Association of hsa-miR-145 overexpression in human testicular cells with male infertility

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Abstract. MicroRNAs (miRs) have crucial functions in spermatogenesis and implications for male infertility. In the present study, *Homo sapiens* (hsa)-miR-145 was designed and cloned into the eukaryotic expression plasmid pGenesil-1. The recombinant plasmids were transfected into Hs 1.tes normal testicular cells and NTERA-2 testicular cancer cells. Quantitative polymerase chain reaction of hsa-miR-145 indicated that pGenesil-1-miR-145 effectively upregulated the expression of hsa-miR-145 *in vitro*. hsa-miR-145 overexpression inhibited the mRNA and protein expression of sex-determining region Y Box 9 in Hs 1.tes cells. The proliferation rates of NTERA-2 cells transfected with pGenesil-1-miR-145 were significantly decreased. High expression levels of miR-145 promoted cell apoptosis in NTERA-2 cells. The results revealed that altered hsa-miR-145 expression in testicular cells affects the regulation of target genes associated with male infertility.

Introduction

Approximately 12-15% of couples of reproductive age worldwide are sterile and approximately half of these cases are due to male factors (1). In at least one-third of male infertility cases, the cause is elusive (2) and is referred to as idiopathic infertility. Idiopathic male infertility predominantly manifests as oligozoospermia or azoopermia. When more than 30% of the spermatozoa have DNA damage, the fecundity of an individual decreases significantly *in vivo*. Thus, screening sperm for DNA damage is necessary in male patients with idiopathic infertility and patients pursuing assisted reproduction (3).

MicroRNAs (miRNAs/miRs) are non-coding RNA molecules approximately 22 nucleotides long, which regulate post-transcriptional gene expression by binding to the 3' untranslated region (UTR) of their target mRNA (4,5). miRNAs affect a series of physiological activities involved in cell proliferation through their combination with target genes, causing mRNA degradation or translation inhibition. Recent studies in the field of reproduction indicate that miRNA is an important regulator of spermatogenesis. A study on the Dicer-dependent pathway demonstrated that miRNA loss has pernicious effects on male fertility (6). A further study indicated that Dicer-deleted primordial germ cells and spermatogonia exhibit proliferation disorders, and spermatogenesis is suppressed during the early stage of proliferation and early differentiation in Dicer-deleted testis (7). These studies suggested a role for certain miRNAs in reproduction. For instance, miR-383 is a negative regulator of cell proliferation, miR-383-pRb pathways are associated with spermatogenesis (8), and miRNA-372 and miR-373 are oncogenes in testicular germ cell tumors (9).

miR-145, located at 5q32-33, is an anti-oncogene, which targets several tumor-associated genes that are involved in tumor growth, metastasis and tumor angiogenesis. miR-145 regulates the expression of the pluripotency factors OCT4, sex-determining region Y Box (SOX)2 and -9 as well as KLF4, which also have oncogenic features (10). Sachdeva *et al* (11) reported that miR-145 inhibits the expression of proto-oncogene c-Myc via p53. Another study by Chiyomaru *et al* (12) illustrated that miR-145 expression is markedly restrained in tumor organization, wherein FSCN1 is overexpressed.

The transcription factor SOX9 is located at 17q24.3-q25.1 and is expressed in the heart, brain, kidneys, prostate, testicles and other organs in human adults. The SOX9 gene is expressed in the brain and testis, as well as during the resting or reproductive stages of the perichondrium of the fetus. SOX9 mutations are associated with sex reversal and SOX9 affects the development of bones and testicles through expression in mesoblastomas.

According to a report on miRNA chips, miR-145 is downregulated among altered miRNA in the testicular tissues of patients with non-obstructive azoospermia (NOA) (13). Among

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all species, miR-145 contains a unique seed sequence which is conserved in Xenopus, zebrafish and various mammals (14,15). In living organisms, *Homo sapiens* (hsa)-miR-145 is abundant in the uterus, ovaries, testes, prostate and heart, which are all mesoderm-derived tissues (16). A previous study also revealed that SOX9 expression differs between normal and infertile samples based on DNA arrays, and SOX9 is required for Sertoli cell maturation and normal spermatogenesis (17). However, the association between SOX9 and miR-145 in reproductive cells has remained to be elucidated, and the function of hsa-miR-145 in human testes requires further study. In the present study, the testicular embryonic carcinoma cell line NTERA-2 (NT2) and a normal testis cell line, Hs 1.tes (18,19) were used to investigate the function of hsa-miR-145 during spermatogenesis by identifying its target genes.

Materials and methods

Database analysis. Through miRBase (http://www.mirbase.org/) (20), it was confirmed that the sequence of hsa-miR-145 was 16-GUCCAGUUUUCCCAGGAAUCCCU-38. A search using TargetScan (http://www.targetscan.org/), miRBase and pictar (http://pictar.mdc-berlin.de/) revealed that the putative 3'UTR of SOX9 did not complement miR-145 (21) (Table I)."

Construction of miR-145 expression plasmid. A total of two single-stranded DNA sequences were designed based on the hsa-miR-145 sequence in the miRBase database. The hsa-miR-145 expression recombinant and a control plasmid were constructed by the cloning of annealed oligonucleotides of hsa-miR-145 (sense, 5'-GATCCCCGTCCAGTTTTCCCAGGAATCCCTTTTTTTGTCGACA-3' and antisense, 5'-AGCTTGTCGACAAAAAAAGGGATTCCTGGGAAAACTGGACGGG-3'), or control (sense, 5'-GATCCCCTTCTCCGAACGTGTCACGTATTATTTTTTGTCGACA-3' and antisense, 5'-AGCTTGTCGACAAAAAATAATACGTGACACGTTCGGAGAAGGG-3') into pGenesil-1 plasmid. The recombinants were transformed into *Escherichia coli* DH5α (TransGen, Beijing, China) competent cells, which were cultured in lysogeny broth (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl; Beyotime Institute of Biotechnology, Shanghai, China) at 37˚C. Restriction enzymes *Sal*I and *Pst*I were used in the initial survey and DNA sequencing was conducted to identify the correct plasmid (22). DNA sequencing was completed by Sangon Biotech Co., Ltd (ABI3730xl; Shanghai, China).

Cell culture. NT-2 [American Type Culture Collection (ATCC), Manassas, VA, USA; CRL-1973™] and Hs 1.tes (ATCC; CRL-7002™) were grown in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) with high glucose, supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Gaithersburg, MD, USA) and 1% antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin). The cells were cultured at 37˚C with 5% CO_2.

Recombinant plasmid transfection. Transfection of NT-2 and Hs 1.tes cells was performed using Lipofectamine 2000 reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. When the cell density in the six orifice plates reached 80%, the cells were transfected with 5 ng pGenesil-1-miR-145 or 5 ng pGenesil-1-miR-NC in each orifice. The plasmids with pGenesil-1-miR-145 or pGenesil-1-miR-NC were extracted from *E. coli* using an Endofree plasmid minikit (Omega, Tarzana, CA, USA). Epifluorescence imaging of cells was carried out on an inverted epifluorescence microscope (Nikon Ti-E fluorescent microscope; Nikon, Tokyo, Japan). At 48 h after transfection, the cells were collected for RNA and protein extraction to be used for further analyses.

RNA isolation. Total RNA was isolated from the collected NT-2 or Hs 1.tes cells using a Total RNA kit II (Omega) according to the manufacturer's instructions. Spectrophotometry (Eppendorf BioSpectrometer® basic; Eppendorf, Hamburg, Germany) was used for RNA quantification. Optical density (OD) was measured at 260 and 280 nm. The OD260/OD280 ratio was used to estimate RNA purity, and the RNA with OD ratios of 1.8-2.0 were used for subsequent studies (23).

miRNA detection. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis of miR-145 was performed in analogy with methods used in previous studies (24,25). PCR was performed on cDNA generated from 2 μg total RNA using the protocol of the All-in-One™ miRNA RT-qPCR Detection kit (GeneCopoeia, Rockville, MD, USA). A 25-μl reaction mixture containing 2 μg total RNA, 1 μl 2.5 U/μl polymerase A, 1 μl RTase mix and 5 μl reaction buffer was prepared according to the manufacturer's instructions. The cycle parameters for the RT reaction were 37˚C for 60 min and 85˚C for 5 min. Subsequently, 0.4 μl RT product was PCR-amplified in 20 μl qPCR reaction mixture containing 10 μl 2X All-in-One qPCR mix (GeneCopoeia), 0.2 μM All-in-One qPCR Primer hsa-miR-145 (GeneCopoeia) and the hsnRNA U6 control (GeneCopoeia). The amplification parameters for RT and qPCR were set according to the manufacturer's instructions of the All-in-One miRNA RT-qPCR Detection kit using a Slan-96S real-time PCR system (Shanghai Hongshi Medical Technology Co., Ltd, Shanghai, China). The amplification parameters were as follows: 95˚C for 3 min, followed by 40 cycles of 95˚C for 10 sec, 60˚C for 20 sec and 72˚C for 16 sec. hsnRNA U6 was used as a control to normalize the expression levels of each miRNA. The relative expression levels were obtained using the $2^{-\Delta\Delta CT}$ method, with the relative gene expression=$2^{-(\Delta Ct\ sample-\Delta Ct\ control)}$. All experiments were performed in triplicate.

RT-qPCR analysis. To determine the expression levels of SOX9 (NC_018928.1) in Hs 1.tes cells, RT-qPCR was performed using the All-in-One First-Strand cDNA Synthesis kit and All-in-One qPCR Mix (GeneCopoeia), followed by RT-qPCR using SYBR Green (GeneCopoeia). GAPDH (NM_002046.4) was used as the control. A 25-μl reaction mixture containing 1 μg total RNA, 10 μM random primer, 1 U/μl RNase inhibitor, 8 U/μl M-MLV RTase, 1 mM dNTP and 5 μl reaction buffer was prepared according to the manufacturer's instructions. The cycling parameters for the RT reaction were 37˚C for 60 min and 85˚C for 5 min. Subsequently, a reaction mixture containing 10 μl 2X All-in-One qPCR Mix and the appropriate primers was added to 2 μl cDNA template to a final reaction volume of 20 μl. The amplification parameters

Table I. Target validation of the SOX9 3'UTRs by TargetScan, miRBase and Pictar.

Gene	Target sites	Position
SOX9	5' … UUUUUGUUGAAAACAAACUGGAA 3'..… UCCCUAAGGACCCUUUUGACCUG	1402-1409

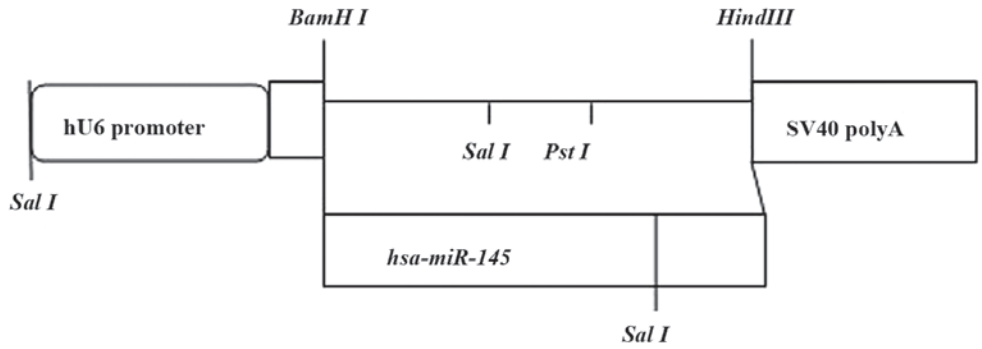

Figure 1. Structure of plasmid pGenesil-1 vector. miR-145 or control sense encoding template was inserted between the *Hin*dIII and Bam*HI* restriction sites. hsa, *Homo sapiens*; miR, microRNA; polyA, polymerase A.

were 95˚C for 5 min, followed by 30 cycles of 95˚C for 10 sec, 62.8˚C for 20 sec and 72˚C for 16 sec. PCR was performed using a Slan-96S real-time PCR system. Data were analyzed using the $2^{-\Delta\Delta CT}$ method. The primers (Sangon Biotech Co., Ltd) used for RT-qPCR were as follows: SOX9 forward, 5'-TGGTCTTTAACTCTGACCGTTACCT-3' and reverse, 5'-TATTCCGGATCTTAATCAGAGAAAGTG-3'; GAPDH forward, 5'-ACGGATTTGGTCGTATTGGGC-3' and reverse, 5'-CTCGCTCCTGGAAGATGGTGAT-3'.

The expression levels of OCT4 (GenBank ID, NM_002701.4), SOX2 (NM_003106.3), c-Myc (NM_002467.4), KLF4 (NM_004235.4) and FSCN1 (NM_003088.3) in NT-2 cells were determined using an All-in-One First-Strand cDNA synthesis kit and an All-in-One qPCR mix (GeneCopoeia). The RT reaction and RT-qPCR were operated as described above. The melting temperature for OCT4, SOX2, c-Myc and KLF4 was 60˚C and the melting temperature for FSCN1 was 65˚C. GAPDH was used as a control and data were analyzed using the $2^{-\Delta\Delta CT}$ method. The primers (Sangon Biotech Co., Ltd) used for RT-qPCR were as follows: OCT4 forward, 5'-CTGGGTTGATCCTCGGACCT-3' and reverse, 5'-CACAGAACTCATACGGCGGG-3'; SOX2 forward, 5'-CCCAGCAGACTTCACATGT-3' and reverse, 5'-CCTCCCATTTCCCTCGTTTT-3'; c-Myc forward, 5'-CGTCTCCACACATCAGCACAA-3' and reverse, 5'-TCTTGGCAGCAGGATAGTCCTT-3'; KLF4 forward, 5'-CAGCTCCCCAGCAGGACTACC-3' and reverse, 5'-CATCTGAGCGGGCGAATTTC-3'; FSCN1 forward, 5'-CTGGCTACACGCTGGAGTTC-3' and reverse, 5'-CTGAGTCCCCTGCTGTCTCC-3'. All experiments were performed in triplicate.

Western blot analysis. GAPDH was used as the internal control. The cell lysates were used for protein extraction. Infected cells were washed three times with ice-cold phosphate-buffered saline (Beyotime Institute of Biotechnology) and the cells were resuspended in 100 μl radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology). Cells were incubated on ice for 30 min, centrifuged at 15,000 x g and the supernatant was collected. Following measuring the protein concentration using an ELISA, the equivalent denatured protein samples were mixed with loading buffer. The samples were resolved using 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane via the wet transfer method. Following blocking for 1 h in 5% nonfat dried milk (Yili Industrial Group Co., Ltd, Inner Mongolia, China) in Tris-buffered saline and Tween 20 (TBST; Amresco, Solon, OH, USA) at room temperature, the blots were incubated overnight with rabbit polyclonal anti-SOX9 (BS1597; Bioworld Technology, Nanjing, China; diluted 1:1,000) or mouse monoclonal anti-GAPDH (200306; Zen Bioscience, Chengdu, China; diluted 1:5,000) antibodies at 4˚C for 15 . Following washing three times in TBST, the blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; diluted 1:2,000) at room temperature for 1 h. The blots were visualized using Immobilon Western chemiluminescent HRP substrate (Millipore, Billerica, MA, USA) following the manufacturer's instructions. A Kodak 440CF imaging system (Kodak, Tokyo, Japan) was used to visualize the blots.

Cell proliferation assay. Growth inhibition of NT-2 cells was determined via MTT cell viability assays using MTT Cell Proliferation kit (Solarbio, Beijing, China). The NT-2 cells were cultured in 96-well plates at an initial number of $1x10^4$ cells/well in DMEM supplemented with 10% FBS. Following transfecting the recombinant plasmids for 48 h at 37˚C, 10 μl MTT reagent was added and allowed to react for 4 h in an incubator. A total of 110 μl formazan reagent was added, which was allowed to react for 10 min. Substrate cleavage was monitored at 490 nm via ELISA. Experiments were performed in triplicate.

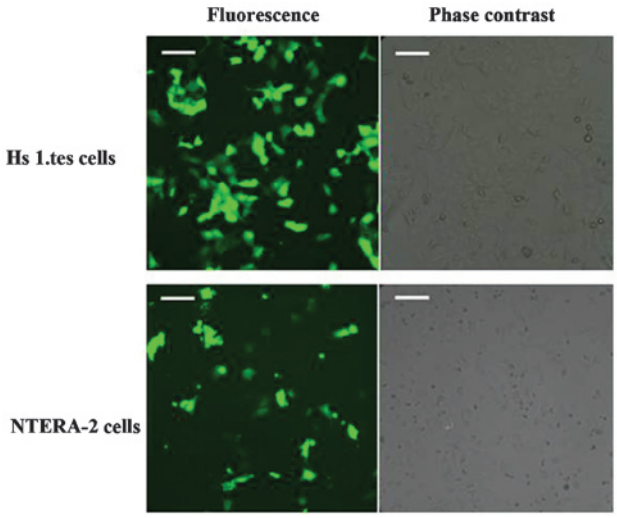

Figure 2. Fluorescent and light microscopy images of Hs 1.tes cells and NT-2 cells within 24 h after transfection. Scale bar, 100 μm.

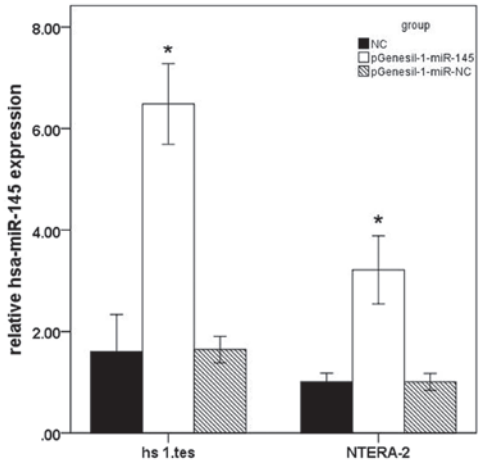

Figure 3. Relative miR-145 levels in NT-2 cells and in Hs 1.tes cells. Transfection was performed for 48 h. Three independent experiments were performed and values are expressed as the mean ± standard deviation. *P<0.05, compared with the NC. NC, negative control; miR, microRNA; hsa, *Homo sapiens*.

Cell apoptosis assay. Cell apoptosis was detected using Annexin V-allophycocyanin (APC)/7-aminoactinomycin D (7-AAD) double staining and flow cytometric analysis. NT-2 cells were cultured in six-well plates. The NT-2 cells were transfected with pGenesil-1-miR-145 and pGenesil-1-miR-NC. After 48 h, the cells were collected and manipulated according to the instructions of the Annexin V-APC/7-AAD apoptosis detection kit (KeyGen Biotech Co., Ltd, Nanjing, China). A total of 500 μl binding buffer was added to make a cell suspension. 5 μl Annexin V-APC was added, followed by 5 μl 7-AAD. The mixture was allowed to react in the dark for 15 min at room temperature. After 1 h, the mixture was analyzed via flow cytometry (BD FACSCalibur; BD Biosciences, San Jose, CA, USA).

Statistical analysis. The data were analyzed using SPSS 19.0 software (IBM, Armonk, NY, USA). A t-test was used for statistical comparisons between groups, whereas a one-way analysis of variance was used for comparisons among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

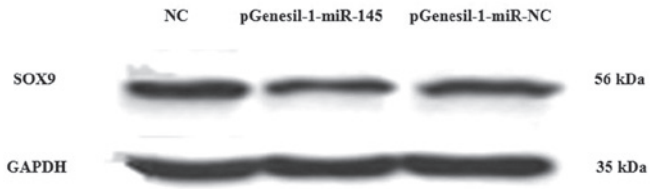

Figure 4. Hs 1.tes cells were transfected with pGenesil-1-miR-145 or pGenesil-1-miR-NC for 48 h. Cells of transfection groups and control were harvested for measurement of SOX9 protein expression using Western blotting. GAPDH was used as an internal control. Quantitation of the SOX9 protein level was performed using Quantity One software. SOX9, sex-determining region Y Box gene 9; NC, negative control.

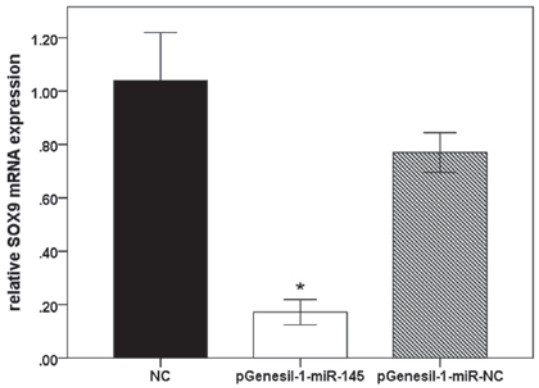

Figure 5. Hs 1.tes cells transfected with pGenesil-1-miR-145, pGenesil-1-miR-NC and following 48 h, mRNA levels of SOX9 were measured using reverse-transcription quantitative polymerase chain reaction. GAPDH was used as an internal control. Three independent experiments were performed and values are expressed as the mean ± standard deviation. *P<0.05, compared with the NC. NC, negative control; miR, microRNA.

Results

Identification of miR-145 expression vector and control construct. Plasmid pGenesil-1 is a eukaryotic expression vector containing a U6 promoter, an enhanced green fluorescent protein (EGFP) gene and an anti-kanamycin gene. Multiple cloning sites were located behind U6, including the restriction enzyme sites *Bam*HI, *Hin*dIII, *Sal*I, and *Pst*I. *Sal*I and *Pst*I are between *Bam*HI and *Hin*dIII. Double digestion was used with restriction enzymes *Bam*HI and *Hin*dIII to obtain a linear fragment, which was inserted into SalI to verify the recombinant plasmids (Fig. 1). The correct plasmid was identified via electrophoresis. *Sal*I digestion produced a 350-bp fragment, whereas *Pst*I digestion did not. DNA sequencing confirmed that the plasmids were reconstructed successfully. The samples were retested through sequencing by Sangon Biotech (Shanghai, China).

Transfection. The pGenesil-1-miR-145 and pGenesil-1-control constructs with enhanced green florescence protein were transfected into the NT-2 cell line and the Hs 1.tes cell line, respectively. The constructs exhibited green fluorescence

Table II. Expression levels of OCT4, SOX2, c-Myc, KLF4 and FSCN1 mRNA.

Gene/group	NC	pGenesil-1-miR-145	pGenesil-1-miR-NC
OCT4	1.00±0.14	0.60±0.15[a]	0.80±0.05
SOX2	1.11±0.29	0.22±0.05[a]	0.84±0.10
c-Myc	1.00±0.07	0.43±0.14[a]	0.89±0.14
KLF4	1.02±0.24	0.78±0.15	0.97±0.27
FSCN1	1.01±0.21	0.15±0.08[a]	0.99±0.17

NTERA-2 cells were transfected with pGenesil-1-miR-145, pGenesil-1-miR-NC and then mRNA levels were measured using reverse transcription quantitative polymerase chain reaction at 48 h. GAPDH was used as an internal control. Three independent experiments were performed and values are expressed as the mean ± standard deviation. [a]P<0.05, compared with the NC. NC, negative control.

Table III. NTERA-2 cell proliferation inhibition by transfection with recombinants for 48 h.

Group	Absorbance (A)	Inhibition ratio
NC	0.22±0.07	-
pGenesil-1-miR-145	0.12±0.03[a]	45.45%
pGenesil-1-miR-NC	0.21±0.09	4.45%

Inhibition ratio=(1-average absorption value in experimental group/average absorption value in NC group)x100%. Values are expressed as the mean ± standard deviation of nine experiments. [a]P<0.05, when compared with the NC. NC, negative control.

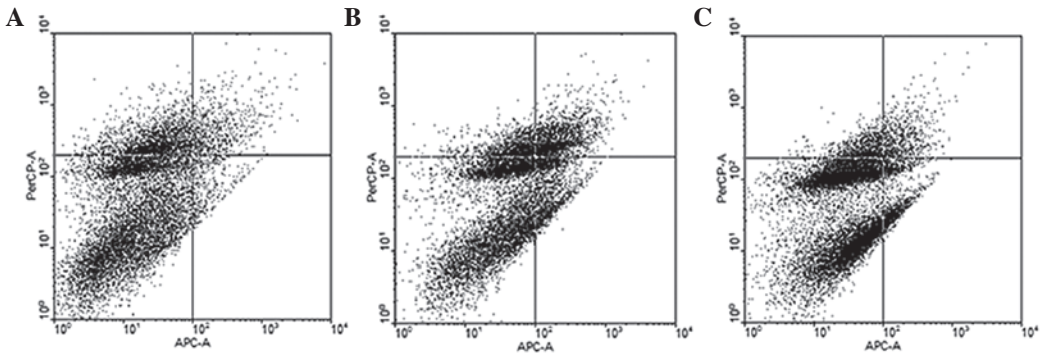

Figure 6. Assessment of apoptotic rates using Annexin V-APC/7-AAD double staining and flow cytometric analysis. (A) NC (normal controls) group. (B) pGenesil-1-miRNA-145 group. (C) pGenesil-1-NC group. (X axis indicates APC staining for viable apoptotic cells, and the Y axis indicates 7-AAD staining for non-viable apoptotic cells). APC, allophycocyanin; 7-AAD, 7-aminoactinomycin D.

under inverted fluorescence microscopy. Fluorescence was observed under fluorescence microscopy in cells containing pGenesil-1-miR-145 and pGenesil-1-miR-NC within 24 h after transfection. No fluorescent signal was observed in the blank control group (Fig. 2).

miR-145 overexpression in pGenesil-1-miR-145-transfected cells. The pGenesil-1-miR-145-transfected groups and pGenesil-1-miR-NC-transfected groups were analyzed at 48 h after transfection. miR-145 expression of the NT-2 and Hs 1.tes cells in the control and the transfected groups were analyzed via RT-qPCR (Fig. 3). The mRNA levels were normalized to the internal control U6. Statistical analysis revealed that the miR-145 expression levels in the NT-2 and Hs 1.tes cells in pGenesil-1-miR-145 groups were higher than those in the control and the pGenesil-1-miR-NC groups (P<0.05). pGenesil-1-miR-NC had no effect on miR-145 expression in NT-2 and Hs 1.tes cells (P>0.05, vs. control).

miR-145 inhibits SOX9 expression in Hs 1.tes cells at the mRNA and protein level. To demonstrate that miR-145 acts as an inhibitor of SOX9 protein expression, pGenesil-1-miR-145 and pGenesil-1-miR-NC were transfected into Hs 1.tes cells. Western blot analysis revealed that SOX9 protein levels were markedly lower in the cells with miR-145 overexpression than those in the negative control (Fig. 4). The relative protein expression of SOX9 in Hs 1.tes cells was as follows: NC group, 0.88±0.02; pGenesil-1-miR-145 group, 0.59±0.01; and pGenesil-1-miR-NC group, 0.82±0.03. RT-qPCR analysis demonstrated that the SOX9 mRNA levels were decreased in the cells with miR-145 overexpression (Fig. 5). The relative mRNA expression of SOX9 in Hs 1.tes cells

was as follows: NC group, 1.03±0.73; pGenesil-1-miR-145 group, 0.17±0.02; and pGenesil-1-miR-NC group, 0.77±0.03. SOX9 had no marked change in the mRNA and protein levels in the pGenesil-1-miR-NC-transfected cells. Thus, the results demonstrated that miR-145 inhibited the protein and mRNA expression of SOX9 in Hs 1.tes cells.

miR-145 inhibits the expression of OCT4, SOX2, c-Myc, KLF4, and FSCN1 at the mRNA level in NT-2 cells. To investigate the effect of miR-145 in germ cell neoplasms, it was determined whether miR-145 inhibited the expression of endogenous OCT4, SOX2, KLF4, c-Myc and FSCN1 in NT-2 cells. miR-145 was upregulated and the mRNA levels of these genes among three groups were detected. RT-qPCR revealed that the mRNA levels of OCT4, SOX2, c-Myc and FSCN1, but not KLF4, were decreased in the pGenesil-1-miR-145 group (Table II). However, no significant differences were detected in the expression of these genes in pGenesil-1-miR-NC-transfected cells.

Inhibition of proliferation by miR-145 in NT-2 cells. To analyze the biological effect of upregulated miR-145 expression, the growth of NT-2 cells treated with pGenesil-1-miR-145 was investigated using an MTT assay (Table III). At 48 h after transfection, it was observed that pGenesil-1-miR-145 significantly reduced the growth of Hs 1.tes and NT-2 cells. By contrast, the growth in the control group exhibited no significant changes.

Promotion of NT-2 cell apoptosis by miR-145. The results of the flow cytometric analysis are shown in Fig. 6. The apoptotic rate was 9.53±0.63% in the NC group, 30.22±0.56% in the pGenesil-1-miR-145 group and 12.54±1.89% in the pGenesil-1-miR-NC group. The apoptotic rate was significantly higher in the pGenesil-1-miR-145 group compared with that in the NC group.

Discussion

miRNAs are small, endogenous molecular regulators of gene expression that have critical roles in the body (26,27). In early studies on the biological characteristics of miRNAs, the pH1-RNApuro plasmid was used as a recombinant construct for upregulating miRNA expression (22). The pGenesil-1 plasmid was selected, which carries a U6 promoter, a kanamycin resistance gene and an EGFP gene, which is used for selection following transfection. The structure of pGenesil-1-miR-145 was determined via DNA sequencing and restriction enzyme digestion. RT-qPCR detection indicated that miR-145 is upregulated in the pGenesil-1-miR-145-transfected group, which suggested that pGenesil-1-miR-145 should be used for further investigation.

Spermatogenesis is a complex process, which involves the development of spermatogonial stem cells into highly differentiated spermatozoon. This process includes two stages: Active proliferation of spermatogonia and meiosis of spermatocytes. Certain studies have demonstrated the accelerated germ cell apoptosis and decreased mitotic activity of spermatogonia in infertile men during the spermatocyte stage (28,29). A recent study revealed that normal active spermatogonia in testes are arrested at the pachytene stage (30). This spermatogenic failure may be due to meiotic failure, but the mechanism of genetic defects in spermatogenesis remains to be elucidated (31). A microarray assay was used to demonstrate the altered expression of different miRNAs, including miR-145 in the testes of patients with NOA. The microarray assay revealed that aberrant miRNA expression is associated with male infertility. As a fertility biomarker, comparison of the DNA array of abnormal human testis samples with normal human testis samples exhibited SOX9 gene overexpression (17). The transcription factor SOX9, which is required for Sertoli cell maturation and normal spermatogenesis, regulates steroidogenic factor-1 promoter activity in Sertoli cells (32). Studies on chondrogenic differentiation demonstrate that miR-145 is a negative regulator through directly targeting SOX9 during the early stage of chondrogenic differentiation (33). Altered hsa-miR-145 expression in testicular cells is involved in the regulation of target genes associated with male infertility. In the present study, recombinant plasmids were constructed, which express pGenesil-1-miR-145 to transfect normal Hs 1.tes cells. The results of the RT-qPCR and western blot analyses demonstrated that miR-145 suppressed SOX9 mRNA and protein expression in transiently transfected Hs 1.tes cells compared with that in pGenesil-1-miR-NC-mediated cells and untreated Hs 1.tes cells ($P<0.05$). The present results coincide with those of previous studies, which indicated that miR-145 downregulates SOX9 protein expression. SOX9 mRNA expression was also decreased in the present study. miR-145 inhibition during SOX9 transcription was greater than that during translation. This difference may be due to miR-145 regulating the expression of numerous genes, which subsequently inTERAct with SOX9.

Previous studies have revealed that miR-145 is downregulated in numerous types of human cancer and its transfection reduces cell proliferation in tumor cell lines (34-39). Earlier studies revealed that miR-145 directly targets the proto-oncogene c-Myc and insulin receptor substrate-1, which are associated with cell proliferation (11,40). In bladder cancer, miR-145 directly controls FSCN1, which functions mainly in cortical cell protrusions, which mediate inTERActions between cells and the extracellular matrix, cell-to-cell inTERActions and cell migration. FSCN1 also forms cytoplasmic microfilamentous bundles, which contribute to cell architecture and intracellular movements (12). In the present study, pGenesil-1-miR-145 was transfected into NT-2 cells and it was demonstrated that miR-145 overexpression significantly reduces the growth of NT-2 cells. Flow cytometric analysis revealed that the apoptotic rate increased in the pGenesil-1-miR-145 group. RT-qPCR revealed that the mRNA levels of c-Myc and FSCN1 decreased in the pGenesil-1-miR-145 transfection group.

miR-145s function by directly targeting the pluripotency factors OCT4, SOX2 and KLF4 to inhibit the pluripotency of stem cells and control embryonic stem cell differentiation. miR-145 and OCT4 form a double-negative feedback loop and the promoter of miR-145 is inhibited by OCT4 (10,41). In the present study, RT-qPCR revealed that miR-145 inhibited the mRNA expression of OCT4 and SOX2, but not that of KLF4. Earlier studies revealed that the genes, which preserve

stem cell properties, including OCT4, SOX2, KLF4 and Nanog, have oncogenic characteristics and are involved in tumor development. These results demonstrated that miR-145 directly regulates the biological properties of tumor cells, as well as regulating cancer stem cells.

In conclusion, recombinant pGenesil-1-miR-145 significantly inhibited the mRNA and protein expression of SOX9 in human testicular cells. These data have important implications in studies on spermatogenesis. Differentially expressed molecules may be used as biomarkers to provide insights into the mechanisms underlying male infertility. The results of the present study may assist in developing a gene therapy for azoospermia. miR-145 inhibits the expression of oncogenes, such as OCT4, SOX2, c-Myc and FSCN1, as well as the proliferation of testicular embryonal carcinoma cells. Thus, upregulating miR-145 expression is a potential alternative treatment for testicular germ cell tumors. Further investigation into the inTERActions of miR-145 with other target genes are required to understand the occurrence and development of spermatogenesis and the suppression of malignant germ cell tumors.

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