

Long-term Culture of Human SSEA-4 Positive Spermatogonial Stem Cells (SSCs)

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Abstract

Recently we and two other groups have shown that human spermatogonial stem cells (SSCs) have the potential to become pluripotent *in vitro* in defined culture conditions and to differentiate into cells of the three embryonic germ layers. This discovery could open new avenues for autologous cell-based therapy in degenerative diseases, bypassing the ethical and immunological problems related to the human embryonic stem cells. In addition, human SSCs could be used to treat infertility in cancer survival children. However, in order to reprogram SSCs into pluripotency, or to preserve them for repopulation of infertile testes, the first and limiting step is to have access to a highly purified human SSC population that could be multiplied and efficiently cultured *in vitro* maintaining their molecular and cellular characteristics. Although various studies have attempted to identify molecular markers of human SSCs, to date there is still limited information related to the specific markers that could be used for their isolation and optimized purification that allows long-term *in vitro* culture of isolated human SSCs.

Here using SSEA-4 as an optimal marker for isolation of a subpopulation of SSCs, we show that SSEA-4 positive cells express the highest level of SSC genes compared to other subpopulations isolated with different markers, and can be maintained in culture for over 14 passages which we were unable to obtain with other SSCs markers including GPR125 and ITGA6. In addition, we have established a new technology for cell sorting and long-term culture of human SSC-SSEA-4 positive cells that maximizes the purity and viability of the sorted cells. Our findings are crucial and could be used for the most efficient isolation, purification and long-term culture of SSCs for clinical applications in regenerative medicine, or for preparation of human SSCs for autologous treatment of infertility in cancer survival children.

Keywords: Human spermatogonial stem cells; Stem cell isolation; Long-term culture; Cell-based therapy; Infertility.

Abbreviations: SSEA-4: Stage-specific embryonic antigen-4; SSCs: Spermatogonial Stem Cells; iPSCs: induced pluripotent stem cells; gPSCs: Germline Pluripotent Stem Cells; ESCs: Embryonic Stem Cells; T-SP: Testis Side Population; GDNF: Glial cell line-Derived Neurotrophic Factor; GFRA1: GDNF family receptor alpha1; ITGA6: Integrin $\alpha 6$; CD9: CD9 antigen (p24); GPR125: G-protein-coupled receptor 125; MACS: Magnetic-Activated Cell Sorting; FGFR3: Fibroblast Growth Factor Receptor 3; DSG2: Desmoglein 2; WRTC: Washington Regional Transplant Consortium; HBSS: Hank's Buffered Salt Solution; GSC medium: Gremlins Stem Cell medium; SFM: Serum Free Medium; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; Ct: Cycle threshold; PBS: Phosphate buffered saline; HRP: Horseradish peroxidase; UCH-L1: Ubiquitin Carboxyl-terminal esterase L1

Introduction

Spermatogonial stem cells (SSCs) self renew and produce a large number of differentiated sperm lifelong in male [1]. Longtime considered as unipotent, capable of only producing sperm, SSCs have been recently shown to be pluripotent, able of differentiating into three primary germ layers [2-4] similar to embryonic stem (ES) cells [5]. Although at its early stages, this approach will open new avenues for generating an alternative source of pluripotent cells for therapeutic implication in degenerative diseases, by bypassing the ethical and immunological issues related to the use of human embryonic stem cells. In addition, SSCs could be considered as a prominent source for treatment of infertility in cancer survival children and therefore their isolation, optimized purification and long-term culture are valuable for clinical application [6-10].

Advanced progress has been made in characterization and culture of rodent SSCs [11-19]. Selection of mouse testis cells with anti-b1- or anti- $\alpha 6$ -integrin antibody, but not anti-c-kit antibody, produced cell populations with a significantly enhanced ability to colonize recipient testes and generate donor cell-derived spermatogenesis [19]. It has been demonstrated that immature mouse testis contains a "side-population" (T-SP), which is Sca-1^{pos}, Ep-CAM^{pos}, EE2^{pos}, $\alpha 6$ -integrin^{pos}, and αv -integrin^{neg}. A 13-fold enrichment in SSC activity was observed when sorted T-SP cells from ROSA 26 mice were transplanted in busulfan-treated mouse testis [20]. The SSCs development is controlled, in part, by factors in the stem cell niche, located on the basement membrane of seminiferous tubules situated among Sertoli cells. Transgenic loss-of-function and over-expression models showed that the dosage of glial cell line-derived neurotrophic factor (GDNF), produced by Sertoli cells, regulates cell fate decisions of undifferentiated spermatogonial cells including the stem cells for spermatogenesis [13]. The GDNF signals through a receptor complex that includes GDNF family receptor alpha1 (GFRA1), which is expressed by SSCs. The GFRA1-

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positive cell fraction co-expresses other markers of SSCs, including ITGA6 and CD9, and is significantly depleted of KIT-positive cells and therefore can be used to isolate, enrich and characterize mouse SSCs. The transplantation of GFRA1-positive cells into seminiferous tubules of recipient mice confirmed that a subpopulation of SSCs expresses GFRA1, but also that the stem cell pool is heterogeneous with respect to the level of GFRA1 expression [21]. Interestingly, POU5F1-positive cells were enriched nearly 15-fold in the GFRA1-selected fraction, possibly suggesting heterogeneity of developmental potential within the stem cell pool [21]. Another recent report demonstrated that GPR125, an orphan adhesion-type G-protein-coupled receptor was expressed in adult mouse spermatogonial stem cells [17]. Despite extensive investigation in rodent SSCs, there is limited information related to human SSCs and their specific markers. Recently, we and a few other groups have investigated the characterization and isolation of human SSCs and have suggested a few markers that are expressed in these cells [22-24]. The isolation of the cells with magnetic-activated cell sorting (MACS) with GPR125 antibody showed that these cells presented the phenotype of SSCs and co-expressed ITGA6, THY1, and GFRA1 [23]. In a recent publication it was demonstrated that GFRA1 was heterogeneously expressed in human A_{dark} and in A_{pale} spermatogonia, the earliest spermatogonia [22]. In addition, a whole genome approach screening for biomarkers of spermatogonia within the human testis has revealed that the surface markers FGFR3 and DSG2 might be used for isolation and enrichment of human SSCs [24]. Furthermore, very recently a paper was published showing that SSEA-4 is a marker of human SSCs [25]. These publications are mostly based on immunostaining and/or gene expression data, and propose markers that might be adequate for isolation, culture, expansion and differentiation of SSCs. Another recent study has addressed long-term culture of human SSCs obtained from obstructive and non-azoospermia using GFRA1 and CD9 as selective markers [26]. However, long-term culture of human SSCs remain challenging due to the difficulties in maintaining the SSCs in culture in conditions that support their self-renewal, molecular and cellular characteristics and pluripotent potential. The recent publication by Conrad et al. [4] using ITGA6 ($\alpha 6$ -integrin) has shown that this marker could be used to isolate, culture and reprogram the SSCs into germline pluripotent stem cells (gPS) cells that represent similar gene expression pattern and differentiation capacities to those of embryonic stem (ES) cells. In our previous paper we have cultured and reprogrammed adult human germ cells without any selection [2]. Recently, after several attempts to isolate and culture ITGA6-positive SSCs, our experiments revealed that ITGA6 is highly expressed in myofibroblasts allowing the selection of large numbers of fibroblasts as well (Supplementary Figure 1) which makes the SSC culture very difficult since the fibroblasts overgrow in the serum containing medium and deprive the growth of the small number of selected SSCs. Furthermore, the gene expression analysis revealed a fibroblast gene expression pattern that was largely different to that of ES cells. These experiments were repeated three times to confirm the authenticity of our observations. Therefore, we concluded that ITGA6 might not be an optimal marker for selection and long-term culture of SSCs *in vitro*. Furthermore, a brief communication recently appeared in Nature by Ko et al. [27] confirming that the gene expression pattern of haGSC from Conrad paper is similar to that of human fibroblasts but different to that of hES cell.

Consequently, we investigated the expression of other markers for isolation of SSCs for long-term culture. We have isolated SSC subpopulations from testes of organ donors with several surface

markers that are proposed to be the surface markers of human SSCs in recent publications [4,23]. Here using various antibodies and MACS, we have isolated different cell populations and have compared their gene expression to identify the population that highly expresses the genes of interest. By proposing a new methodology that permits the most efficient isolation of SSCs, we have observed that stage-specific embryonic antigen-4 (SSEA-4) can be used for isolation, long-term culture and reprogramming of human SSCs, therefore represents the most optimal marker for selection of SSCs proposed to date. We therefore propose a new methodology for isolation and long-term culture of human SSCs and move forward the use of these cells one-step closer to the clinical application in cell-based regeneration therapy.

Materials and Methods

Procurement of testes from organ donors

For the use of human testes from deceased organ donors in our research, written approval from the next of kin was obtained by the Washington Regional Transplant Consortium (WRTC; Annandale, VA), as described previously [23,28]. Prior to surgery, the donors were maintained on a heart-lung machine with artificial respiration. During surgery, the abdominal organs were first dissected away from the posterior abdominal wall. Blood flow was stopped after intravenous injection of heparin followed by aortic "cross clamping" in the thorax and in the lower abdomen. Immediately after clamping, the aorta was perfused with Viaspan (DuPont Pharma), an organ preservation solution. Therefore, all abdominal organs as well as the testes were preserved in Viaspan. The testes were removed after a 10-min perfusion, packed in cold Viaspan, and sent to the Georgetown University Medical Center by courier within 1-2 h of surgery. In our laboratory, the testes were immediately placed aseptically in Dulbecco modified Eagle medium (DMEM) containing 1× antibiotic/antimycotic (Gibco, Carlsbad, CA). The data generated for this project was obtained from three donors (14, 34 and 45 years old).

Isolation and culture of SSEA-4(+) cells from human testes

Testes were digested with 10ml of digestion solution per 1gr of tissue for 45min at 34°C with 100rpm rotation. The digestion solution consisted of 2.5mg/ml collagenase XI (SIGMA, St Louis, MO), 1.25 mg/ml dispase (Gibco) and 2mg/ml DNase I (SIGMA) in Hank's Buffered Salt Solution (HBSS). The undigested tissue fragments were removed by filtration through a 100µm cell strainer (BD Falcon, San Diego, CA). To remove the adherent somatic cells, the resulting cell suspension was incubated at 34°C on Fetal Bovine Serum (FBS) - coated dishes in Germline Stem Cell (GSC) medium [29] for 24hrs and the non-attached cells were transferred to a 50ml tube and washed with Cell Dissociation buffer (Invitrogen) to inhibit formation of cell aggregates. The cells were resuspended in 1ml RBC Lysis buffer (BD Biosciences, San Diego, CA) for 30sec to selectively lyse the red blood cells in the preparation. After RBC lysis, the cells were washed in HBSS and incubated with 2mg/ml dispase for 5min to digest any remaining cell aggregates. Dispase reaction was terminated with DMEM-10% FBS and the cells were resuspended in HBSS and filtered through 100µm and 40µm cell strainers. Dead cells were removed using the Dead Cell Removal Kit (Miltenyi Biotec, Cambridge, MA). Briefly, 200µl of Micro-Beads were added to the cell pellet and incubated for 15 min at room temperature. The mixture was applied to a medium sized (MS) MACS column, which was placed in the magnetic field using the MiniMACS separation unit (Miltenyi Biotec). Live cells were collected in the flow-through in 1ml of binding buffer (Miltenyi Biotec). The removal of dead cells was

confirmed by Trypan Blue staining. For the immunosorting of the live cells from human testis, 200 μ l FcR blocking Reagent (Miltenyi Biotec) and 5 μ g of biotinylated anti-human/mouse SSEA-4 (R&D Systems) were added to 700 μ l cell suspension of approximately 2×10^7 cells and incubated for 30min at 4°C. Magnetic micro-beads conjugated to anti-biotin (20 μ l, Miltenyi Biotec) and 10 μ l FcR blocking Reagent (Miltenyi Biotec) were added in the cell suspension at 100 μ l final volume and incubated for 15min at 4°C. The labeled cells were washed on the beads twice and then passed through an MS column for sorting. Typically, from an isolation using a whole human testes, starting with a testicular cell suspension of 10^9 cells, we obtained 500,000 SSEA-4(+) cells (0.0005%). The SSEA-4(+) and the SSEA-4(-) cells were then used for culture or RNA isolation or immunostaining.

Cell culture media and passaging

For the culture of SSEA-4(+) and SSEA-4(-) cells we used the germline stem cell (GSC) medium, slightly modified from the one used by Ko et al. [29] for the culture of adult mouse germline stem cells. It consists of StemPro-34 Serum Free Medium supplemented with 1x StemPro supplement, 1x N2 supplement, 2mM L-glutamine, 1% ES-qualified FBS, 1x penicillin/streptomycin (all purchased from Invitrogen, Carlsbad, CA), 6mg/ml D-(+)-glucose (SIGMA), 30mg/ml pyruvic acid (Invitrogen), 0.1% (v/v) DL-lactic acid (SIGMA), 5mg/ml BSA (SIGMA), 50mM b-mercaptoethanol (SIGMA), 1x MEM Vitamins (Invitrogen), 30ng/ml beta-estradiol (SIGMA), 60ng/ml progesterone (SIGMA), 20ng/ml human EGF (R&D Systems, Minneapolis, MN), 10ng/ml human bFGF (R&D Systems), 10ng/ml human GDNF (R&D Systems), 10^3 units/ml human LIF (Millipore, Billerica, MA).

The de-differentiation medium used for the reprogramming of the *POU5F1*-promoter-GFP expressing cells was hES medium: DMEM-F12 supplemented with 20% Knockout Serum Replacement, 1x Non Essential Amino Acids, 1mM glutamine (all purchased from Invitrogen), 50mM b-mercaptoethanol (SIGMA) and 4ng/ml bFGF.

The SSEA-4(+) and the SSEA-4(-) cells were cultured separately in GSC medium on hES-qualified Matrigel with reduced growth factors (BD Biosciences) at a cell density of 50,000 cells per cm². The medium was changed every 2-3 days. Colonies, consisting of round-shaped cells formed after 1 week in culture. The cells were passaged manually for the first time 1 month after the initiation of culture, triturated by pipetting and transferred to a new dish. After passage 1, the following passages were performed enzymatically; after the colonies were pipetted off the dish, they were incubated with 2mg/ml dispase (Gibco) and 1mg/ml collagenase Type IV (Gibco) in HBSS for 10 minutes at 37°C. The enzymatic digestion resulted in a mixture of single cells and partial digested colonies, consisting of 5-10 cells. Colony numbers were counted before the enzymatic digestion and cell numbers were counted after the digestion using a hemacytometer. Immediately after the enzymatic digestion, nine volumes of serum containing medium (DMEM with 10% FBS) were added, and the cells were spun (1000 rpm/5 min) and re-suspended in GSC medium. The medium was changed every 2 days and the cells were passaged every 10-15 days.

RNA Isolation and qRT-PCR

Total RNA was extracted from the cells using the RNeasy Mini Kit (Qiagen, Germantown, MD) according to the manufacturer's protocol. The RNA samples were treated with RNase-free Dnase I (Qiagen) to remove traces of genomic DNA. The concentration and quality of total RNA was determined using the NanoDrop-1000 Spectrophotometer.

For the quantitative RT-PCR analyses, the SuperScript® III First-Strand Synthesis System (Invitrogen) was used to perform reverse transcription with 500 ng of the oligo-dT primer and 50-500 ng of total RNA from each sample. Reactions were performed in duplicates; RT enzyme was added in one of the two, the other serving as a negative control for the presence of genomic DNA in the samples. Primers were designed for real-time qPCR using the Primer Quest tool (www.idtdna.com/Scitools/Applications/Primerquest/, Integrated DNA Technologies, Coralville, IA). All primers had similar length (22-24 nucleotides), ~50% GC content and melting temperatures (T_m) were 58-60°C. To avoid false positive results due to contamination by genomic DNA, we chose to design the primers spanning at least one intron in the genomic sequence, which was possible in most cases (Supplementary Table 1). Real-time qPCR was carried out in triplicates on an ABI7900HT (Applied Biosystems, Foster City, CA). Reactions were performed in 96-well plates with a final volume of 25 μ l. Polymerase chain reactions contained 1x QuantiTect SYBR Green PCR master mix (Qiagen), 6 pmol of each primer, and 1 μ l of the Reverse Transcription reaction. Cycling parameters shared for all genes were 95°C for 15 min, followed by 40 cycles at 94°C for 15 sec, 58°C for 30 sec, and 72°C for 30 sec. Finally, a temperature-determining dissociation step was performed at 95°C for 15 sec, 60°C for 15 sec, and 95°C for 15 sec at the end of the amplification phase. For all primer combinations, PCR product authenticity was assessed by gel electrophoresis. To normalize the data from different RNA samples, the human glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was used as an internal control. Quantitative PCR data were collected by SDS 2.2 software (Applied Biosystems) and used to estimate the cycle threshold (Ct) for each reaction replicate. Once Ct for all replicates was obtained the amplification efficiency (*E*) was determined for each primer combination, as $E = 1(-1/\text{slope})$, where the slope was estimated plotting the Ct in a serial dilutions of cDNA. To compare the mRNA levels among different samples, the normalized expression (NE) of each target gene (assayed in triplicates) was related to *GAPDH* reference gene expression levels by the formula: $NE = (E_{\text{target}})^{Ct_{\text{target}}}/(E_{\text{ref}})^{Ct_{\text{ref}}}$. For each gene, differences in expression levels among the different samples were analyzed by a one-way analysis of variance (ANOVA). Differences were considered statistically significant at $P < 0.05$.

Immunostaining

Human testes were fixed in 4% paraformaldehyde and embedded in paraffin prior to sectioning. Cells were also fixed in 4% paraformaldehyde prior to staining. Sections and cells were permeabilized for 5 min with 0.5% Triton-X in 1x PBS and blocked in 1% BSA, 0.1% Tween-20 and 5% normal serum, in 1x PBS. Primary antibodies (Supplementary Table 2) were diluted 1:100 in FACS buffer (0.1% BSA in 1xPBS), incubated for 16hrs at 4°C and washed 3 times for 5 min in 1x PBS. In case the secondary antibodies were conjugated to horseradish peroxidase (HRP), the endogenous peroxidase activity was quenched with 3% hydrogen peroxide. Secondary antibodies were incubated for 1-2hrs at room temperature. DAPI was used to stain the nuclei. The stained cells or testis sections were observed for epifluorescence using an Olympus Fluoview 500 laser-scanning microscope. Negative control experiments, without primary antibody, were performed in parallel.

Microarray analysis

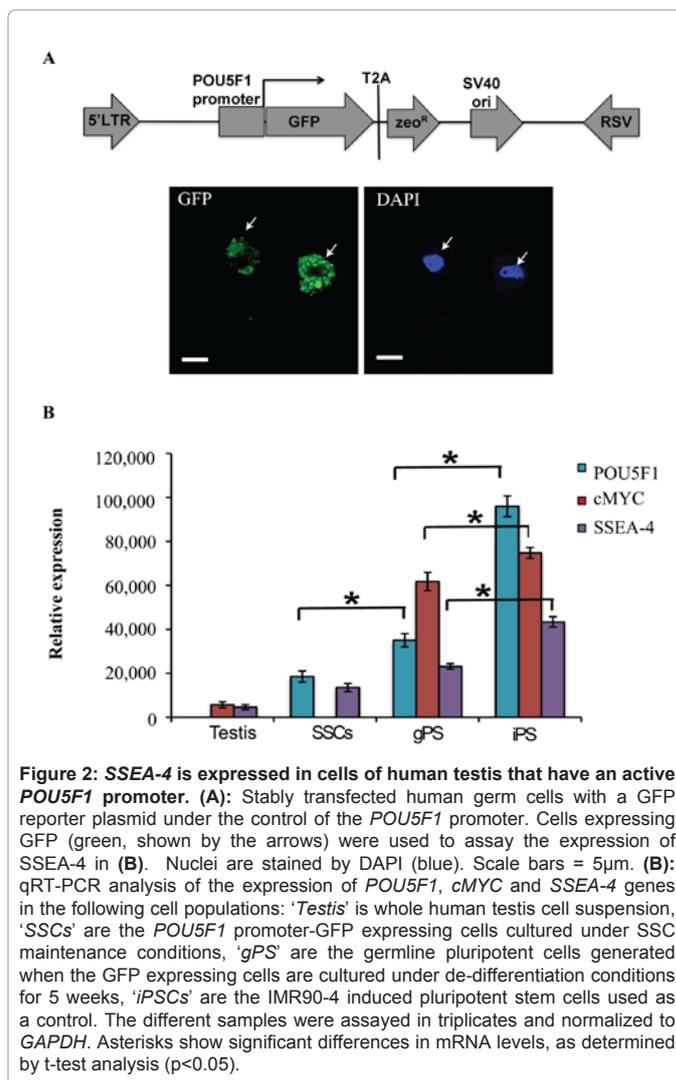
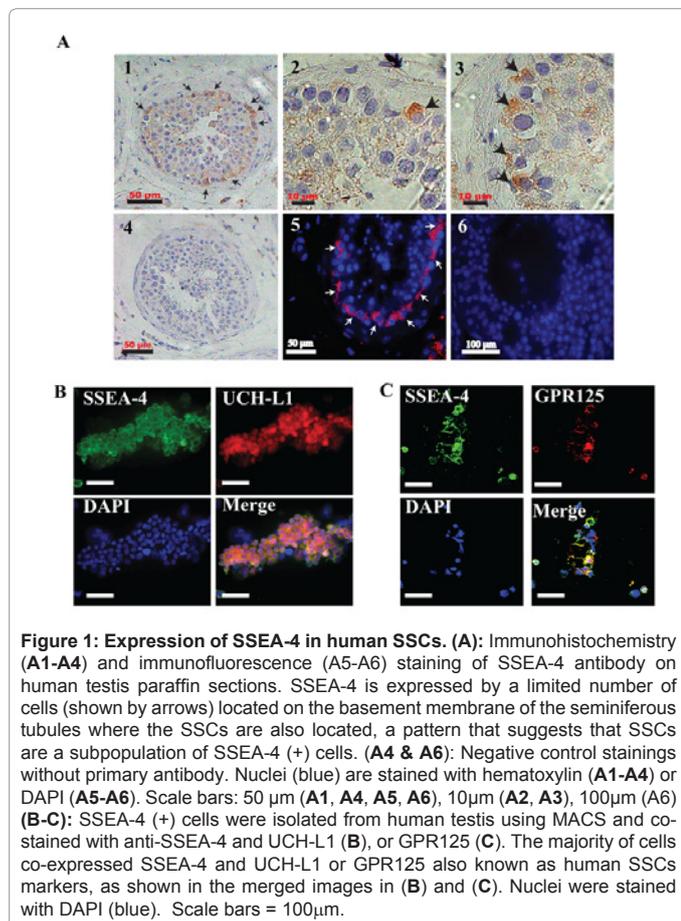
The ITGA6 positive cells were isolated from human testis as described previously [4]. Total RNA (2 μ g) of these cells was used for gene expression analysis using the human whole genome arrays from

Affymetrix (Santa Clara, CA) and following the standard protocols that have been used for the analysis of various NIH induced pluripotent (iPS) and embryonic stem (ES) cell lines. The data were analyzed and compared to the expression profiles of the iPS cell lines NIH12 (passage 19), NIH17 (passage 12) and the WA07 human ES cell line, using the Genespring GX software (Agilent Technologies, Santa Clara CA).

Results

SSEA-4 is a surface marker of human SSCs

The Stage Specific Embryonic Antigen A-4 (SSEA-4) is a surface marker of undifferentiated embryonic stem cells [30]. We and others have demonstrated that SSEA-4 is expressed in specific cells of the basal membrane of seminiferous tubules (Figure 1A). Here we propose that the SSCs are a sub-population of the SSEA-4-expressing cells in the human testis. To test this, we performed Magnetic Cell Sorting (MACS) with the SSEA-4 antibody to isolate the SSEA-4-positive - SSEA-4(+) - cells from freshly isolated germ cells derived from human testis of organ donors and analyzed the SSEA-4(+) cells by double staining for SSEA-4, and the Ubiquitin Carboxyl-terminal esterase L1 (UCH-L1), the G-Protein coupled Receptor 125 (GPR125), both of which have been characterized as SSC markers in the mouse and/or human [23]. We have obtained three testes from three different donors to complete our data. Immunostaining showed co-expression of SSEA-4 and UCH-L1 (Figure 1B) or SSEA-4 and GPR125 (Figure 1C) in these cells, showing that SSEA-4 could indeed be considered as a marker of SSCs.



Stably transfected human germ cells with POU5F1 promoter - GFP reporter co-express SSEA-4 and exhibit gene expression pattern similar to iPS cells in reprogramming media

To confirm that the SSEA-4(+) cells contain the SSCs, we generated a stably transfected line of human germ cells carrying a retroviral construct expressing the green fluorescence protein (GFP) under the control of the POU5F1 promoter. Thus, the cells with an active POU5F1 promoter, which are the SSCs, were expressing GFP (Figure 2A). In addition, we cultured the POU5F1 promoter-GFP cells under both SSC maintenance (GSC medium) and de-differentiation (hES medium) conditions, as described in Material and Methods and analyzed the expression of POU5F1, cMYC and SSEA-4 in both cases, using qRT-PCR (Figure 2B). Under SSC maintenance conditions, the POU5F1 promoter-GFP cells (SSCs) expressed basal levels of POU5F1 and SSEA-4 but the expression of cMYC could not be detected. Under de-differentiation conditions, the resulting germline pluripotent stem (gPS) cells expressed increased levels of POU5F1, SSEA-4 and cMYC, which were 50-80% the expression levels of the same genes in induced pluripotent (iPS) cells used as a control.

SSEA-4(+) subpopulation of human germ cells express the highest levels of proposed SSC and stem cell markers

Using qRT-PCR analysis on the RNA from cells isolated from human testis by MACS with antibodies to SSEA-4, EpCAM, GPR125 and ITGA6, we showed that the SSEA-4 (+) subpopulation was highly enriched in cells expressing reported SSC markers (i.e., *PLZF*, *GPR125*) or the hESC marker *POU5F1*, as compared to the levels of expression in the whole testis and therefore contained a higher number of SSCs, compared to cell populations of germ cells isolated with antibodies to GPR125, ITGA6 and EpCAM (Figure 3). In addition, our data show that ITGA6 is not a specific marker of SSCs and therefore cannot be used for enrichment of human SSCs, since myofibroblasts also express ITGA6.

The SSEA-4(+) cells isolated from human testis form germ cell-like colonies that are sustained in long-term culture

We cultured the SSEA-4(+) cells from the human testis in GSC medium over a period of 4 months. The cells formed grape-shaped colonies, typical for SSC culture (Figure 4E & 4F). Colony formation was observed as early as 6 days after the initiation of culture (Figure 4B & 4C). During the first week of culture, the majority of cells were round-shaped, forming aggregates with similar morphology to germ cell colonies. However, the number of cells at that early stage (Figure 4B & 4C) does not represent the number of SSCs in the culture, since many of the initially round-shaped cells acquired a fibroblast-like morphology and adhered strongly to the dish. These fibroblast-like cells might be testicular myofibroblasts that probably were included in the preparation of SSEA-4(+) cells and later proliferated at a higher rate than the actual stem cells. These myofibroblasts served as a supportive cell layer for the colonies. After the first 4 weeks of culture they proliferated and finally covered the culture surface, supporting the formation of more colonies of SSEA-4(+) cells (Figure 4E & 4F). After the first 4 weeks, the cells were passaged at a dilution of 1:2, for the first time manually, by pipetting the colonies off the myofibroblasts. Afterwards, we passaged the colonies enzymatically (Materials and Methods) at the same dilution, every 10-15 days. The number of colonies

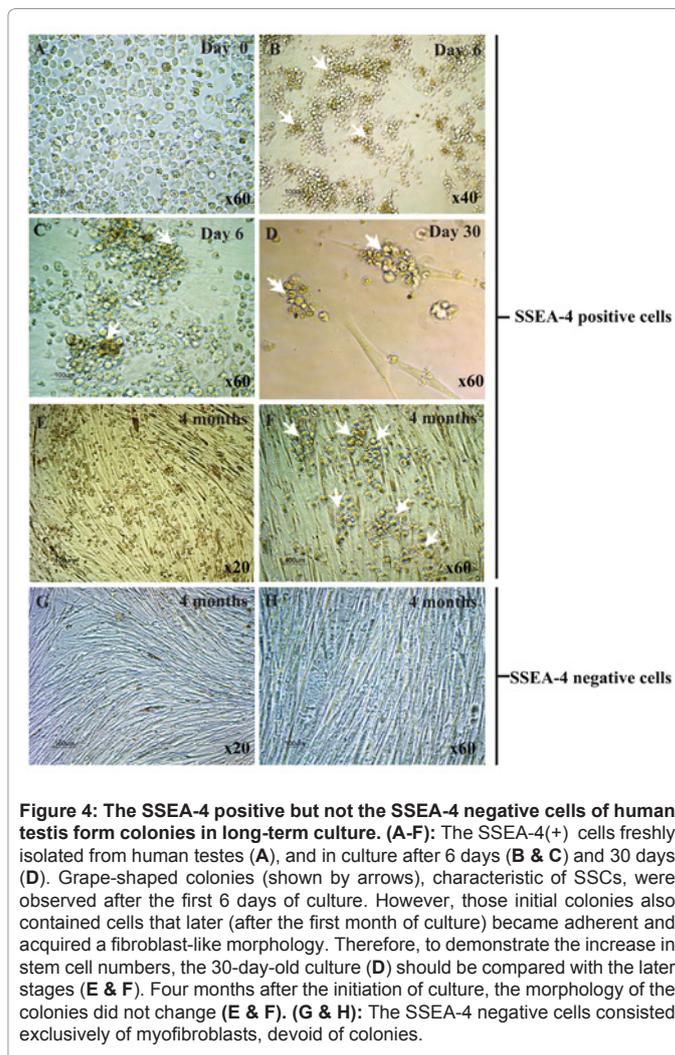


Figure 4: The SSEA-4 positive but not the SSEA-4 negative cells of human testis form colonies in long-term culture. (A-F): The SSEA-4(+) cells freshly isolated from human testes (A), and in culture after 6 days (B & C) and 30 days (D). Grape-shaped colonies (shown by arrows), characteristic of SSCs, were observed after the first 6 days of culture. However, those initial colonies also contained cells that later (after the first month of culture) became adherent and acquired a fibroblast-like morphology. Therefore, to demonstrate the increase in stem cell numbers, the 30-day-old culture (D) should be compared with the later stages (E & F). Four months after the initiation of culture, the morphology of the colonies did not change (E & F). (G & H): The SSEA-4 negative cells consisted exclusively of myofibroblasts, devoid of colonies.

per cm² was counted every week before the 1st passage and every two weeks afterwards. A linear increase in the number of colonies per cm² was observed, starting with 5 (±1) colonies at week 1 and increasing up to 104 (±8.7) colonies at week 16 (Figure 5A). The number of cells per colony was also calculated at week 5 and every two weeks afterwards, after each enzymatic passaging of colonies. We observed a 2x increase in the number of cells per colony over the period of nine weeks, starting with 46 (±4.0) at week 5 and increasing up to 103 (±2.1) at week 16. It should be noted that cell number before week 5 would not be indicative of stem cell number in our culture, as many of the round-shaped cells shown in Figure 4B and Figure 4C at 6 days of culture later formed fibroblasts. Therefore, we started counting cell numbers after the first month of culture (Figure 4D) when the myofibroblasts had already formed. Furthermore, during passaging, although the colonies were pipetted off the myofibroblast layer, we could not avoid a small degree of contamination by the fibroblasts, that supported the colonies in the next passage. It is noteworthy that the culture of SSEA-4 negative cells under the same conditions resulted in a layer of proliferating myofibroblasts, which lacked any colony formation (Figure 4G & 4H). This observation further supports our hypothesis that the SSEA-4(+) cells contain SSCs.

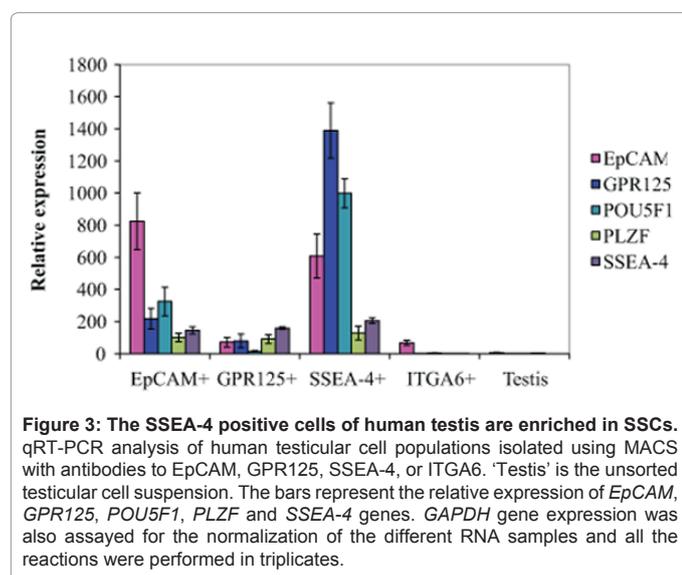


Figure 3: The SSEA-4 positive cells of human testis are enriched in SSCs. qRT-PCR analysis of human testicular cell populations isolated using MACS with antibodies to EpCAM, GPR125, SSEA-4, or ITGA6. 'Testis' is the unsorted testicular cell suspension. The bars represent the relative expression of *EpCAM*, *GPR125*, *POU5F1*, *PLZF* and *SSEA-4* genes. *GAPDH* gene expression was also assayed for the normalization of the different RNA samples and all the reactions were performed in triplicates.

The SSEA-4(+) cells of human testis maintain their ‘SSC’ identity in long-term culture

We then analyzed the expression of SSC and hESC markers after 5 months of culture and compared their expression levels to those of freshly isolated cells, by qRT-PCR analysis. We have observed an enrichment of GPR125 (+), SSEA4 (+) and PLZF (+) cells by two times after 5 months culture demonstrating that SSEA-4 is an optimal marker

for isolation and enrichment of human SSCs (Figure 6). In addition, we also assayed the cultured SSEA-4(+) cells for the expression of other pluripotency factors (i.e., SOX2, NANOG, LIN28, KLF4 and cMYC) that were not expressed in the freshly isolated cells. The expression of these genes was also undetectable after culture of the SSEA-4(+) cells in the SSC maintenance medium (Figure 6). In conclusion, our results suggest that the SSEA-4(+) cells maintain the gene expression pattern of SSCs under our culture conditions.

Discussion

Human spermatogonial stem cells have recently been the center of attention for their pluripotent potential that qualifies them as an alternative source of cells for autologous organ regeneration therapy in degenerative diseases [2-4,31-33]. In order to efficiently reprogram the SSCs into gPS cells, or to use the SSCs for restoration of fertility in cancer treated children, it is crucial to identify the specific markers that allow the enrichment of human SSCs that could be then cultured and maintained *in vitro*.

Here we propose a new method and an optimal marker for generating single cell suspensions from human testicular tissue and thus increasing the efficiency of cell sorting using SSEA4 as a surface marker. Based on our experience, the current method used for human testicular tissue digestion [4], a modified version of a protocol that works most efficiently in 6-day-old mice testes [34], is not as efficient in human testis resulting in incomplete digested tissue, high number of cell aggregates, and increased percentage of cell death. These factors negatively influence the efficiency of cell sorting, resulting in relatively low purity of the isolated cell populations from human testis.

We identified the SSC marker that could not only be used for efficient isolation of human SSCs, but would also select the subpopulation of SSCs that could be maintained and expanded *in vitro*.

Recent publications have tried to relate the information available in rodent to human SSCs. GFRA1 has been shown to be a marker for mouse SSCs and progenitor cells [12,13]. A recent publication has reported distinct GFRA1-positive subpopulations in men. MACS enriched GFRA1(+) cells threefold in monkey and fivefold in human. Interestingly, a high degree of morphological heterogeneity among the GFRA1+ cells from human testes was observed. Therefore, MACS using anti-GFRA1 antibodies was proposed to be an enrichment strategy for spermatogonia from monkey and human testes [35]. GPR125, THY1 and ITGA6 have also been identified as markers for isolation of mouse SSCs [17,19,21,36-38]. In addition, it was reported that THY1 is a conserved marker of undifferentiated spermatogonia in the pre-pubertal bull testis [39]. These observations revealed that SSCs share some, but not all, phenotypic and functional characteristics with other stem cells [18]. One publication has suggested that GPR125-positive cells are putative human SSCs based on phenotypic characterization [23]. Another report has proposed that the surface markers FGFR3 and DSG2 may facilitate the isolation and enrichment of human stem cell and/or progenitor spermatogonia [24]. These observations although informative, fall short in providing deeper information on the genetic characteristic of these cells, and their ability of self-renewal and long-term maintenance in culture. Another study has reported that human testicular cells could be cultured and propagated up to 15 weeks [40]. However, the authors culture a mixed population of testicular cells and not the isolated SSCs.

It is noteworthy that the optimal culture of SSCs is challenging due its dependence on the use of the specific surface marker for selection,

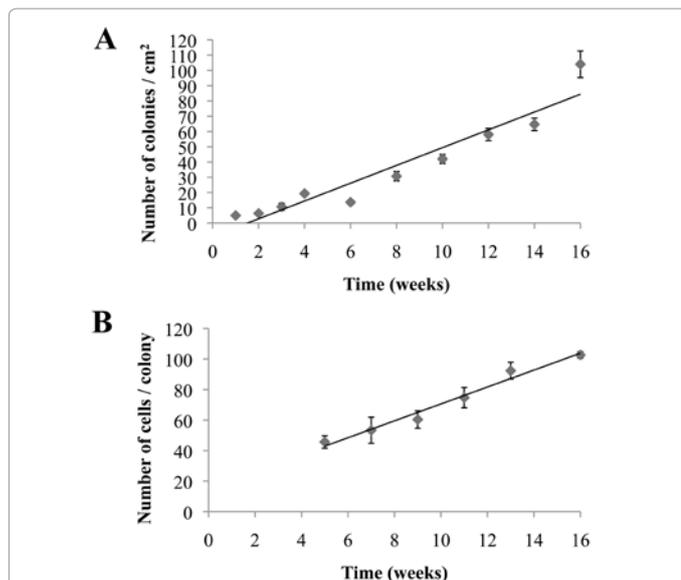


Figure 5: Increase of the number of colonies per cm² and the number of cells per colony in the culture of SSEA-4(+) cells. (A): The number of colonies was counted in 3 wells of a 24-well dish before passaging and the mean average and standard deviation were calculated. (B): The number of cells was counted on a hemacytometer after enzymatic passaging of colonies. The mean average and standard deviation were calculated by the cell numbers on three 4X4 grids of the hemacytometer.

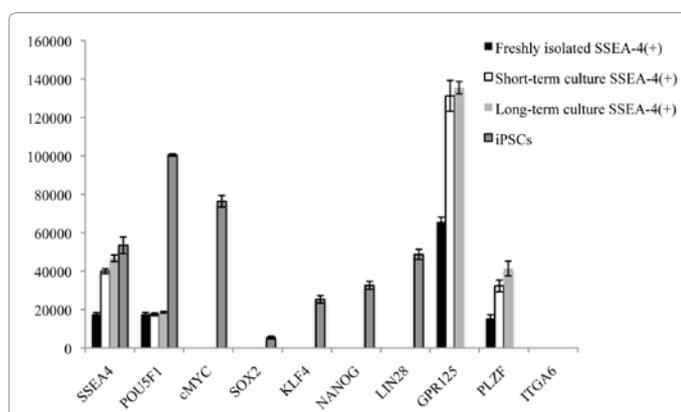


Figure 6: The SSEA-4 positive cells of human testis maintain their SSC identity in long-term culture. qRT-PCR analysis of the expression of the pluripotency and hESC marker genes (SSEA-4, POU5F1, cMYC, SOX2, KLF4, NANOG, LIN28), the SSC marker genes (GPR125, PLZF) and spermatogonial marker gene ITGA6. The bars represent the relative levels of expression of the corresponding genes in the following cell populations: SSEA-4(+) cells freshly isolated from human testis with MACS, SSEA-4(+) cells that have been cultured for 1 month in SSC-maintenance media (short-term culture), SSEA-4(+) cells that have been cultured for 5 months in SSC-maintenance media (long-term culture) and induced pluripotent stem cells (iPSCs), used as control. The different samples were assayed in triplicates and normalized to GAPDH.

and the use of the appropriate culture medium. Considering the importance of this research and its possible implication in clinical therapy, and little information available for selection, expansion and long-term culture of human SSCs, we have investigated other surface markers that will select the best subpopulation of cells for long-term culture of human SSCs in terms of morphology and gene expression.

SSEA-4 is expressed upon the surface of human teratocarcinoma stem cells [41,42], human embryonic germ cells, adult primate testes [42,43] and human ES [44,45]. Co-expression of SSEA-4 and the pluripotency stem cell marker POU5F1 has been detected in human fetal testes [46]. In adult non-human primate testes, SSEA-4 expression by spermatogonia has been reported [47]. In addition, distinct populations of CD90(+) ITGA6(CD49f)(+) CD117(-) (Triple Stained) cells and a small population of SSEA-4(+) cells which are both localized at the basement membrane of seminiferous tubules have been described in the testes of adult non-human primates [43]. Both SSEA-4(+) and Triple Stained cells express germ cell and SSC-specific markers and show high telomerase activity; however, only adult Rhesus monkey SSEA-4(+) testis cells appear to contain functional and actively dividing SSCs that can repopulate recipient mouse testes following spermatogonial transplantation [43]. Very recently, while we were preparing this manuscript, a paper on the characterization of human SSCs was published [25], confirming our results that SSEA-4 is a marker for these cells. Indeed, it was demonstrated that the SSEA-4 expressing cells of human testes could efficiently repopulate the busulfan treated testes of nude mice, suggesting that they are enriched in stem cells. However, the SSC subpopulations isolated in this study were not cultured *in vitro*.

By immunostaining we show that SSEA-4 is expressed in human germ cells that are located in the basement membrane of seminiferous tubules. Using a new methodology we have isolated and enriched the SSEA-4(+) cells and have investigated the expression of other putative human SSC markers on this subpopulation and their potential for long-term culture. We have shown that SSEA4 (+) cells also co-express UCH-L1 and GPR125 that are reported as markers of human SSCs. We have also used other known mouse and reported human SSCs markers for isolation and enrichment of SSCs subpopulations and have compared their gene expression pattern, self-renewal ability, to those of SSEA-4(+) cells. We have found that SSEA-4(+) cells can be long-term cultured unlike the GPR125(+) and ITGA6(+) cells, and showed the gene expression of human SSCs after more than three months in culture. By generating stably transfected germ cells that express GFP under the control of active *POU5F1* promoter, we have demonstrated that the GFP positive cells also express SSEA-4. These cells after being cultured in de-differentiation media express *POU5F1*, *cMYC* and *SSEA-4* at levels similar to those of iPS cells, therefore can be reprogrammed to gPS cells. Our proposed technique can be used for isolation of an enriched SSEA-4(+) population that contains the SSCs and that can be most efficiently reprogrammed to gPS cells for clinical applications in degenerative diseases and could be maintained in culture for treatment of infertility in cancer survival children.

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