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ORIGINAL ARTICLE *Andrology*

Identification and characterization of repopulating spermatogonial stem cells from the adult human testis

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BACKGROUND: This study was conducted to identify and characterize repopulating spermatogonial stem cells (SSCs) in the adult human testes.

METHODS: Testes biopsies from obstructive azoospermic patients and normal segments of human testicular tissue were used. Flow cytometry, real-time PCR and immunohistochemical analysis were performed. Purified human spermatogonia were transplanted into busulfan-treated recipient mouse testes and integrated cells were detected by human nuclear protein antibody co-localized with stem cell and germ cell markers.

RESULTS: Testicular biopsies collected from obstructive azoospermic men showed similar morphology and distribution of markers to the normal human testes. Flow cytometry showed distinct populations of stage-specific embryonic antigen-4 (SSEA-4), CD49f and CD90 positive cells in the adult human testes. SSEA-4 (+) cells showed high expression levels of SSC-specific genes and high levels of telomerase activity. Extensive colonization of human cells in the mouse testes indicates the presence of highly enriched populations of SSCs in the SSEA-4 (+) sorted cells. All the HNP (+) cells in the mouse testes were positive for germ cell marker dead box mRNA helicase and only half of them were dimly positive for c-kit. In addition, subpopulations of human spermatogonia that colonized mouse testes were positively stained for CD49f, GPR-125, Nanog and Oct-4 indicating the existence of population of cells among human spermatogonia with SSC and pluripotent characteristics.

CONCLUSIONS: This study clearly demonstrates that repopulating human SSCs have phenotypic characteristics of SSEA-4^{+}, CD49f^{+}, GPR-125^{+}and c-Kit $^{neg/low}$. The results have direct implications for enrichment of human spermatogonia for further culture and germ cell differentiation studies.

Key words: surface markers / repopulating / spermatogonial stem cells / human / testes

Introduction

Spermatogonial stem cells (SSCs) maintain spermatogenesis by self-renewal and continuous production of spermatozoa throughout life. Histological and ultra structural studies revealed that in non-primate mammals, the A_s (A single) spermatogonia are considered to be the stem cells of spermatogenesis (Huckins, 1971; Oakberg, 1971; de Rooij, 1973). Upon division of the A spermatogonia, the daughter cells either migrate away from each other and become two new stem cells or stay together through an intercellular bridge and become A-paired (A_{pr}) spermatogonia. The A_{pr} spermatogonia develop further into chains of 4, 8 or 16 A-aligned (A_{al}) spermatogonia. The A_{al} spermatogonia differentiate into A1 spermatogonia and after six mitotic divisions result in A2, A3, A4 and, finally, B spermatogonia, which give rise to spermatocytes at the last mitotic division.

Unlike rodents, in human and other primates classical histological studies of nuclear morphology indicate that two types of undifferentiated spermatogonia are present on the basement membrane of testicular seminiferous epithelium, designated as A_{dark} and A_{pale}

spermatogonia (Clermont and Leblond, 1959; Heller and Clermont, 1963; Schulze *et al*., 1986; Zhengwei *et al*., 1997; Simorangkir *et al*., 2005). Morphological characterization of SSCs on testicular biopsies from patients having undergone semicastration for malignant tumors and radio- and chemotherapy showed that the stem cells of the human testis most likely are the A_{pale} spermatogonia (Schulze, 1979), recent studies in adult Rhesus monkey testes also revealed that A_{dark} spermatogonia represent a reserve stem cell population which rarely divide and are activated following cytotoxic insult, whereas A_{pale} spermatogonia are active stem cells that undergo regular self-renewing divisions to maintain spermatogenesis under normal circumstances (Ehmcke *et al*., 2005; Hermann *et al*., 2007; Ehmcke and Schlatt, 2008). This indicates that both human and primate spermatogenesis have similar ontogeny, thus characteristics of the subpopulations of SSCs between these two species might be similar.

Recently, the phenotypic and molecular characteristics of SSCs in the adult primate testes have been identified (Herman *et al*., 2009; Maki *et al*., 2009). Knowledge about the characteristics of human spermatogonia is very limited. Up to now, expression of CD49f, CD133, GFR-α1, GPR-125, MAGE-4, PLZF, SSEA-4 and CD90 in human spermatogonia (Costaya *et al*., 2004; Conrad *et al*., 2008; Dym *et al*., 2009a; He *et al*., 2010) has been reported. However, the identity and characteristics of SSCs in the adult human testes are poorly understood. In this study, different populations of spermatogonia in the adult human testes were characterized. In addition, enriched populations of human spermatogonia were transplanted into the recipient mouse testes and the identity of the repopulating human spermatogonia in the recipient mouse testes was investigated.

Materials and Methods

Tissue preparation and cell isolation

Testicular biopsies were obtained from 29 patients undergoing the TESE (testicular biopsy and testicular sperm extraction) procedure. Testicular tissues devoid of tumor contamination were obtained from patients who underwent an orchiectomy and were generously donated by two patients. Prior to tissue collection, IRB approval was obtained from all patients consenting to investigation of discarded tissue. A small portion of tissue was extracted and used in this study. Testicular tissues and biopsies were surgically removed, placed in phosphate-buffered saline (PBS) (Cellgro, Herndon, VA, USA) supplemented with penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) and transported and maintained on ice for as little as 2 h to overnight. Testicular tissue samples were taken for histology and molecular biological analysis. Seminiferous tubules of the remaining tissue were finely minced and digested with collagenase A (1 mg/ml) (Roche, Indianapolis, Indiana, USA) and DNase (10 U/ml) (Invitrogen) in a reciprocating 37°C water bath for 15 min. After collagenase digestion, the undigested tissue was allowed to settle and cells in the supernatant were removed. The undigested tissue was further digested in an enzyme cocktail consisting of 1.5 mg/ml collagenase A (Roche), 1.5 mg/ml hyaluronidase type V (Sigma, St. Louis, MO, USA), 0.5 mg/ml trypsin (Worthington Biochemical Corporation, Lakewood, NJ, USA) and 10 U/ml DNase (Invitrogen) in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) in a reciprocating 37°C water bath for 20 min. After straining out remaining undigested tissue, isolated cells were centrifuged at 400*g* for 10 min. Cell pellets were re-suspended in MEM + HEPES + 5% fetal bovine serum (FBS) (Invitrogen, Hyclone, Logan, UT, USA) and placed in tissue culture coated 15 cm dishes (VWR, West Chester, PA, USA) in a Forma Series 2, 5% CO_2 humidified incubator (Thermo Electron Corporation, Waltham, MA, USA) until flow cytometric analysis. Testicular biopsy samples were only digested in the enzyme cocktail and were generally used for only one purpose due to their small size.

Flow cytometry and magnetic cell sorting

For cell surface characterization and sorting, cells were stained with selected stem cell markers used for characterization of primate SSCs (Maki *et al*., 2009) including CD90-FITC, CD49f-PE and CD117 and SSEA-4 alexa 488 (Supplementary data, Table S1). Cells were stained for 30 min in MEM + HEPES (Invitrogen) + 5% FBS (Hyclone) (complete medium) on ice, washed once, and re-suspended in complete medium and kept on ice until flow analysis. CD117 staining required a second step with an Alexa 635 conjugated anti-rabbit polyclonal antibody used at 1:500 dilution. Flow analysis was accomplished on an InFlux Cell Sorter (BD Cytopeia, Seattle, WA, USA). Alexa 488, Fluorescein (FITC) and phycoerythrin (PE) fluorochromes were excited with a 488 nm 200 mW laser (Coherent, Santa Clara, CA, USA). Alexa 488 and FITC emissions were collected with 530/40 band pass filters and PE emission was collected with a 580/30 band pass filter. Allophycocyanin (APC) and Alexa 635 was excited with a 638 nm 25 mW laser (Coherent) and emission was collected with a 670/40 band pass filter. Negative controls were unlabeled cells collected in each channel to account for autofluorescence background. Matched IgG isotypes of FITC, PE and APC were used to account and adjust for non-specific binding. For magnetic sorting, cells (up to 200×10^6) were suspended in DMEM+ 10% FBS and SSEA-4-biotin (Millipore, Temecula, CA, USA) was added (1:200) and was incubated on ice for 1 h. Labeling buffer containing PBS, bovine serum albumin (BSA) (0.5%) and 2 mM EDTA is prepared and degassed for 10 min. Labeling buffer was added to the SSEA-4 stained cells and centrifuged at 400*g* for 10 min. SSEA-4 cells re-suspended in 1.8 ml of buffer and 200 μl of streptavidin microbeads (Miltenyi Biotec, Auburn, CA, USA) was added. Also 100 μl of SSEA-4-FITC (Millipore) conjugated antibody was added to be able to check the purity of the magnetically separated cells by flow cytometry and incubated at 4°C for 20 min. Ten milliliters of buffer added to the tube, centrifuged at 400*g* for 7 min. Cells were then re-suspended in 1 ml of buffer, passed through a 40 μM strainer and then passed through a Magnetic Field (Miltenyi). SSEA-4 positive cells attached to the magnetic field were collected according to the manufacturer's instructions.

Histological and immunohistochemical staining

Tissues were fixed overnight in 4% paraformaldehyde (PFA; Electron Microscopy Science, Hatfield, PA, USA) and transferred into 20% sucrose (Sigma) for overnight equilibration. Tissues were frozen in OCT compound (VWR) and cryosections were prepared at 8 μm thickness and stored at −80°C. For histology, sections were washed in PBS and stained with Mayer's Modified Hematoxylin (American Master Tech Scientific, Lodi, CA, USA) for 5 min followed by a wash with distilled water for 5 min. Sections were then stained with Eosin Y (Fisher Scientific, San Diego, CA, USA) for 1 min, washed with distilled water for 5 min and mounted using an aqueous mounting medium (PolySciences Inc., Warrington, PA, USA). The sections were then analyzed using bright field microscopy. For immunohistochemical staining, testicular sections were blocked and permeabilized using 0.1% Triton-X (Sigma)/2% BSA (Sigma)/5% Sheep Serum (Abcam, Cambridge, MA, USA). Slides were then stained with germ cell and SSC-specific antibodies as described in Supplementary data, Table S1. For each primary antibody, a matched Isotype IgG was used as negative control. 4′6′-Diamidino-2 phenylindole (DAPI) (Invitrogen) was used for nuclear visualization. Following multiple

washes in distilled water (Cellgro), cells were preserved using aqueous fluorescent preservative (PolySciences Inc.). Slides were analyzed using an Olympus BX-61 microscope with SlideBook™ imaging software. For quantification studies, ~25 tubule cross sections per slide were counted and the data from four slides were pooled together and presented in this study. In addition to tissue, SSEA-4 sorted cells and non-sorted cells were fixed in 4% PFA (Electron Microscopy Science) and re-suspended in 100 mM sucrose (Sigma) at ~25 000 cells/10 μl. Cells were pippetted onto pre-coated ornithine/lysine (Sigma) glass slides (Surgipath, Richmond, IL, USA) at 10 μl/drop and placed on a 37°C hot plate until all drops dried. Slides were stored at −80°C before analysis.

RNA extraction and real-time PCR analysis

Total cellular RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations. The isolated RNA was then transcribed to cDNA using the Quantitect RT kit (Qiagen Inc.) and later purified with the QIAquick PCR purification kit (Qiagen Inc.). For each RT–PCR reaction, 20 ng of cDNA template was used in a 25 μl reaction volume with HotStar Taq Plus (Qiagen Inc., Valencia, CA, USA) and respective primers (Table I). All targets were amplified for 30 cycles. Amplification products were identified by size on a 2% agarose gel. For QRT–PCR, 5 ng of cDNA template was used in a 25 μl reaction volume with Quantitect Sybr Green PCR master mix (Qiagen Inc.) and run on a BioRad iCycler. Each sample was assayed in triplicate and normalized to a glyceraldehyde-3-phosphatedehydrogenase control.

Telomerase assay

The SYBR Green real-time quantitative telomeric repeat amplification protocol was adapted from Wege *et al.* (2003). Tissue or cell pellets were washed once in PBS and re-suspended and homogenized in a prepared lysis buffer containing 1× Chaps lysis buffer (Millipore, Billerica, MA, USA) and 400 U/ml RNaseOut Inhibitor (Invitrogen) at a volume of 1000 cells/μl. After 25 min of incubation on ice, the cell lysates were centrifuged at maximum speed for 10 min at 4°C. The supernatant was then transferred to a new micro centrifuge tube and the protein concentrations were determined at A280 nm with the ND-1000 spectrophotometer (Nanodrop). Reactions were done in 25 μl volumes containing 500 ng protein lysate, Quantitect SYBR Green PCR mix (Qiagen), 1 μg TS primer, 0.5 μg ACX primer and nuclease-free water. For every reaction plate assayed, each sample was tested in triplicate along with a no template control (lysis buffer), a positive control (embryonic stem cells, ESC) and a standard curve prepared from Human ESC (hESC) protein lysates (1000, 200, 40, 8 and 1.6 ng). Using the iCycler iQ5 (Bio-Rad, Hercules, CA, USA), the reactions were incubated for 20 min at 25°C, for 15 min at 95°C, and amplified in 40 PCR cycles for 30 s at 95°C and 90 s at 60°C. The threshold cycle values (C_t) were determined from semi-log amplification plots (log increase in fluorescence versus cycle number) and compared with the standard curve. The software default setting for the threshold is 10 times the mean of the standard deviation of the fluorescence reading of each well over the first 10 cycles, excluding cycle 1. Telomerase activity was expressed as a percentage relative to Human ESCs.

SSC transplantation

SSC transplantation technique was used to test the functionality of cell populations by colonization in recipient mice testes. Eight-week-old immune deficient Athymic Nude-*Foxn1*^{nu} male mice (Harlan, Indianapolis, IN, USA) were treated with a single intraperitoneal busulfan injection (40 mg/kg, Sigma) and were used as recipients ($n = 6$). One month after busulfan treatment, 0.3–0.8 × 10^6 SSEA-4+ magnetically enriched adult human testicular cells were transplanted into the seminiferous tubules of recipient mice ($n = 3$) via rete testis injection as described before (Ogawa *et al.*, 2000). Three other recipients were transplanted with the same concentration of freshly isolated non sorted human testicular cells. Four weeks after transplantation, the mice were sacrificed and the testes were fixed in 4%PFA and cryosections were made as described above. The identity of human SSCs in the mouse testes was recognized using human nuclear protein (HNP) antibody directly conjugated to Alexa Fluor 488 in combination with other stem cell or germ cell markers. All animal experiments were conducted in accordance with the National Research Council's Guidelines for the Care and Use of Laboratory Animals.

Statistical analysis

Except otherwise indicated all the experiments were repeated three times. Two sample student *t*-test and analysis of variance test were used for statistical analysis and $P < 0.05$ was considered as significant.

Results

Isolation and enrichment of spermatogonia

Cells isolated from testicular biopsies collected from obstructive azoospermic men showed similar morphology and distribution of spermatogenic cells to the normal human testes indicating that spermatogenesis is in progress in these patients. On average, 0.5 × 10^6 cells were isolated from each sample with a viability of 87%. Spermatogonia were morphologically detectable among the other cells as round cells with a large nucleus to cytoplasm ratio, one to three nucleoli and cytoplasmic inclusions (Fig. 1C and D). For enrichment of spermatogonia, dissociated cells from adult human testicular tissues were analyzed for various cell surface markers using flow cytometry. The expression profile of adult human testicular cells stained with various stem cell markers is presented in Supplementary data, Fig. S1. Among the surface markers used, stage-specific embryonic antigen-4 (SSEA-4) that has been shown to be expressed on the surface of actively dividing spermatogonia in the adult Rhesus monkey testes (Maki et al., 2009) was abundantly expressed in the human testes. We tested human testicular cells isolated from both testicular biopsies and donated tissues and found that on average 13.3 (±1.4)% of the cells express SSEA-4 on their surface. Another subset of spermatogonial markers used in this study were CD49f (alpha-6-integrin), CD90 (Thy-1), CD117 (c-Kit) and in combination CD49f+ CD90+ and CD117− (Triple Stain). In the adult human testes, we found that on average 25 (±2.5)% of cells express CD49f and 13 (±5)% were CD90 positive. Only a very small (<1%) population of Triple Stained cells was found in the human testes (Fig. 1E and F).

Histological and immunohistochemical staining of testicular sections

Histological examination revealed that testicular biopsies have a similar morphology when compared with the donated tissues after hematoxylin-eosin staining (Fig. 1A and B). Human testicular tissues were taken for immunohistochemical examination to better understand the distribution and marker expression of human SSCs. We found SSEA-4 and CD49f, also known as alpha-6-integrin, to have

Table I Primers used for quantitative RT–PCR analysis of the SSC-specific genes.

Name of gene	5′-sequence	3′-sequence	DNA size (bp)
c-Kit	AGGTGACACTATAGAATAGCACGGTTGAATGTAAGGCT	AGGTGACACTATAGAATAGCACGGTTGAATGTAAGGCT	151
GFRa1	AGGTGACACTATAGAATATCAGCAAGTGGAGCACATTC	GTACGACTCACTATAGGGAAGCATTCCGTAGCTGTGCTT	256
PLZF	AGGTGACACTATAGAATATTCATCCAGAGGGAGCTGTT	GTACGACTCACTATAGGGACCTCGTTATCAGGAAGCTCG	155
c-Ret	AGGTGACACTATAGAATAACATTGCCCAGCAACTTAGG	GTACGACTCACTATAGGGAGGTGGCTCCTTTCTCAACTG	219
GPR125	AGGTGACACTATAGAATACTTGGCGCAGATGTGATAGA	GTACGACTCACTATAGGGAGAAAAGTTGGCTGCTTCCAC	215
Dppa5	AGGTGACACTATAGAATAGAAAGTTCCCGAAGACCTGA	GTACGACTCACTATAGGGAACTGGAGCATCCACTTGGTC	252
hTERT	AGGTGACACTATAGAATATTGTCAAGGTGGATGTGACG	GTACGACTCACTATAGGGAGGCTGGAGGTCTGTCAAGGT	227

c-Kit, Kit receptor; GFRa1, Glial cell line-derived neurotrophic factor (GDNF) receptoe alfa-1; PLZF, Promyelocytic leukemia zink factor; c-Ret, Tyrosine kinase receptor for GDNF; GPR125, G-protein coupled receptor 125; Dppa5, Developmental pluripotency associated 5; hTERT, Human Telomerase Reverse Transcriptase.

interesting staining patterns in adult human testes. According to histological quantifications, almost all of the cells adjacent to the basement membrane of seminiferous tubules expressed CD49f (28.7 ± 1.2 per tubule cross section). Also, many of the germ cells along the basement membrane of seminiferous tubules expressed SSEA-4 (18 ± 1 per tubule cross section), a pluripotent marker found in human embryonic stem (ES), embryonic germ (EG) cells (Shamblott *et al.*, 1998; Heins *et al.*, 2004). Interestingly, the majority (88.3%) of SSEA-4 positive cells co-localized with CD49f. Co-localization of CD90 and dead box mRNA helicase (VASA) revealed that CD90 was mainly localized in germ cells at the luminal part of the tubular cross sections, in peritubular and interstitial cells. Very few cells at the basement membrane of the seminiferous tubules co-localized VASA and CD90. Co-labeling of CD90 and CD49f showed that almost all the CD49f did not co-localize CD90 (Fig. 2). As expected we found that germ cells and Leydig cells showed c-Kit immunoreaction on their plasma membrane. Only 25% of SSEA-4 positive cells co-localized with c-Kit suggesting the possibility of the existence of two different populations among SSEA-4 positive cells in adult human testes. In contrast, co-localization of CD49f and c-kit showed that the majority of the CD49f positive cells at the basement membrane of seminiferous tubules co-express c-Kit. All the SSEA-4 positive cells were also positive for germ cell marker VASA while only 50% of CD-49f positive cells showed VASA staining indicating that CD-49f is also expressed at the surface of the testicular somatic cells. Contrary to this, luteinizing hormone receptor (LH-r) was not expressed on VASA positive cells in the seminiferous tubules, but appeared to stain the cytoplasm of Sertoli and Leydig cells in human testes, indicating that LH-r expressed only on the somatic cells and not germ cells in adult human testes (data not shown). There was also a clear population of SSEA-4 positive cells at the basement membrane of human testes co-expressing Nanog and Oct-4 indicating the existence of a population of cells among human spermatogonia with pluripotent characteristics. To further confirm our flow cytometric analysis and immunohistochemical staining on testis sections, after cell isolation we sorted SSEA4+ cells and stained them with SSC markers GFR-α1 and PLZF. There were significantly higher percentages of GFR-α1 and PLZF positive cells in SSEA-4 sorted cells when compared with the non-sorted cells, indicating that they are enriched in self-renewing SSCs (Fig. 3).

Real-time PCR and telomerase assay

Gene expression analysis was performed on SSEA-4 positive and negative cells to test for SSC-specific expression. All of the genes including c-Kit, GFRα-1, PLZF, c-RET and GPR-125 were expressed at least 3-fold and up to 7-fold greater in the SSEA4 positive population. Moreover, higher expression level of h-TERT in SSEA-4 sorted cells indicates their high level of telomerase activity and their proliferation capability. SSEA-4 positively sorted cells were compared with non-sorted cells from the testis against hESCs for telomerase activity. The non-sorted cells showed an average of 10.4% telomerase activity when compared with hESCs (100%), while SSEA-4+ cells had about five fold more expression (54.6%) when compared with the non-sorted cells, which is ∼2-fold less than hESC telomerase expression level. This supports the finding of up-regulated h-TERT expression and suggests at least prolonged replication capabilities in SSEA-4 positive cells (Fig. 3).

SSC transplantation

On average, 6.8 HNP positive cells were found per tubular cross section in mice transplanted with SSEA-4 mangetically sorted cells; While only 0.15 HNP positive cells were detected when non-sorted cells transplanted in the mouse testes. This indicates that magnetic sorting for SSEA-4 enriched population of human SSCs to 40–50-folds. Co-localization studies were performed on mouse testes that had been transplanted with enriched population of human spermatogonia to unravel markers expressed on the surface of human spermatogonia repopulating in the mouse testes. A summary of the localization of these markers is presented in Table II. Using HNP together with SSEA-4, CD49f and c-Kit, we have found that of the cells that are positive for HNP, 28.1 ± 3.6% were positive for CD49f, and 14.2 ± 3.6% were positive for SSEA-4 and 49.8 ± 2.9% were dimly positive for c-Kit. Furthermore, we observed that only a small population of the SSEA4+ cells was also positive for c-Kit. Co-localization studies with CD49f and SSEA-4 have demonstrated that 95.6 ± 1.6% of SSEA-4+ cells were also positive for CD49f. Considering these results, and the fact that CD49f co-localizes with HNP 2-fold more than SSEA-4 does with HNP, we reason that two populations of CD49f+ cells, CD49f+ SSEA4+ and CD49f+ SSEA-4−, are SSCs that can repopulate the recipient testis. It should also be

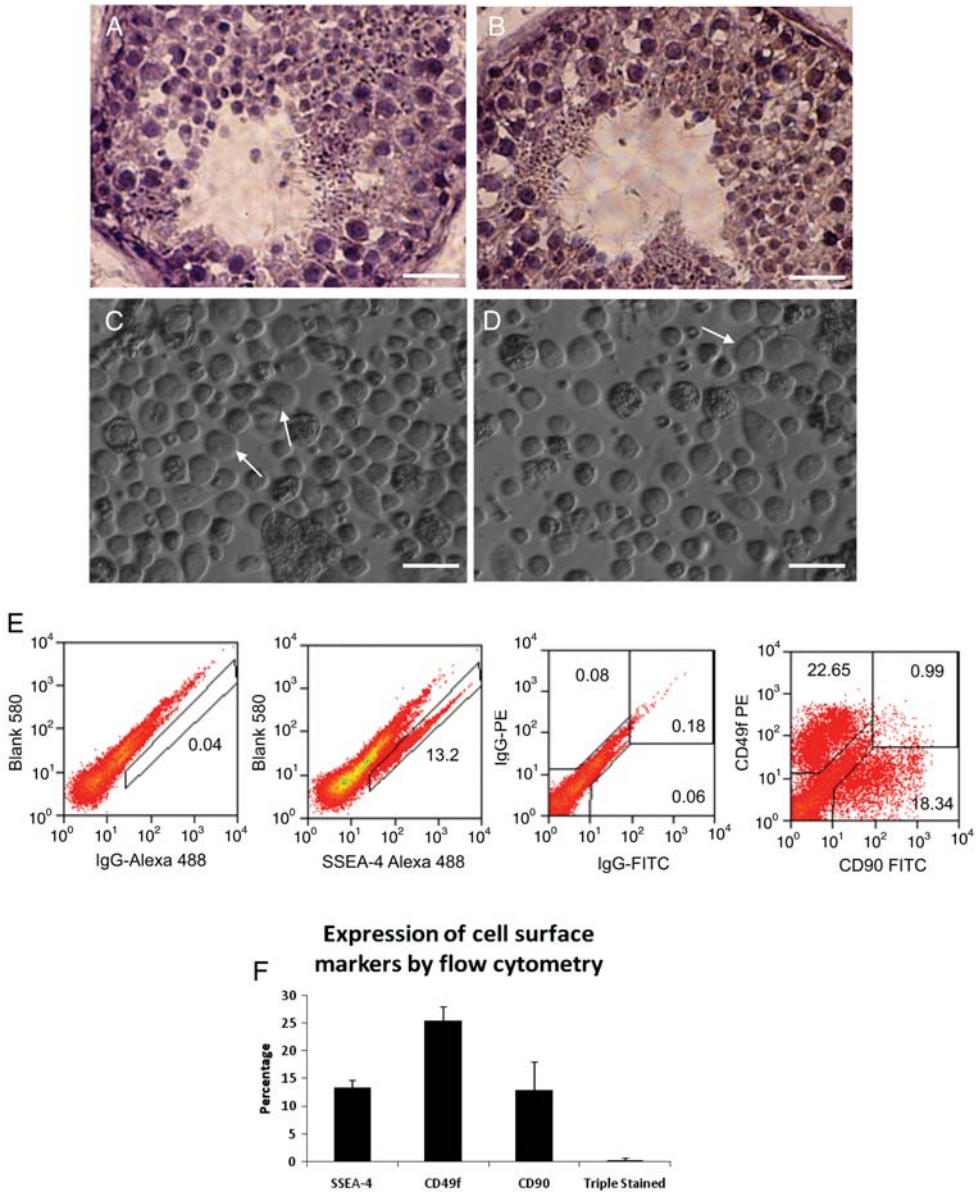

Figure 1 Morphology and cell surface marker analysis of adult human testicular cells. Note the morphology of the testes obtained from the obstructive azoospermic men (**A**) were similar to normal human testes (**B**). Also after isolation cells with similar morphologies were obtained from both normal testes (**C**) and testes collected from azoospermic patients (**D**). Note spermatogonia were present in both testes isolates and could be identified as round cells with a large nucleus: cytoplasm ratio, one to three nucleoli and cytoplasmic inclusions (arrows). (**E**) Flow cytometry analysis of surface markers SSEA-4, CD49f and CD90 in isolated cells from adult human testes. Distinct populations of SSEA-4, CD49f and CD90 positive cells were found in the adult human testicular biopsies and a very small population of double Stained cells for CD49f and CD90 was found in the adult human testes. The presented SSEA4 Alexa 488 was displayed in a two-dimensional plot and uncompensated against the 580 channel to aid in visualizing the cells from the auto fluorescent events. Histogram representation of four independent flow analyses is presented in (**F**). Scale bars: A and B, 50 μm; C and D, 25 μm.

noted that CD49f+ cells only account for about a quarter of the integrated cells, meaning that about 75% of the SSCs that have integrated have yet to be characterized with a surface marker that is feasible with flow cytometry. Co-localization study of HNP with other cell surface markers revealed that human spermatogonia colonized in the mouse testes do not express CD-29 also known as Beta-1 integrin, a marker that is expressed on the surface of mouse SSCs. Also there was no expression for Tumor Representing Antigen 1-60 (TRA-1-60), a surface marker expressed in pluripotent cells including human ES and human EG cells (data not shown). However, 42.8% of HNP cells co-localized with GPR-125 indicating this marker is expressed at the surface of a population of repopulating human

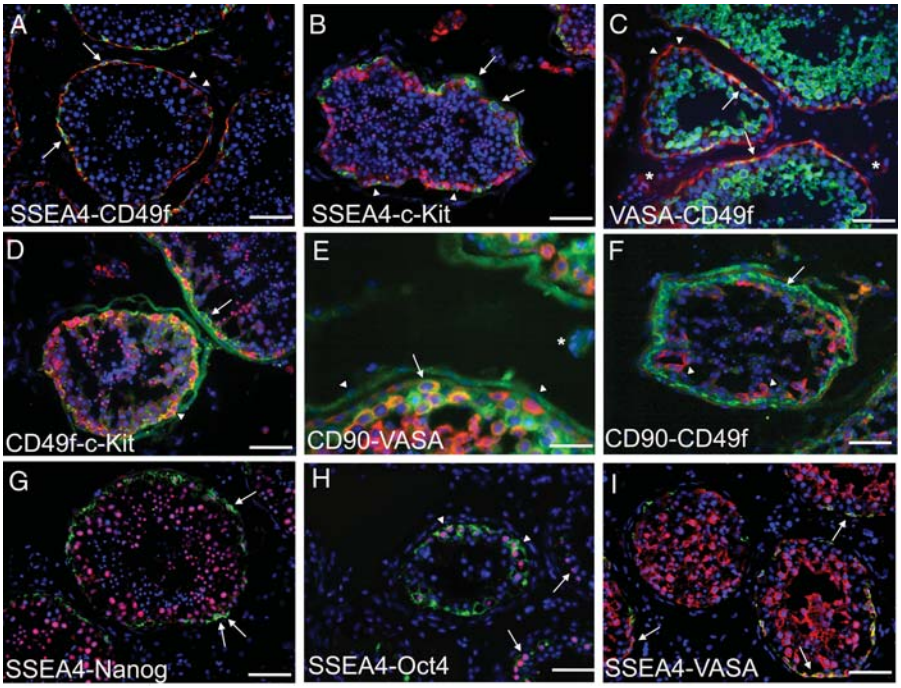

Figure 2 Immunohistochemical localization of germ cell and stem cell markers in adult human testes. Co-localization of SSEA-4 (green) and CD49f (red) at the basement membrane of seminiferous tubules (**A**). CD49f was abundantly expressed on cells at the basement membrane of seminiferous tubules. Note SSEA-4 is specifically localized in a subpopulation of spermatogonia at the basement membrane of the seminiferous tubules and are presumably the SSCs. All SSEA-4 positive cells were also positive for CD49f (arrows), while there were some CD49f positive cells that were SSEA-4 negative (arrowheads). C-Kit was found in both cells located at the basement membrane of seminiferous tubules and in more advanced germ cells (**B**). Co-localization of SSEA-4 (green) and c-Kit (red) revealed that most of the SSEA-4 positive cells were c-Kit negative (arrows) and some possesed c-Kit on their surface (arrowheads). (**C**) Co-localization of CD49f (red) and VASA (green) revealed that while most of the CD49f positive cells at the basement membrane of tubular cross sections were VASA positive (arrow), there were also cells that were not positive for germ cell marker VASA (arrowheads). Also some cells in the interstitial tissue were positively stained for CD49f (asterisks). (**D**) Co-localization of CD49f (green) with c-Kit (red) showed that the majority of CD49f positive cells at the basement membrane of tubular cross sections were also positively stained for c-Kit (arrowhead) and there were some CD49f positive cells that were not c-Kit positive (arrow). (**E**) Co-localization of CD90 (green) and VASA (red). Note CD90 is predominantly expressed in germ cells in the lumen of the seminiferous tubules, peritubular (arrowhead) and interstitial cells (asterisk). Very few cells at the basement membrane were positively stained for VASA and CD90 (arrow). (**F**) Co-localization of CD90 (red) with CD49f (green). Note that CD49f positive cells were mainly localized at the basement membrane of seminiferous tubules (arrow), while CD90 positive cells were predominantly at the luminal part of the tubules (arrowheads). (**G**) Expression pattern of Nanog in adult human testes was similar to c-Kit and it was present in both undifferentiated and differentiated germ cells. Co-localization of SSEA-4 (green) with Nanog (red) showed that some of the SSEA-4 positive cells in adult human testes were Nanog positive (arrows). (**H**) Oct-4 was specifically localized at the basement membrane of the seminiferous tubules (arrows). Co-localization of stem cell marker SSEA-4 (green) with Oct-4 (red) also showed that some of the SSEA-4 positive cells along the basement membrane of human testes were positively stained for pluripotent marker Oct-4 (arrow heads). (**I**) Co-localization of VASA (red) with SSEA-4 (green) showed that the majority of SSEA-4 positive cells are VASA positive (arrows). Scale bars: 60 μm; E: 30 μm.

SSCs. Only half of the SSEA-4+ cells repopulating in the mouse testes co-localized GPR-125 indicating that GPR-125+ SSEA-4+ cells and GRP-125+ SSEA-4- cells are both repopulating spermatogonia. We also found that 20–30% of the HNP positive cells co-localized with pluripotent markers Nanog and Oct-4 indicating that about one-third of repopulating human spermatogonia might have pluripotent characteristics (Fig. 4).

Discussion

This study clearly demonstrates that SSCs in the adult human testes have phenotypic and molecular characteristics distinct from the mouse and similar, but not identical to primate SSCs. First, we studied the localization and expression of selected markers in the human testes sections and isolated cells. Our immunohistochemical study showed that among the markers tested, SSEA-4 is specifically expressed at the surface of human SSCs. All SSEA-4 positive cells were located at the basement membrane of the seminiferous tubules and co-localized the germ cell marker VASA. This is very similar to the previous observations in the adult primate testes (Muller et al., 2008; Maki *et al*., 2009). However, the percentage of SSEA-4 positive cells in the adult human testes was much higher in the human (13%) than the monkey (2%) testes. Our molecular biological analysis also revealed that SSEA-4 sorted cells have higher

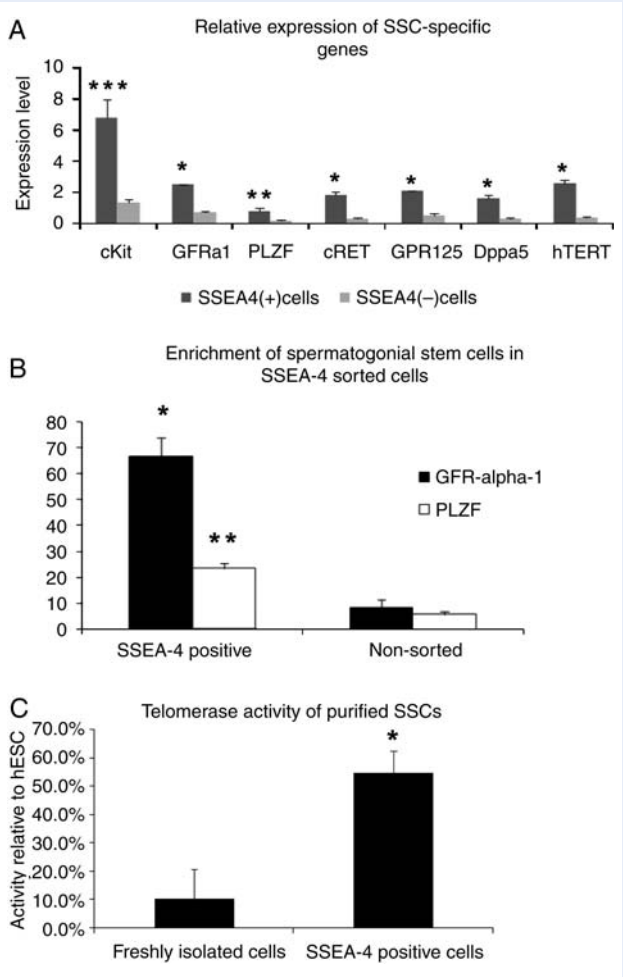

Figure 3 Molecular and phenotypic characteristics of an enriched population of spermatogonia isolated from adult human testes. (**A**) SSEA-4 positive cells showed significantly higher expression levels of SSC-specific genes including GFR-α1, C-Ret, GPR-125 and hTERT when compared with the negative cells. (**B**) SSEA-4 sorted cells contained significantly more GFR-α1 ($P < 0.0001$) and PLZF ($P < 0.001$) positive cells when compared with the non-sorted cells. (**C**) Telomerase activity of SSEA-4 positive cells was also significantly ($P < 0.01$) higher than SSEA-4 negative cells. *$P < 0.01$; **$P < 0.05$; ***$P < 0.001$.

expression levels of all the SSC-specific genes and a high level of telomerase activity indicating the presence of SSCs in this population.

Previous studies showed that SSCs isolated from mouse (Shinohara *et al.*, 2000) and adult primate (Maki *et al.*, 2009) testes express CD49f and CD90 and are negative for CD117. Expression of CD49f, CD90 and SSEA-4 in human testes and cells has already been reported (Conrad *et al.*, 2008; He *et al.*, 2010). Müller *et al.* (2008) did not find SSEA-4 staining in the human testes. This might be due to the technical differences used in different studies. In our study as well as Conrad *et al.* (2008) cryosections were used for immunolocalization studies while Müller *et al.* used paraffin sections. It is possible that during the paraffinization and deparaffinization procedures the integrity of proteoglycan SSEA-4 in human testes may have been compromised. Our immunohistochemical study revealed that CD49f in human testes was mainly localized along the basement membrane of seminiferous tubules suggesting that this marker is expressed in both the undifferentiated as well as the differentiating spermatogonia. Also, expression of some CD49f positive cells outside seminiferous tubules indicates that CD49f, although expressed in human spermatogonia, is not a specific marker and cannot be used alone for enrichment of SSCs from human testes. Our flow cytometry analysis confirmed our immunohistochemical staining and showed that there are distinct populations of cells within the adult human testes that positively stained for CD49f or CD90. However, in contrast to primate, there was only a very small population of cells positive for both CD49f and CD90 present in the adult human testes. Similar to SSEA-4, the percentage of CD49f and CD90 positive cells also was much higher in the adult human testes when compared with the monkey testes.

Table II Localization of germ cells and stem cell markers in human SSCs repopulating in the mouse testes as detected by HNP staining.

Marker	Co-localization with HNP (%)
VASA	100
CD117 (c-kit)	49.8 ± 2.9
GPR-125	42.8 ± 2.6
LH-R	0
CD49f	28.1 ± 3.6
CD29	0
SSEA-4	14.2 ± 3.6
Nanog	28.3 ± 1.5
Oct-4	21 ± 2.3
TRA1-60	0

Our flow cytometric analysis revealed that there are about 13% CD117 (c-Kit) positive cells in the adult human testes. Immunohistochemical staining showed localization of c-kit in many cells located at the basement membrane of seminiferous tubules as well as the cells in the luminal compartment of the human testes. Knowledge about the localization of c-kit in human testes is limited and contradictory. While localization patterns of c-Kit protein in human testes, similar to our results has been reported by Sandlow *et al.*, 1996; 1997), histological examination of samples obtained from prostate cancer patients showed c-kit staining mainly in the interstitial area of human testes (Rodriguez *et al.*, 2008). Recently, Unni *et al.* (2009) reported stage-specific localization and expression of c-kit in undifferentiated spermatogonia in adult human testes, while He *et al.*, (2010) did not find expression of c-kit in human spermatogonia.

Co-localization of c-kit with CD49f in our study revealed that the majority of CD49f positive cells are c-kit positive. While only 25% of SSEA-4 positive cells along the basement membrane of tubular cross sections dimly stained for c-kit. These results indicate that most likely the undifferentiated populations of human spermatogonia including SSCs are c-kit negative. Histological and transplantation

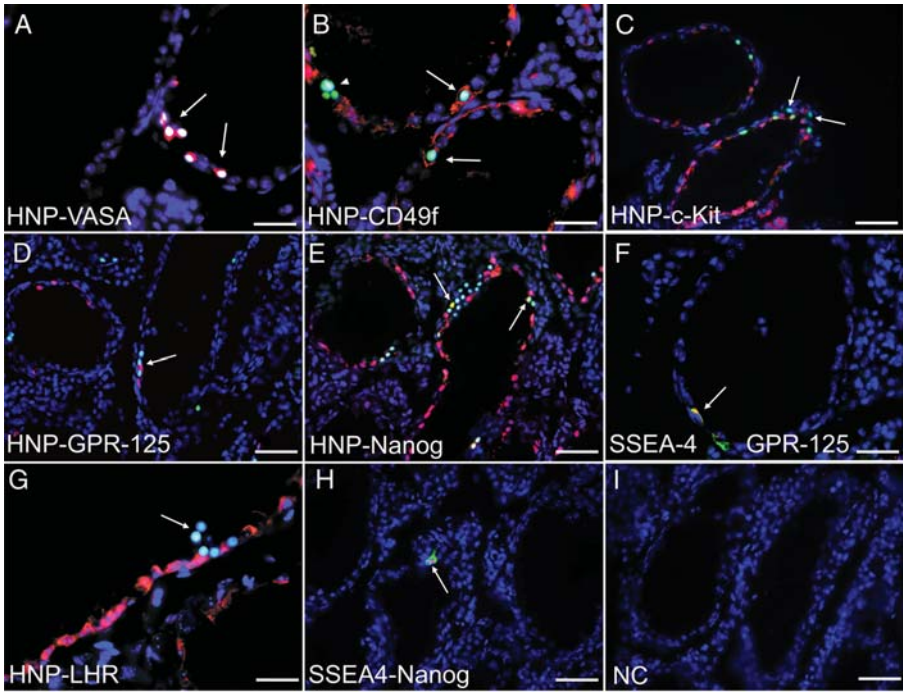

Figure 4 Expression of specific markers in human SSCs repopulating in the mouse testes. The identity of human cells in the mouse testes was detected by HNP antibody directly conjugated to Alexa Fluor 488 and shown in green and all the other markers shown in red. (**A**) Note all the human cells that colonized mouse testes were positively stained for germ cell-specific marker VASA (arrows). (**B**) Some of the human cells at the basement membrane of the mouse testes co-localized CD49f (arrows) and some were negative for this marker (arrowhead). (**C**) Co-localization of SSEA-4 with c-Kit showed that majority of the SSEA-4 positive cells in the mouse testes were either c-Kit negative (arrows) or express low level of c-Kit (arrowheads). (**D**) Among human cells colonized in the mouse testes some co-localized with GPR-125 (arrow). (**E**) Co-localization of HNP with Nanog showed that only a subpopulation of the HNP cells repopulating mouse testes were Nanog positive (arrows). (**F**) Co-localization of stem cell marker SSEA-4 with SSC marker GPR-125 showed that some of the SSEA-4 positive cells repopulating in recipient mouse testes co-localized GPR-125 (arrow). (**G**) Double staining of HNP with leuteinizing hormone receptor showed that none of the human cells repopulating recipient mouse testes were positive for this marker (arrow). (**H**) Also co-localization of SSEA-4 with Nanog showed that only some of the SSEA-4 positive cells in the mouse testes express Nanog (arrow). No staining was observed in the negative control sections (**I**). Scale bars: A, B and G, 30 μm; C–F, H and I, 60 μm.

studies in the mouse also indicates that SSCs are c-Kit negative (Yoshinaga *et al*., 1991; Schrans-Stassen *et al*., 1999; Shinohara *et al*., 2000).

In our previous study in the adult primate we found that SSEA-4 positive cells are the actively dividing population of SSCs capable of repopulating recipient mouse testes (Maki *et al*., 2009). Using the same strategy and in order to answer this important question, we purified human spermatogonia by SSEA-4 magnetic sorting and transplanted SSEA-4 positive cells into the busulfan-treated recipient mouse testes. SSEA-4 positive cells were found at the basement membrane of the majority of mouse seminiferous tubules following transplantation indicating the presence of functional SSCs in this population. In addition, our RT–PCR analysis revealed that SSEA-4 sorted cells have a high expression level of SSC-specific genes. Moreover, SSEA-4 positive cells were significantly enriched in GFR-α1 and PLZF when compared with the non-sorted cells. GFR-α1, receptor for GDNF, and PLZF are both specific markers for SSCs and are involved in SSC self-renewal (Costoya *et al*., 2004; Oatley and Brinster, 2008). This observation further supports our transplantation and gene expression analyses and indicates that SSEA-4 sorted cells are enriched in SSCs.

We also found that a subpopulation of integrated human cells in the mouse testes expressed GPR-125 on their surface, indicating that this marker also is expressed on repopulating human SSCs. Our result is in agreement with He *et al.* (2010) who showed that purified GPR-125 positive cells isolated from adult human testes express SSC markers. Expression of GPR-125 in the mouse (Seandel *et al*., 2008) and human (Dym *et al*., 2009b; He *et al*., 2010) testes sections has also been reported. We further demonstrated that subpopulations of repopulating human SSCs in the mouse testes are positively stained for the pluripotent marker Nanog. Expression of pluripotent markers Nanog and SSEA-4 in some of the spermatogonia indicates that a subpopulation of the repopulating spermatogonia in the adult human testes might have the multipotent ability to differentiate into other cell lineages. Generation of multipotent cell lines from the mouse (Kanatsu-Shinohara *et al*., 2004; Guan *et al*., 2006; Saendel *et al*., 2007; Izadyar *et al*., 2008) and human (Conrad *et al*., 2008; Kossack *et al*., 2008) testes supports multipotentiality of human SSCs suggests clinical application of these cells for regenerative diseases other than the restoration of fertility. On the other hand, immunolocalization of Nanog in human testes was not only limited to the

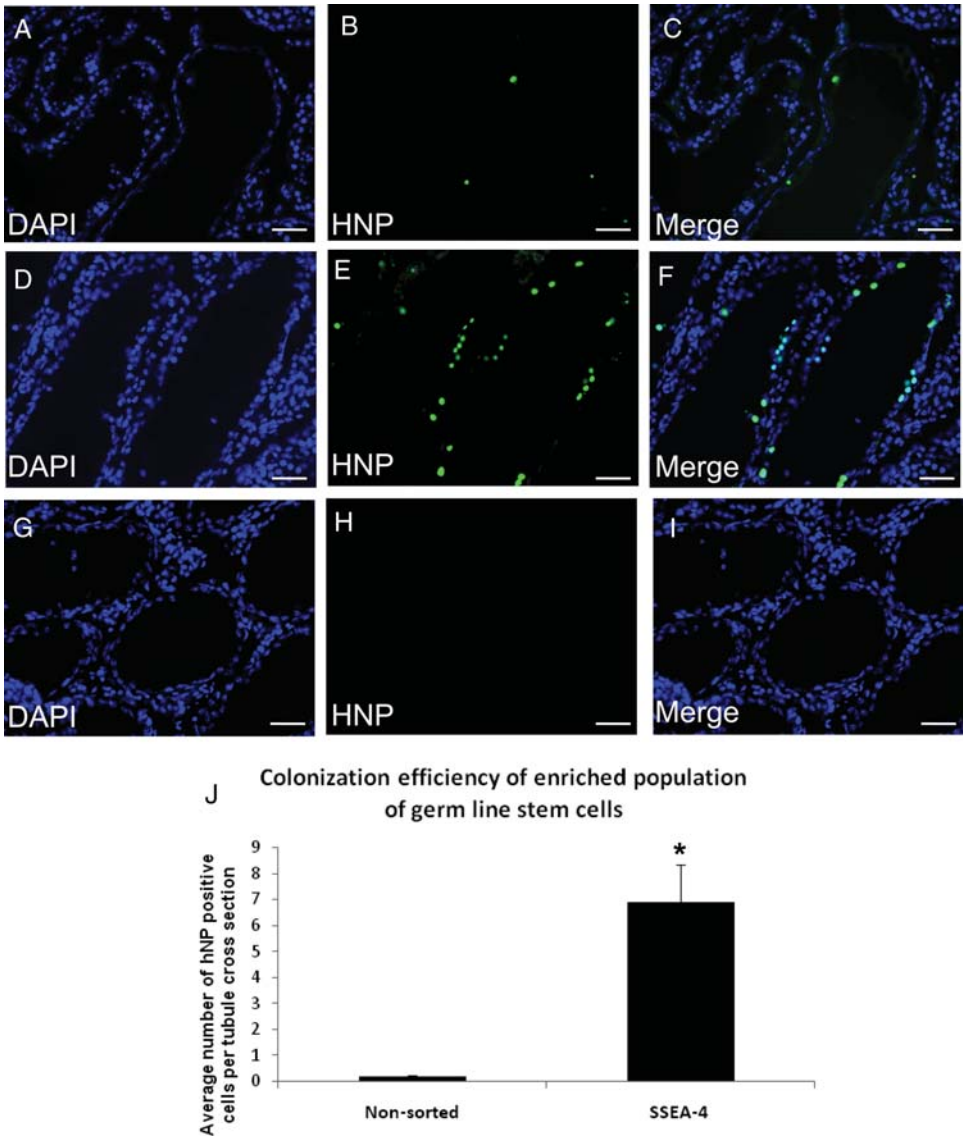

Figure 5 Transplantation efficiency of enriched population of human spermatogonia in the mouse testes. The identity of human cells in the mouse was detected using anti-HNP antibody directly conjugated to Alexa Fluor 488 and shown in green. DAPI was used for nuclear staining and is presented in blue. (**A**–**C**) Representative images of the mouse testes transplanted with non-sorted freshly isolated cells from human testes. Note only few HNP positive cells are found in the recipient testis. (**D**–**F**) Representative images of the mouse testes transplanted with magnetically sorted SSEA-4 positive cells. There are many HNP positive cells lining along the basement membrane of seminiferous tubules of the recipient testis. Representative images of sham transplanted mouse testes are shown in (**G**–**I**). Note there are no HNP positive cells found in the sham group. Scale bars: 60 μm.

undifferentiated spermatogonia but was also localized in all the germ cells even in the lumen of the seminiferous tubules. This observation is very similar to the adult primate testes (Maki *et al*; 2009) suggesting a different role for transcription factor Nanog in the advanced germ cells. The nature of such a role for Nanog is yet to be determined, however, it has been reported that pluripotent marker Oct-4 is a survival factor for germ cells and its down-regulation will result in apoptosis and cell death rather than differentiation (Kehler *et al*., 2004).

Our transplantation study also demonstrated niche compatibility between the human and mouse seminiferous tubules for SSC colonization. Interestingly, the percentage of SSEA-4 (14%) positive cells and CD49f (28%) positive cells in the recipient mouse testes was very similar to that of the human testes (13% for SSEA-4 and 27% for CD49f). This indicates that human SSCs have the ability to colonize and repopulate empty mouse testes at a level very similar to its natural environment suggesting that mouse testes have provided a favorable environment for the colonization of human SSCs. However, any development further than limited spermatogonial proliferation was not found neither in this study nor in previous studies using bovine, porcine, primate or human SSCs (Dobrinski *et al*., 2000; Nagano *et al*., 2001; Honaramooz *et al*., 2002; Izadyar *et al*., 2002; Nagano *et al*., 2002). Our observations for the first time clearly demonstrates

that although mouse testes cannot provide an appropriate environment to support complete spermatogensis from higher species, its basement membrane of seminiferous tubules has the ability to selectively attract and house human SSCs in the manner very similar to the human testes.

In summary, this study demonstrates that repopulating SSCs in the adult human testes have phenotypic characteristics of SSEA-4+, CD49f+, CD90+, GPR-125+ and c-Kit$^{neg/low}$. About one-third of repopulating spermatogonia express Oct-4 and Nanog indicating the existence of populations of SSCs in the adult human testes with pluripotent characteristics. The results have direct implications for isolation and purification of SSCs from adult human testes for clinical applications, culture expansion or differentiation purposes. In addition, expression of pluripotent markers in subpopulations of human SSCs indicates the potential application of these cells for cell replacement therapy and tissue regeneration.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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