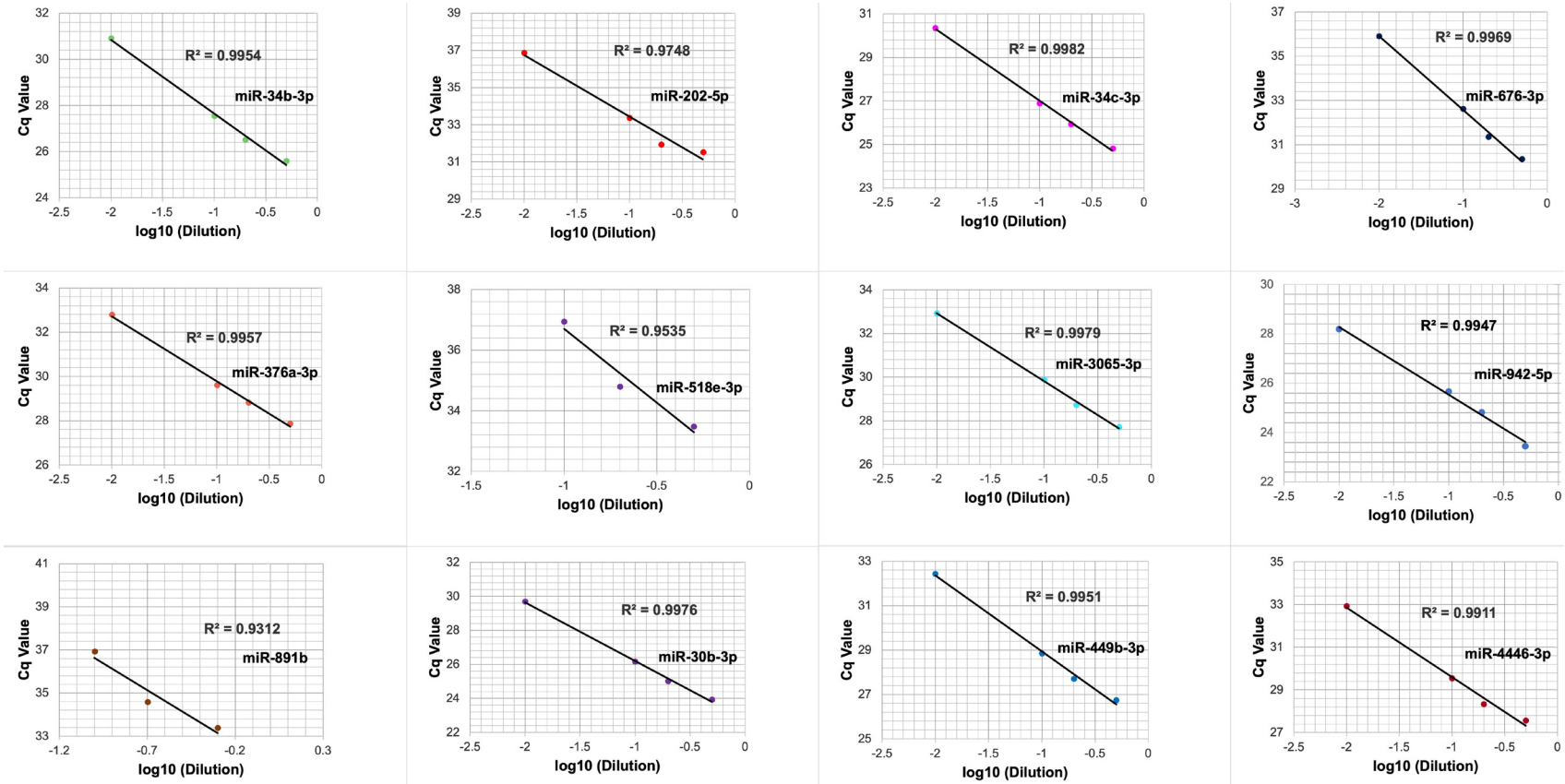

Figure S3 The standard curve and R2 of each microRNA qPCR.

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Table S3 Micro-TESE outcomes of NOA patients with different histological diagnosis

Micro-TESE outcome (case number)	Histopathological diagnostic result			
	Sertoli-cell-only syndrome	Maturation arrest	Hypo-spermatogenesis	Normal
Good (n=18)	0	0	6	12
Fair (n=47)	3	38	6	0
Poor (n=57)	40	17	0	0

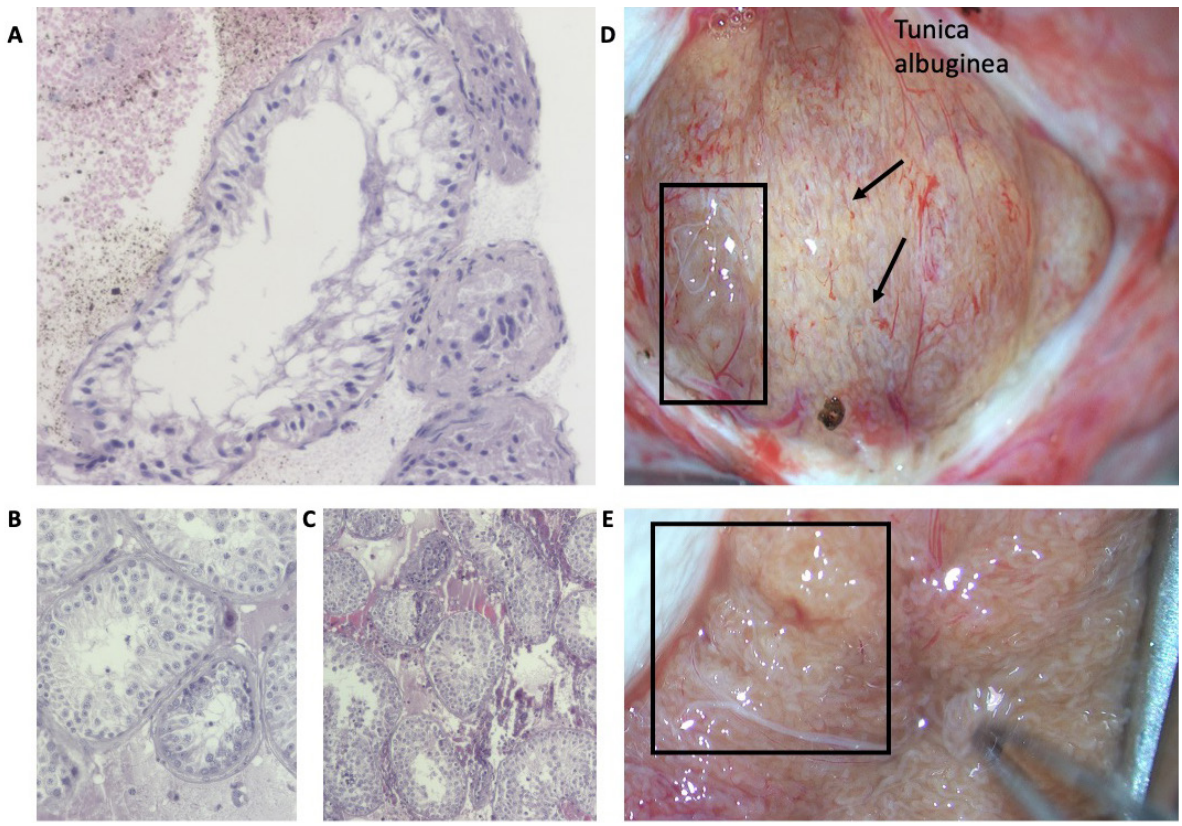

Figure S4 Histological diagnosis and micro-TESE outcome of enrolled patients. (A) HE staining of Sertoli-cell-only Syndrome (40-fold magnification). (B) HE staining of Maturation Arrest (20-fold magnification). (C) HE staining of hypospermatogenesis (20-fold magnification). (D) An isolated region of micro-TESE from the "Fair" group, and the histopathological diagnosis was "Sertoli-cell-only" Syndrome (25-fold magnification). The region in the black box shows tubules with normal spermatogenesis. The adjacent testicular tissue contains tubules with no spermatogenesis pointed by black arrows. (E) An isolated region of micro-TESE from the "Good" group, and the histopathological diagnosis was "Maturation Arrest" (25-fold magnification). The region in the black box shows tubules with normal spermatogenesis.

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