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Supplemental Information

Generation of Haploid Spermatids with Fertilization and Development Capacity from Human Spermatogonial Stem Cells of Cryptorchid Patients

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1. Supplemental Experimental Procedures

Immunohistochemistry

For immunohistochemistry, testis sections of cryptorchid patients and OA patients were de-paraffinized, hydrated with series of graded alcohols, and treated with 3% H_2O_2 to block the endogenous peroxidase activity according to the procedure as described previously (He et al., 2009). After blocking with 10% normal serum, the sections were incubated with MAGEA4 (a kind gift from Professor Giulio C. Spagnoli, University Hospital of Basel, Switzerland), SCP3 (Abcam), normal mouse or rabbit IgG (Santa Cruz Biotechnology Inc.), or PBS. For the detection of MAGEA4, peroxidase-conjugated goat anti-mouse IgG (Envision detection kit, DAKO) was used as the secondary antibody. Sections were washed twice for 10 min with PBS and incubated with diaminobenzidine (DAB). After immunostaining, testis sections were counterstained with hematoxylin (Vector Laboratories) and examined under a light microscope. To detect SCP3 and IgG expression, goat anti-mouse or rabbit Alexa Fluor 594 (red)-labeled secondary antibody (A11072, Invitrogen) was employed, and DAPI (4, 6-Diamidino-2-phenylindole) was used to stain the nuclei of the cells in the testis. The sections were observed for epifluorescence under a fluorescence microscope (Leica).

Quantitative Real-time PCR & RT-PCR

Total RNA was extracted from various kinds of cells and testicular tissues using TRIzol (Invitrogen). After DNase I treatment to remove potential contamination of genomic DNA, 2 μg of total RNA were reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Quantitative PCR reactions were performed using Power SYBR® Green PCR Master Mix and a 7500 Fast Real-Time PCR System (Applied

Biosystems, Tokyo, Japan). The primer sequences of genes used for RT PCR and real-time PCR were designed and listed in Table S3 and Table S4, respectively. To quantify the PCR products, we used the comparative Ct (threshold cycle) method as described previously (He et al., 2007). The threshold of cycle values was normalized against the threshold value of human housekeep gene *GAPDH*. The results were expressed as mean ± SEM. Differences in normalized expression values between samples were analyzed using one way ANOVA statistical test or the unpaired t test. RT-PCR analysis of the chosen genes was performed according to the procedure as described previously (He et al., 2008), and PCR using water but without cDNA served as negative controls.

Immunocytochemistry

For immunocytochemical analysis, the freshly isolated male germ cells and the differentiated cells from human SSCs without or with RA and SCF treatment were fixed in 4% paraformaldehyde (PFA) in PBS and permeabilized with 0.4% Triton/X-100. Blocking with 10% serum was performed for 1 hour prior to incubation with primary antibodies. The cells were incubated with primary antibodies against UCHL1 (AbD Serotec), GFRA1 (Abcam), SCP3 (Abcam), Protamine 2 (Santa Cruz), or Acrosin (Santa Cruz) for 2 hours at room temperature and followed by goat anti-rabbit Alexa Fluor 594 (red)-labeled secondary antibody (A11072, Invitrogen) for 1 hour. DAPI was used to label cell nuclei, and images were captured with a fluorescence microscope (Leica).

To evaluate DNA content of differentiated cells from human SSCs treated with SCF and RA, immunofluorescence of the sorted cells by Hoechst was carried out using an antibody to Acrosin. The FACS sorting 1N cells were cytospun onto glass slides, fixed in 4% PFA, and permeabilized with 0.4% Triton/X-100. Blocking with 10% serum was performed for 1 hour prior to incubation with primary antibodies. The cells were incubated with primary antibody

against Acrosin (Santa Cruz) for 2 hours at room temperature and followed by goat anti-rabbit Alexa Fluor 594 (red)-labeled secondary antibody (A11072, Invitrogen) for 1 hour. DAPI was used to label cell nuclei, and images were captured with a fluorescence microscope (Leica).

Immunocytochemistry was performed to exclude parthenogenetic activation of oocytes by detecting human cells in embryos from mouse oocytes fertilized with human round spermatids derived from human SSCs of cryptorchid patients and in the naturally fertilized mouse embryos, using mouse anti-human nuclei antibody (HumNuc) (Millipore), and followed by goat anti-mouse TRITC-labeled secondary antibody (Jackson). Replacement of anti-HumNuc with mouse IgG or PBS in the embryos from mouse oocytes fertilized with human round spermatids was used as negative controls.

Immunofluorescence was carried out to check nuclear movement of the embryos derived from round spermatids of cryptorchid patients at various time points, embryos at 2 hours after injection of PBS to oocytes, and embryos at 2 hours after microinjection of the nucleus of Sertoli cells using antibody to H3K9 trimethylation (Abcam). Embryos were fixed in 4% PFA for 30 min, washed twice with 1% BSA–PBS, and permeabilized 0.1% Triton X-100 overnight at 4°C. The embryos were incubated with anti-H3K9 trimethylation, normal rabbit IgG, or PBS for 2 hours at room temperature and followed by goat anti-rabbit Alexa Fluor 594 (red)-labeled secondary antibody (A11072, Invitrogen) for 1 hour. DAPI was used to label cell nuclei, and images were observed with a confocal microscope (Leica).

Supplemental References

He, Z., Jiang, J., Hofmann, M.C., and Dym, M. (2007). Gfra1 silencing in mouse spermatogonial stem cells results in their differentiation via the inactivation of RET tyrosine kinase. Biol Reprod *77*, 723-733.

He, Z., Jiang, J., Kokkinaki, M., and Dym, M. (2009). Nodal signaling via an autocrine pathway promotes proliferation of mouse spermatogonial stem/progenitor cells through Smad2/3 and Oct-4 activation. Stem Cells *27*, 2580-2590.

He, Z., Jiang, J., Kokkinaki, M., Golestaneh, N., Hofmann, M.C., and Dym, M. (2008). Gdnf upregulates c-Fos transcription via the Ras/Erk1/2 pathway to promote mouse spermatogonial stem cell proliferation. Stem Cells *26*, 266-278.

2. Supplemental Figures and Legends

Figure S1 and Legends

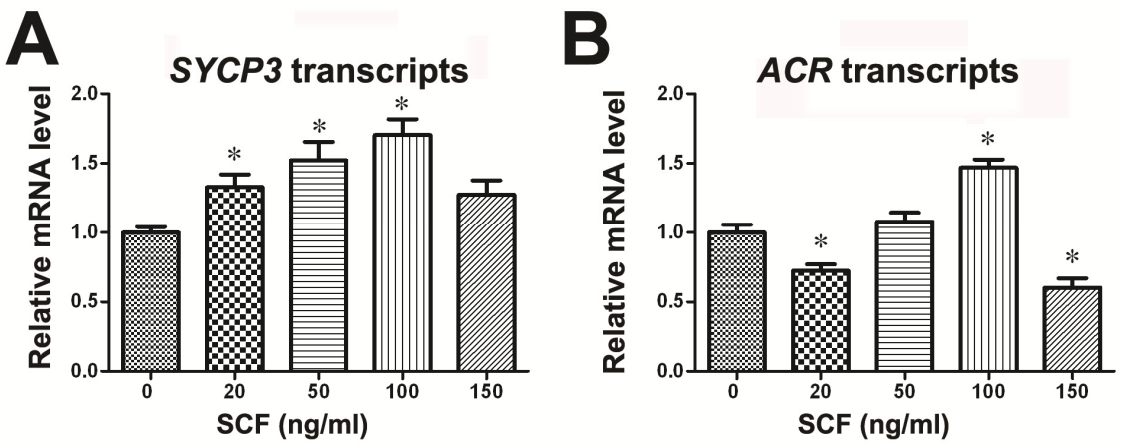

Figure S1. The Effect of Different Concentrations of SCF on the Differentiation of Human SSCs Derived from Cryptorchid Patients, Related to Figure 3. (A and B) RT-PCR showed the expression of *SYCP3* (1.00±0.03, 1.33±0.07, 1.52±0.11, 1.70±0.09, 1.27±0.08, n=3) and *ACR* (1.00±0.05, 0.72±0.04, 1.08±0.05, 1.47±0.05, 0.60±0.05, n=3) in human SSCs treated with 0, 20, 50, 100, and 150 ng/ml of SCF, respectively. Notes: * indicated $p < 0.05$ in RA+SCF treated cells compared with the control.

Figure S2 and Legends

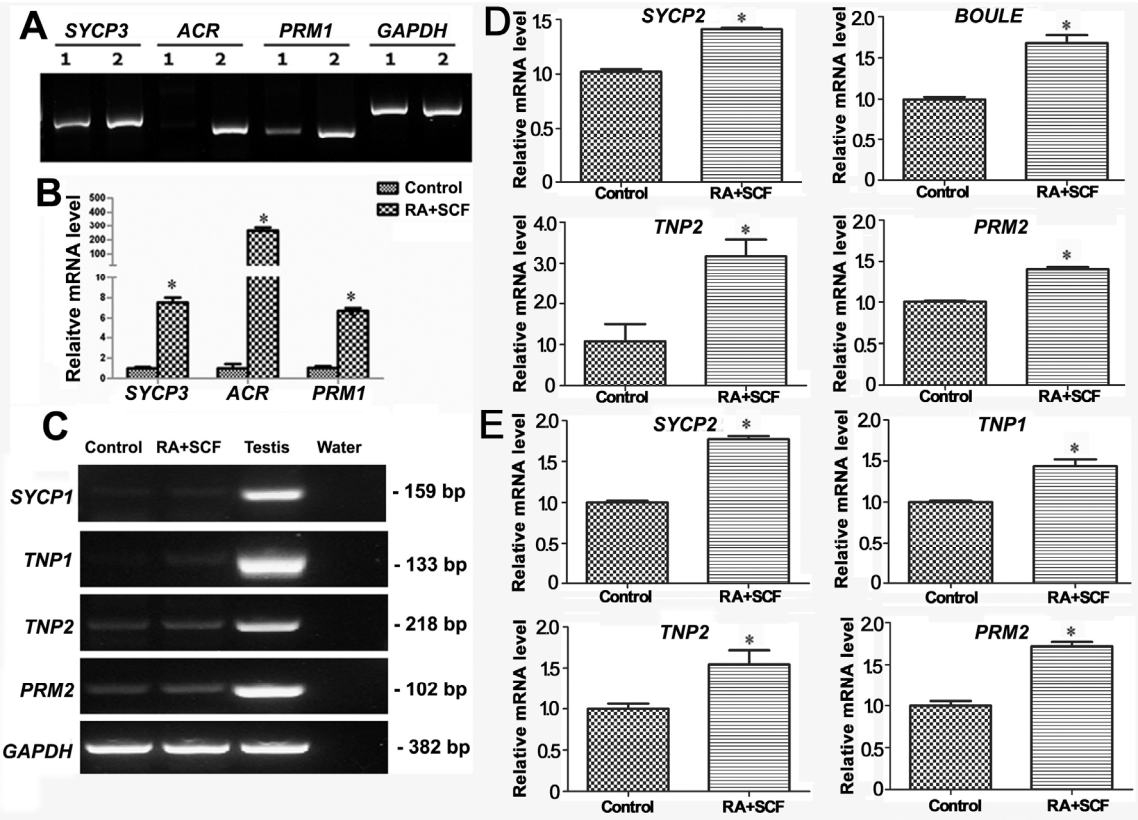

Figure S2. Expression of Meiotic and Post-meiotic Genes in Human SSCs without or with SCF and RA Treatment, Related to Figure 3. (A and B) RT-PCR (A) and real time PCR (B) revealed mRNA expression of *SYCP3* (7.53±0.28, n=3), *ACR* (264.52±12.75, n=3), and *PRM1* (6.71±0.28, n=3) in human SSCs induced by SCF and RA, and transcripts of *SYCP3* (1.00±0.06, n=3), *ACR* (1.00±0.24, n=3), and *PRM1* (1.00±0.09, n=3) in human SSCs without SCF and RA treatment. (C) RT-PCR showed mRNA expression of *SYCP1*, *TNP1*, *TNP2*, and *PRM2* in human SSCs without or with SCF and RA treatment. Human *GAPDH* was used as a loading control of total RNA. RNA from human testis was used as positive controls, whereas PCR with water but without cDNA served as negative controls. (D and E) Real time PCR showed the expression of *SYCP2* (1.40±0.01, n=3), *BOULE* (1.68±0.11, n=3), *TNP2* (2.94±0.31, n=3), and *PRM2* (1.41±0.01, n=3) in cryptorchid patients' SSCs with RA

and SCF treatment compared to SSCs without RA or SCF (control, designed as 1.0) (D), as well as the transcripts of *SYCP2* (1.77±0.04, n=3), *TNP1* (1.44±0.08, n=3), *TNP2* (1.55±0.17, n=3), and *PRM2* (1.72±0.05, n=3) in OA patients' SSCs with RA and SCF induction compared with SSCs without RA or SCF (control, designed as 1.0) (E). Notes: * indicated $p < 0.05$ in RA+SCF treated cells compared with the control.

Figure S3 and Legends

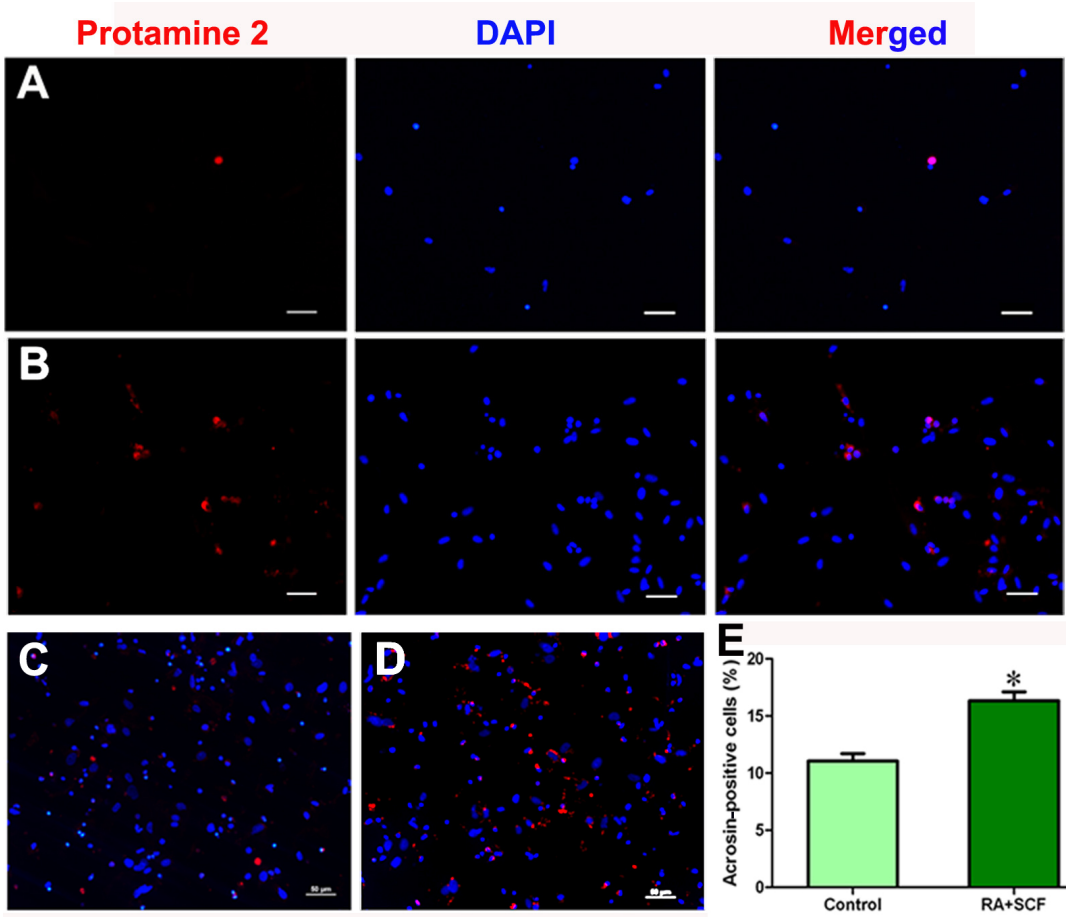

Figure S3. The Expression of Haploid Markers in Human SSCs without or with SCF and RA Treatment, Related to Figure 5. (A and B) Immunocytochemistry displayed Protamine 2 expression of human SSCs without (A) or with SCF and RA treatment (B). (C and D) Immunocytochemistry showed Acrosin expression in human SSCs without (C) or with SCF and RA treatment (D). Scale bars in A-D=50 μm. (E) Quantification of Acrosin-positive cells in human SSCs without SCF and RA treatment (11.06±0.65, n=3) or with SCF and RA induction (16.32±0.78, n=3). * indicated $p<0.05$ in RA+SCF treated cells compared to the control.

Figure S4 and Legends

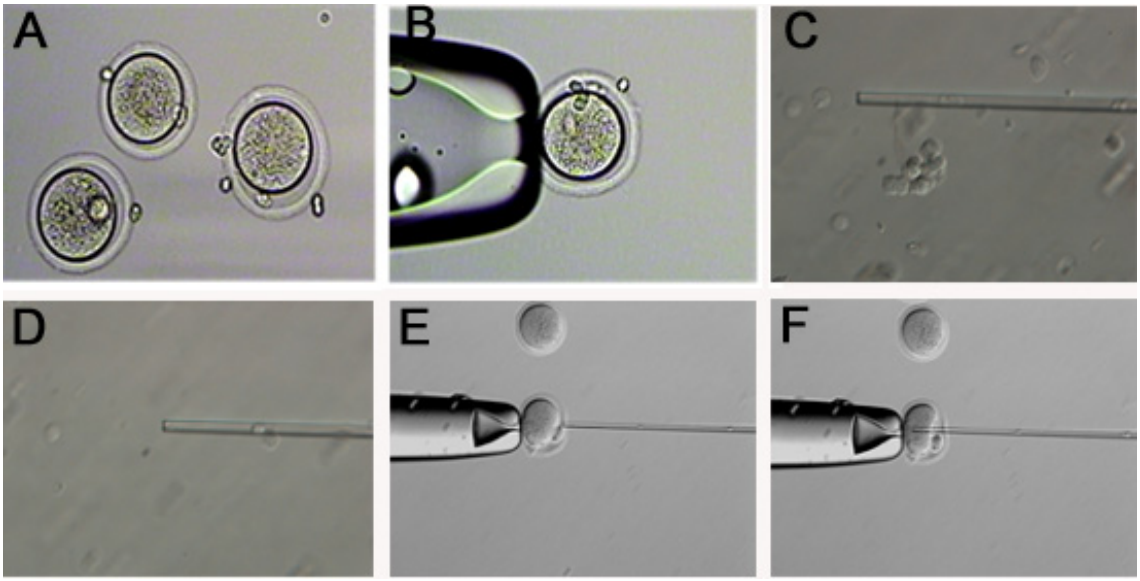

Figure S4. The Procedure of Round Spermatid Microinjection (ROSI) into Mouse Oocytes, Related to Figure 6. Notes: oocyte collection (A), holding of oocyte (B), selection of human round spermatid (C), pick-up of round spermatid (D), before microinjection of round spermatid (E), and ROSI (F).

Figure S5 and Legends

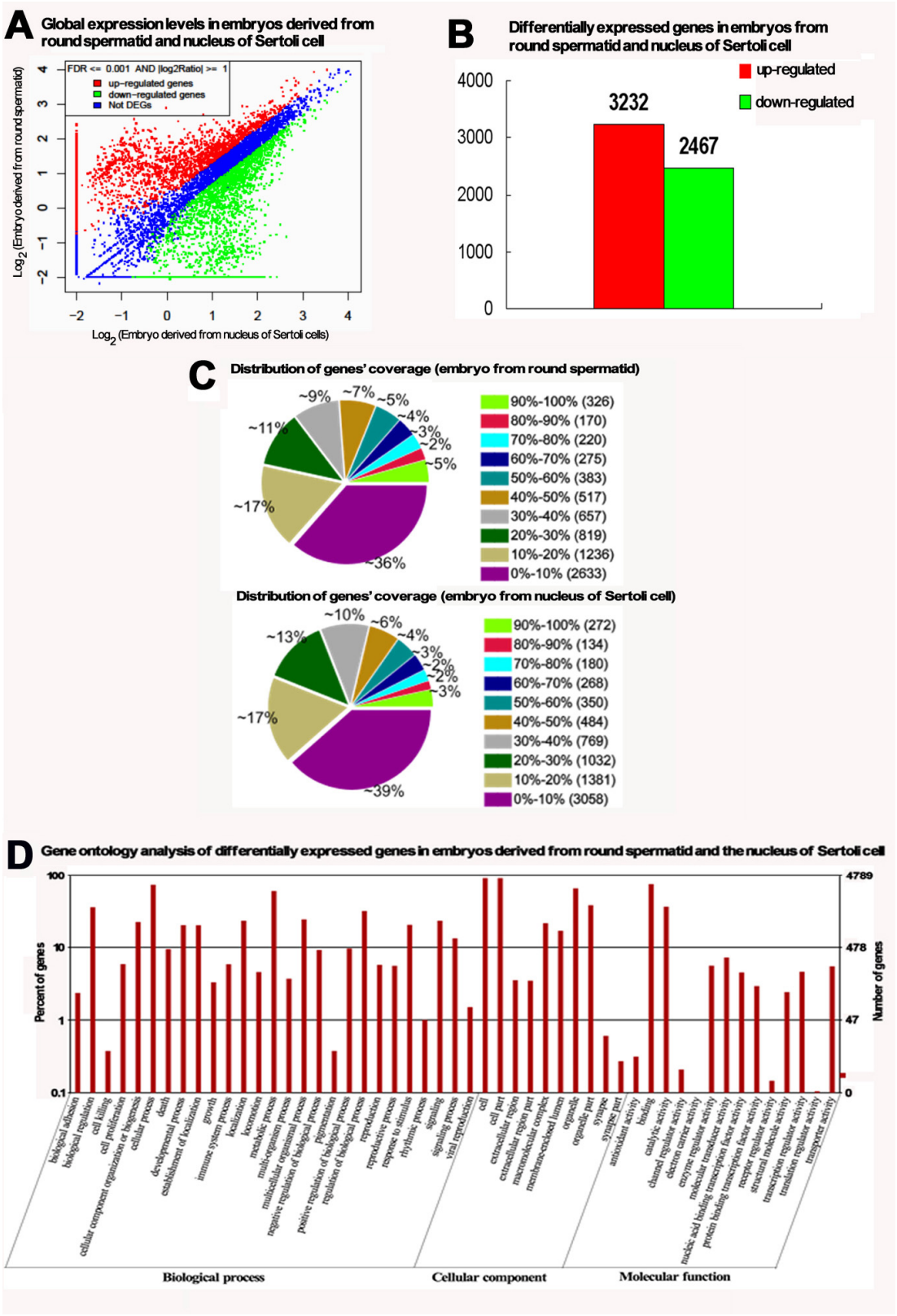

Figure S5. Single-cell RNA Sequencing Analysis Showed Distinct Global Gene Profiles in the Embryos Derived from Round Spermatid and the Nucleus of Sertoli Cell, Related to Figure 6. (A) Scatter plot revealed large scale of gene expression levels in the embryos derived from round spermatid and the nucleus of Sertoli cell. (B) Differentially expressed genes (DEGs) between the embryos derived from round spermatid and the nucleus of Sertoli cell. (C) Distribution of genes' coverage in embryos derived from round spermatids of cryptorchid patients and from the nucleus of Sertoli cells. (D) Gene ontology analysis of the DEGs in the embryos from round spermatid and the nucleus of Sertoli cell.

Figure S6 and Legends

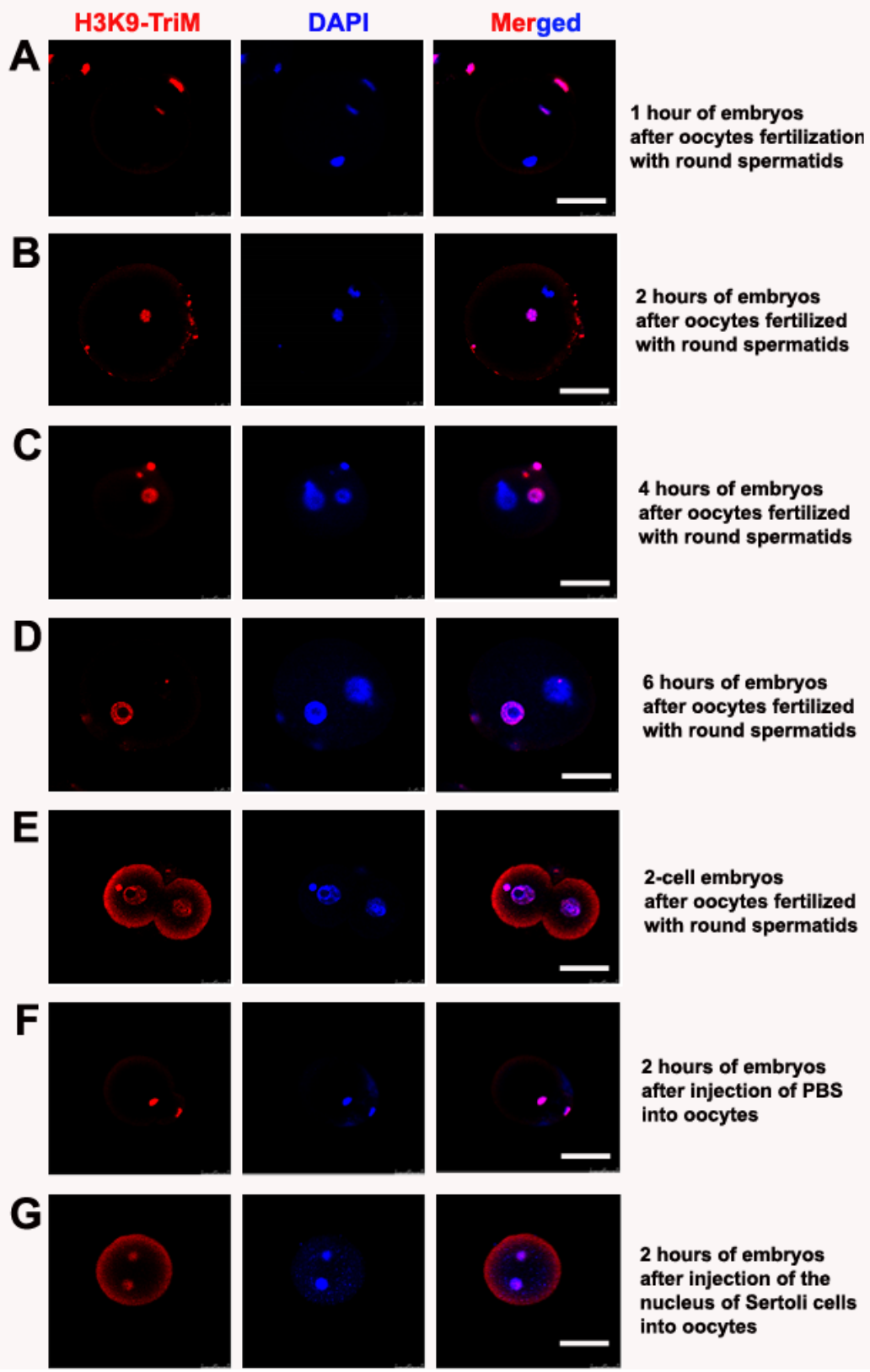

Figure S6. Nuclear Movement of the Embryos Derived from Round Spermatids of Cryptorchid Patients and from PBS or the Nucleus of Sertoli Cell, Related to Figure 6. (A-E) Immunofluorescence revealed dynamic expression of H3K9 trimethylation (H3K9-TriM) in the embryos at various time points derived from round spermatids of cryptorchid patients. Female pronuclei were immunostained for H3K9-TriM, while DAPI was used to show the nuclei of embryos. Scale bars in A-E= 50 μm. (F and G) Expression of H3K9-TriM in embryos derived from PBS (F) or the nucleus of Sertoli cells (G) was also shown. Scale bars in F and G= 50 μm. The data shown in (A-G) were representatives of three patients.

3. Supplemental Tables

Table S1. Clinical Data of 16 Cryptorchid Patients, Related to Figure 1

Parameters	cryptorchid patients (mean ± SEM)	Normal range
Age (years)	28.93±2.25	N/A
Left testicular volume (ml)	7.61±0.72	15-25
Right testicular volume (ml)	5.77±0.59	
FSH (IU/L)	22.28±2.27	0.8-5.1
LH (IU/L)	7.72±0.93	0.8-6.3
T (nmol/L)	6.35±0.86	6.1-27.1
PRL (μg/L)	25.22±10.78	4.5-12.6
E2 (pmol/L)	91.19±25.81	73.4-172.5

Notes: FSH: follicle-stimulating hormone; LH: luteinizing hormone; T: testosterone; PRL: prolactin; E2: estradiol.

Table S2. Development of Embryos from Mouse Oocytes Fertilized with Round Spermatids Derived from Human SSCs of Cryptorchid Patients and OA Patients, Related to Figure 6

Patients	Number of oocytes injected	Number & percentage of oocytes survived	Number and percentage of Embryo stages		
			2-cell	4-cell	8-cell
Cryptorchid patients	60	36 (60%)	22 (61.1%)	6 (16.7%)	2(5.6%)
OA patients	35	20 (57.1%)	18 (90%)	10 (50%)	8 (40%)

Table S3. Primer Sequences of Genes Used for RT-PCR, Related to Figure 3

Genes	Primer sequences	Product size (bp)	Tm(℃)
SYCP1	Forward: 5'-CTGTTGCCCTCATAGACC-3' Reverse: 5'-ACACCTGACTGCTGCTTG-3'	159	50
SYCP3	Forward: 5'-ATCTGGGAAGCCGTCTGT-3' Reverse: 5'-CTGCCTTTGATCTTGTTGTG-3'	325	51
ACR	Forward: 5'-CCAGGAGTATGGTTGAGATG -3' Reverse: 5'-GTGGCTGTTGTACGTGAAGA-3'	212	55
PRM1	Forward: 5'-GAAGTCGCAGACGAAGGA-3' Reverse: 5'-CAAGATGTGGCAAGAGGA-3'	171	53
PRM2	Forward: 5'-CCGGAGCACGTCGAGGTCTA-3' Reverse: 5'-ATGCTGCCGCCTGTGGAT-3'	102	57
TNP1	Forward: 5'-ATGAGGAGGAGCAAGAGC-3' Reverse: 5'-ACAAGTGGGAGCGGTAA-3'	133	51
TNP2	Forward: 5'-TAGTCCACCACCAAAGCG-3' Reverse: 5'-GAACAAGCCAAGGAGTGC-3'	218	53
GAPDH	Forward: 5'-AATCCCATCACCATCTTCC-3' Reverse: 5'-CATCACGCCACAGTTTCC-3'	382	56

Table S4. Primer Sequences of Genes Used for Real-time PCR, Related to Figure S2

Genes	Primer sequence	Product size(bp)	Tm(℃)
SYCP1	Forward: 5'-AACTACTGTCTGCAGCTTGG-3' Reverse: 5'-CATCTCTTCCAGCTCACTTGAT-3'	124	58
SYCP2	Forward: 5'-AAAAGCGAACAACAGAGGCTTCAT-3' Reverse: 5'-CTAGCATGTCCTTAAGAAGCCTGTC-3'	157	61
SYCP3	Forward: 5'-TGCAGGAGTAGTTGAAGATATG-3' Reverse: 5'-CTAGCATGTCCTTAAGAAGCCTGTC-3'	84	55
BOULE	Forward: 5'-GCAAGAAGAGCCTTGTTAATG-3' Reverse: 5'-CCTCAGAAGGTTGCAGGTATAAG-3'	139	58
DMC1	Forward: 5'-CCTCAGAAGGTTGCAGGTATAAG -3' Reverse: 5'-GTGGCTGTTGTACGTGAAGA-3'	159	60
ACR	Forward: 5'-GAAGTCGCAGACGAAGGA-3' Reverse: 5'-GTTTTGCCTGAACCGTAACCC-3'	108	60
TNP1	Forward: 5'-ATGAGGAGGAGCAAGAGC-3' Reverse: 5'- ACAAGTGGGAGCGGTAA-3'	133	58
TNP2	Forward: 5'-AACACTAGTCCACCACCAAAG-3' Reverse: 5'-GCTTGCCTTCCAAGTTCTTTC-3'	118	58
PRM2	Forward: 5'-CCAACACCATGGTCCGATAC-3' Reverse: 5'-TCAGCCCTTGCTCCTCTT-3'	117	58
GAPDH	Forward: 5'- TCACTGTTCTCTCCCTCCGC-3' Reverse: 5'- ACCAAATCCGTTGACTCCG-3'	117	60

4. Ethical Approval & a Sample of the Informed Consent Form

4.1. Ethical Approval

Ethical approval for the experiments of hybrid and nuclear transfer embryos

This letter is aimed to approve ethically the group of Dr. Zuping He and Dr. Zheng Li to perform the experiments of generating human-mouse hybrid embryos and human nuclear transfer embryos, with an objective to evaluate the fertilization and developmental function of human round spermatids derived from spermatogonial stem cells of cryptorchid patients. Human testis biopsies should be obtained with the Informed Consent Form signed by the patients. The hybrid and nuclear transfer embryos could be developed to no more than 8-cell stages, and all embryos should be disrupted after H3K9 trimethylation staining and single cell RNA sequencing analysis.

Institutional Ethical Review Committee

Ren Ji Hospital

School of Medicine

Shanghai Jiao Tong University

4. 2. A Sample of the Informed Consent Form

Informed Consent Form

I _____ agree to participate in research, entitled "Molecular mechanisms regulating meiosis and reconstruction of male infertility", by Dr. Zuping He and Dr. Zheng Li from Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine. The purpose and nature of the study has been explained to me well in writing, and I am participating voluntarily in the research project.

I accept the implementation of testicular sperm extraction or exploration of the vas deferens (anastomosis) surgery. Physicians of Ren Ji Hospital have introduced to me the detailed information about testicular sperm extraction and seminal duct exploratory surgery. I have learned that azoospemia includes obstructive (OA) and non-obstructive azoospermia (NOA), and NOA is caused by spermatogenic failure with no effective treatment methods. However, normal spermatogenesis is present in a few of seminiferous tubules in the testis of NOA patients. Numerous studies have reported that sperm can be obtained by retrieval from testis of some NOA patients for intracytoplasmic sperm injection (ICSI).

For the benefits of personal feelings and scientific research as well as ethical consideration, I agree to use sperm from testicular biopsies for ICSI and donate the remaining testicular biopsies for scientific research, such as in vitro differentiation of spermatogonial stem cells and ICSI with round spermatids.

These donations are completely voluntary and nobody forced me to participate in the study. My participation in this project is completely voluntary. I understand that I will not be paid for my participation. I can withdraw and discontinue participation at any time without penalty.

I have carefully read the consent form. I have obtained answers to all my questions with my satisfaction, and I voluntarily participate in this study.

Agreement:

The nature and purpose of this research have been sufficiently explained to me, and I agree to participate voluntarily in this study. I understand that I am free to withdraw at any time without incurring any penalty.

Signature: _____ Date: _____

Signature of Investigator: _____ Date: _____