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Expression Profile of Genes Identified in Human Spermatogonial Stem Cell-Like Cells Using Suppression Subtractive Hybridization

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ABSTRACT

Spermatogenesis is the process by which testicular spermatogonial stem cells (SSCs) self-renew and differentiate into mature sperm in the testis. Maintaining healthy spermatogenesis requires proper proliferation of SSCs. In this study, we sought to identify factors that regulate the proliferation of SSCs. Human SSC (hSSC)-like cells were isolated from azoospermic patients by a modified culture method and propagated in vitro. After four to five passages, the SSC-like cells spontaneously ceased proliferating in vitro, so we collected proliferating (P)-hSSC-like cells at passage two and senescent (S)-hSSC-like cells at passage five. Suppression subtractive hybridization (SSH) was used to identify genes that were differentially expressed between the P-hSSC-like and S-hSSC-like cells. We selected positive clones up-regulated in P-hSSC-like cells using SSH and functionally characterized them by reference to public databases using NCBI BLAST tools. Expression levels of genes corresponding to subtracted clones were analyzed using RT-PCR. Finally, we confirmed the differential expression of 128 genes in positive clones of P-hSSC-like cells compared with S-hSSC-like cells and selected 23 known and 39 unknown clones for further study. Known genes were associated with diverse functions; 22% were related to metabolism. Fifteen of the known genes and two of the unknown genes were down-regulated after senescence of hSSC-like cells. A comparison with previous reports further suggests that known genes selected, *SPP1*, may be related to germ cell biogenesis and cellular proliferation. Our findings identify several potential novel candidate biomarkers of proliferating- and senescencet-hSSCs, and they provide potentially important insights into the function and characteristics of human SSCs. J. Cell. Biochem. 110: 752–762, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: SSH; hSSC-LIKE CELLS; SPERMATOGENESIS; DIFFERENTIALLY EXPRESSED GENES; SENESCENCE

Adult stem cells are found in many locations throughout the body and provide support for a variety of tissue functions [Kenyon and Gerson, 2007]. The typical adult stem cell is considered multipotent rather than totipotent, as it can generally only give rise to differentiated cell types from the tissue of origin. For example, the hematopoietic stem cell (HSC) is capable of undergoing self-renewing cell divisions in vivo and differentiating at the single-cell level into all mature blood cells to functionally repopulate the hematopoietic system of a myeloablated recipient. A number of other adult stem cells have been identified, although they are not as well characterized as the HSC. Neural stem cells (NSCs) give rise to neurons, astrocytes and oligodendrocytes, the three main types of nerve cells in the adult brain. Mesenchymal stem cells (MSC) differentiate into fibroblasts, adipocytes, osteoblasts, chondrocytes, and skeletal muscle cells. Other cells, such as spermatogonial, corneal and endothelial stem cells, also fulfill the criteria of a stem cell, except that they differentiate into only a single type of cell [Roobrouck et al., 2008].

Spermatogonial stem cells (SSCs) provide the foundation for continual sperm production throughout the lifetime of a male. SSCs first arise in the testis from undifferentiated germ cells called gonocytes, which are themselves derived from primordial germ cells

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(PGCs) that migrate from the urogenital ridge to the embryonic gonad during prenatal development [Oatley and Brinster, 2006]. To overcome male infertility and understand the mechanism of spermatogenesis, our team has previously isolated human spermatogonial stem-cell-like cells (hSSC-like cells) from azoospermic patients and successfully produced haploid germ cells by culturing in vitro for 4–6 weeks [Lee et al., 2006]. However, in contrast to mouse SSCs, which can be maintained for long periods in culture, long-term proliferation of human SSCs in vitro has not been reported. In addition, as obviously stated earlier in our previous study, SSC-like cells could not be maintained beyond a limited number of passages, most likely due to the fact that critical information necessary to reproduce the specific culture conditions required for in vitro proliferation of human SSCs is lacking. In the present study, hence, we performed suppression subtractive hybridization (SSH) technique in order to identify proliferation- or senescent-regulatory genes that were differentially expressed in proliferative hSSC-like cells (P-hSSC-like cells) and senescent hSSC-like cells (S-hSSC-like cells). Using this approach, we identified differentially expressed known and unknown genes in subtracted clones and analyzed the expression levels of these genes in P-hSSC-like and S-hSSC-like cells using RT-PCR. These genes represent potential biomarkers that could act as determinants of the proliferative or senescent state of hSSC-like cells.

MATERIALS AND METHODS

ISOLATION AND IN VITRO CULTURE OF hSSC-LIKE CELLS

Human testicular samples were obtained from male patients with either obstructive azoospermia (OA) or non-obstructive azoospermia (NOA) showing maturation arrest of male germ cells (Fig. 1A,B). They underwent a testicular sperm extraction (TESE)-intracytoplasmic sperm injection (ICSI) program, and, under informed consent, donated remaining tissue for the study. This study was approved by the Institutional Review Board of the CHA Gangnam Medical Center (Seoul, Korea). Subsequent experimental protocols and quality control of the laboratory facility and equipment were also appropriately managed.

Procedures for isolating and culturing hSSC-like cells were modified from our previous report [Lee et al., 2006]. In brief, testicular tissues obtained from OA and NOA patients were placed in 10 ml of enzyme solution A containing 0.5 mg/ml collagenase (Type I; Sigma Chemical Co., St. Louis, MO), 10 μg/ml DNase I, 1 μg/ml soybean trypsin inhibitor (Gibco, Grand Island, NY), and 1 mg/ml hyaluronidase (Sigma) in Ca^{2+}, Mg^{2+} free phosphate-buffered saline (PBS), and incubated for 20 min at ambient temperature (~25°C). After the peritubular cells were removed in low collagenase concentration, the testicular tissues were redissociated in 10 ml of enzyme solution B containing 5 mg/ml collagenase (Gibco), 10 μg/ml DNase I (Sigma), 1 μg/ml soybean trypsin inhibitor (Gibco), and 1 mg/ml hyaluronidase (Sigma) in Ca^{2+}- and Mg^{2+}-free PBS, and incubated for 30 min at 37°C. Next, dissociated testicular cells were plated and grown on gelatin-coated dishes in Dulbecco's modified Eagle's medium (DMEM) containing 15% (v/v) fetal bovine serum (Gibco), 10 μmol/L 2-mercaptoethanol (Gibco), 1% non-essential amino acids (Gibco), 4 ng/ml basic fibroblast growth factor (Invitrogen, Carlsbad, CA), 10 μmol/L forskolin (Sigma), and 1,500 IU/ml human leukemia inhibitory factor (Peprotech, Inc., Rocky Hill, NJ) in a humidified atmosphere of 5% CO_2 in air. Over the following 2–4 weeks, the large multicellular (≥100 cells) colonies that formed on the dish were sequentially subcultured every 2 weeks in StemPro-34 SFM (Invitrogen) supplemented with StemPro Supplement (Invitrogen), 10 μg/ml insulin–transferrin–selenium solution (Gibco), 6 mg/ml D-(+)-glucose, 30 μg/ml pyruvic acid, 1 μl/ml DL-lactic acid (Sigma), 5 mg/ml Bovine Albumin (ICN Biomedicals, Irvine, CA), 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, MEM Vitamin Solution (Invitrogen), MEM Non-Essential Amino Acids Solution (Invitrogen), 10^{-4} M ascorbic acid, 10 μg/ml d-biotin, 30 ng/ml β-estradiol, 60 ng/ml progesterone (Sigma), 20 ng/ml mouse epidermal growth factor (Becton Dickinson, Bedford, MA), 10 ng/ml human basic fibroblast growth factor (Becton Dickinson), 103 U/ml ESGRO (murine leukemia inhibitory factor; Invitrogen), 10 ng/ml Recombinant rat GDNF (R&D Systems, Minneapolis, MN) and 1% (v/v) fetal calf serum (JRH Biosciences, Lenexa, KS).

IMMUNOCYTOCHEMISTRY OF P-hSSC-LIKE CELLS

To investigate the localization and expression levels of spermatogonial stem cell and differentiated germ cell markers in culture, we performed immunocytochemistry as previously described using Oct-4 (Santa Cruz), integrin α6 (Santa Cruz), integrin β1 (Santa Cruz) and c-Kit (Santa Cruz) antibodies [Lee et al., 2006]. Samples were washed three times in DPBS with 5% FBS and fixed in paraformaldehyde (4% v/v in DPBS) for 24 h. For permeabilization, cells were incubated in 0.1% Triton X-100 in DPBS for 1 h. After being washed three times with DPBS, non-specific binding of antibodies was suppressed by incubation in blocking solution (4% normal goat serum in DPBS) for 30 min at room temperature. After being washed three times with PBS, immunocytochemical staining was performed by incubating the fixed samples with primary antibody diluted to 1:200 with DPBS containing 0.1% Tween-20 and 1% BSA for 60 min at room temperature or overnight at 4°C. Immunoreactive protein was then detected using a CY3 or FITC-conjugated secondary antibody diluted to 1:500 with DPBS for 60 min at room temperature. Finally, samples were counterstained with 1 μg/ml 4′,6′-diamidino-2-phenyindiol (DAPI; Sigma). Following multiple washes, dot slides were mounted in Vectashield mounting medium (Vector laboratories, Burlingame, CA). The resulting staining was viewed on an inverted confocal laser scanning microscope (LSM 510; Carl Zeiss, Oberkochen, Germany) with fluorescence at 400× magnification. Micrographs were stored in LSM (Zeiss LSM Image Browser version 2.30.011; Carl Zeiss Jena GmbH, Jena, Germany).

TELOMERASE ACTIVITY

A total of 1×10^5 cells were treated with trypsin-EDTA (LS 015-08, Welgene) and lysed in 200 μl cold lysis buffer (10 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 0.5 M EGTA, 0.5% CHAPS, 10% glycerol, 0.1 mM AEBSF, 5 mM 2-mercaptoethanol). They were incubated on ice for 30 min and centrifuged at 13,000 rpm for 20 min, and the

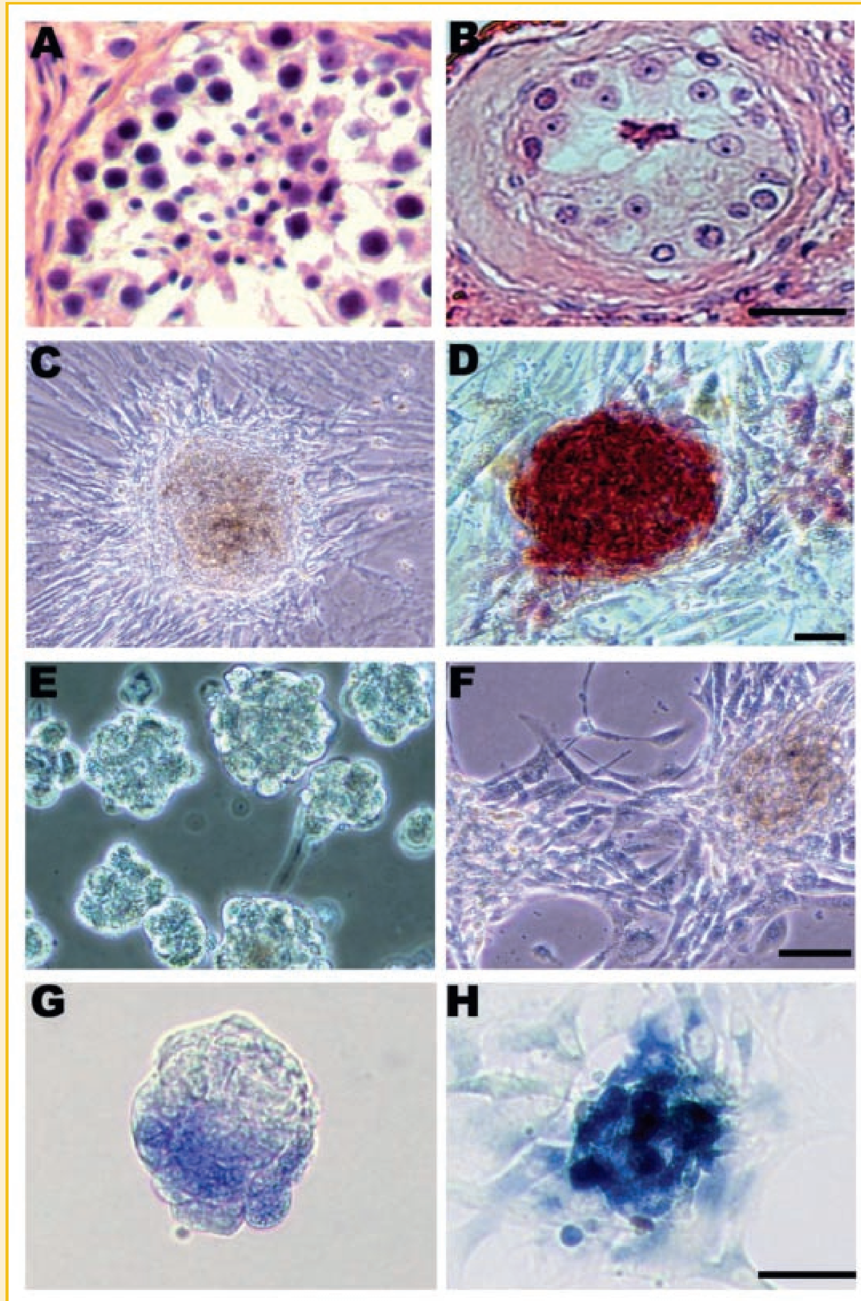

Fig. 1. Human spermatogonial stem cell (hSSC)-like cells isolated from azoospermic patients. A,B: Histological observation of obstructive azoospermic (OA) and non-obstructive azoospermic (NOA) patient showing maturation arrested. C,D: Primary colony formation (hSSC-like cells) by in vitro culture of testicular cells from azoospermic patients and alkaline phosphatase activity. E: Colonies containing proliferating (P)-hSSC-like cells (at passage 2) isolated from primary cultured colonies. F: Colonies containing senescent (S)-hSSC-like cells at passage 5. G,H: SA-β-Gal staining pattern of P- and S-hSSC-like cells. All scale bar = 100 μm.

supernatant was transferred to a new tube. For the telomere PCR reaction, 2 μl of this supernatant was added to 2.5 μL 10X T-PCR buffer (200 mM Tris–HCl (pH 8.3), 15 mM $MgCl_2$, 680 mM KCl, 0.5% Tween-20, 10 mM EGTA), 0.125 μl 10 mM dNTPs, 1 μl TS primer (5′-AAT CCG TCG AGC AGA GTT-3′), 0.2 μl *Ex Taq* (RR001A, Takara), and up to 25 μl of DW and incubated at 20°C for 30 min. Finally, 1 μl CX primer (5′-CCC TTA CCC TTA CCC TTA CCC TAA-3′) was added, and the following PCR reaction was repeated for 31 cycles: 45 s at 94°C, 45 s at 50°C, 1 min at 72°C. Electrophoresis was performed using 15% polyacrylamide gel for 15 min at 100 V and 2 h at 150 V. The gel was stained with SYBR green at RT for 20–30 min.

SENESCENCE-ASSOCIATED β-GALACTOSIDASE (SA-β-GAL) STAINING

SSCs grown on plates were washed with PBS, fixed in 4% formaldehyde for 10–15 min at room temperature, and washed

again with PBS. Cells were incubated at 37°C in staining solution (40 nm citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$, with freshly added 1 mg/ml X-gal; BioVision) for 12 h before being examined as previously described [Kondo et al., 2001].

TOTAL RNA PREPARATION

Total RNA was isolated from P- and S-hSSC-like cells derived from OA ($n = 2$) and NOA patients ($n = 3$) using TRIzol reagent (Molecular Research Center, Cincinnati, OH) and quantified with a DN-1000 Spectrophotometer (Nanodrop Technologies, Inc., Waltham, MA). The A_{260}/A_{280} ratio was in the range of 1.8–2.0. Approximately 1 μg total RNA was electrophoresed on a 1.2% denaturing agarose gel to confirm integrity.

CDNA SYNTHESIS

cDNA was synthesized using the BD SMART™ cDNA Synthesis Kit (BD Biosciences, Clontech) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg total RNA and reverse-transcribed in a 10-μl reaction mixture containing PowerScript Reverse-transcriptase (BD Biosciences) using 3′ BD SMART CDS primer II A (5′-AAG CAG TGG TAT CAA CGC AGG GTA CTT-3′) and BD SMART™ A oligonucleotide primer (5′-AAG CAG TGG TAT CAA CGC AGA GTA CGC GGG-3′). The first-strand cDNA was diluted to a final volume of 40 μl with 1× TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and used to generate second-strand cDNA by long-distance PCR with BD Advantage 2 polymerase mix (BD Biosciences) using PCR primer II A (5′-AAG CAG TGG TAT CAA CGC AGA GT-3′) and following the manufacturer's instructions. The PCR-amplified second-strand cDNA was purified using column chromatography and then digested with 10 U/μl *Rsa*I in a final volume of 50 μl at 37°C for 3 h to obtain blunt-ended fragments. The final concentration of digested second-strand cDNA was 300 μg/μl.

SUPPRESSION SUBTRACTIVE HYBRIDIZATION (SSH)

Purified two-target cDNA was used for SSH using a Clontech PCR-select cDNA Subtraction kit (BD Biosciences) according to the manufacturer's instructions. Tester cDNA (P-hSSC-like cells, Fig. 1E) was ligated to adaptor 1 and adaptor 2R in separate ligation reactions at 16°C overnight using 400 U/μl T4 DNA ligase. Driver cDNA (S-hSSC-like cells, Fig. 1F) was not ligated to adaptors. After ligation, two samples were subjected to hybridization. In the first sample, an excess of driver mRNA was added to each adaptor-ligated tester cDNA in the hybridization buffer, heat-denatured at 98°C for 1.5 min and then allowed to anneal at 68°C for 8 h. Thereafter, the two primary hybridization samples were mixed together without denaturing, and fresh denatured driver cDNA was added to the sample, which annealed at 68°C overnight. This step formed newly hybridized cDNA. After a second hybridization, samples were diluted in 200 μl dilution buffer (20 mM HEPES, 50 mM NaCl, 0.2 mM EDTA) at 68°C for 7 min. This hybrid cDNA was used in PCR reactions to amplify the desired differentially expressed sequences using the 50× Advantage cDNA Polymerase PCR kit (BD Biosciences). First-round PCR amplifications were performed using PCR primer 1, and second-round PCR amplifications were performed using nested primer 1 and nested primer 2R. First-round PCR was performed using the following cycling parameters: 75°C for 5 min, followed by 28 cycles of 94°C for 30 s, 66°C for 30 s and 72°C for 90 s. A volume of 3 μl of primary PCR mixture was diluted in 27 μl H_2O for nested-PCR reactions consisting of 11 cycles at 94°C for 30 s, 68°C for 30 s and 72°C for 90 s. The amplified second-round PCR product was cloned directly into the pGEM-T Easy vector (Promega, Madison, WI). The ligation products were purified using the Wizard Plus SV Miniprep DNA purification system (Promega), and the presence of the insert sequence was verified by digestion with *Eco*R1 (NEB, England) and sequenced using a primer specific to the M13F-pUC on an ABI PRISM 3730XL Analyzer (Macrogen, Korea).

ANALYSIS OF SUBTRACTION EFFICIENCY

Subtracted cDNA products were subject to 18, 23, 28, or 33 cycles of PCR amplification with primers specific to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) in order to evaluate subtraction efficiency. The sense (5′-AAC ACA GTC CAT GCC ATC AC-3′) and antisense (5′-TCC ACC ACC CTG TTG CTG TA-3′) G3PDH primers were provided in the PCR-Select cDNA subtractive Kit. Unsubtracted controls were amplified in parallel to analyze subtraction efficiency.

BIOINFORMATIC ANALYSIS

Sequences obtained from SSH were identified by reference to sequences in the National Center for Biotechnology Information (NCBI) database, using the Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.nih.gov.BLAST).

REVERSE TRANSCRIPTASE–POLYMERASE CHAIN REACTION (RT-PCR) ANALYSIS

RT-PCR was performed to assess the expression of spermatogonial stem cell marker genes in colonies from TESE, specifically, integrin α6, integrin β1 and c-Kit in spermatogonia and spermatocytes. PCR was initiated with denaturation at 94°C for 5 min, followed by 35–40 cycles of 30 s at 94°C, 30 s at 55–57°C, and 30 s at 72°C. A final extension step for 10 min at 72°C completed the amplification reaction, after which the products were separated by 1.5% agarose-gel electrophoresis and verified by automated nucleotide sequencing. Negative controls included mock transcription without mRNA or PCR with distilled, deionized water.

To validate differential expression among the cell lines, we also performed RT-PCR. Full-length cDNA was synthesized using the SuperScript First-strand Synthesis System (Invitrogen). PCR reactions were carried out in a total volume of 25 μl using 1 μl reverse-transcribed samples under the following cycling conditions: 5 min at 94°C for initial denaturation, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, followed by a final extension step at 72°C for 5 min. An aliquot (6 μl) of the PCR products was analyzed in 1% agarose. RT-PCR reactions were repeated at least three times. The Multigauge program (Fujifilm, Japan) was used to measure PCR product density, and cDNA levels were normalized to those of 18s rRNA.

RESULTS

CHARACTERIZATION OF hSSC-LIKE CELLS ISOLATED FROM AZOOSPERMIC PATIENTS AND IN VITRO PROPAGATION

Human SSC-like cells were isolated from the testis of both OA and NOA patients and cultured in vitro (Fig. 1). Primary large colonies were collected after 1–2 weeks of culture (Fig. 1C) and then replated into new dishes after dissociation to generate a high purity of hSSC-like cells. hSSC-like cells re-aggregated into small colonies and slowly proliferated (Fig. 1E), but somatic cells remained attached to the bottom of the culture dish. Cultured cells were passaged every 2 weeks. Proliferation of hSSC-like cells derived from both OA and NOA patients ceased after passage five and finally disappeared after attached to fibroblast-like cells (Fig. 1F). We designated proliferating hSSC-like cells during the first two to three passages as P-hSSC-like cells and senescent hSSC-like cells after passage five as S-hSSC-like cells. These two types of cells were reconfirmed by their intensities of SA-β-gal staining. Only a weak signal of SA-β-gal staining was seen in small colonies of P-hSSC-like cells, but all cells in colonies of S-hSSC-like cells showed a strong signal (Fig. 1G,H). The RT-PCR analysis revealed high levels of Oct4, integrin α6, and integrin β1 but lower levels of c-Kit in P-hSSC-like cells (Fig. 2A). In addition, these P-hSSC-like cells stained positive for alkaline phosphatase (Fig. 2B). However, by passages five, proliferation of hSSC-like cells was not observed, and they became senescent. Expression of spermatogonial stem cell marker genes (Oct4, integrin α6, and integrin β1) as well as telomerase activity was quickly decreased (Fig. 2C,D). These results manifest that hSSC-like cells after passage five displayed a senescent phenotype.

SSH ANALYSIS IN P-hSSC-LIKE AND S-HSSC-LIKE CELLS

To apply SSH in P-hSSC-like and S-hSSC-like cells, we first analyzed the subtraction efficiency using G3PDH as an indicator. G3PDH abundance was reduced in the subtracted population, requiring 28–33 cycles for its detection, whereas the corresponding band appeared earlier in the unsubtracted population (23–28 cycles). Thus, approximately five to seven additional cycles of amplification were required for subtracted cDNAs (Fig. 3), indicating a potential subtracted library size of 2^5 (32)–2^7 (128) in density of G3PDH levels [Jung et al., 2009]. Subtracted cDNAs were cloned into T-vectors and transformed into *E. coli* strain DH5-α. A total of 157 clones were randomly selected and sequenced in one direction using M13F-pUC primers.

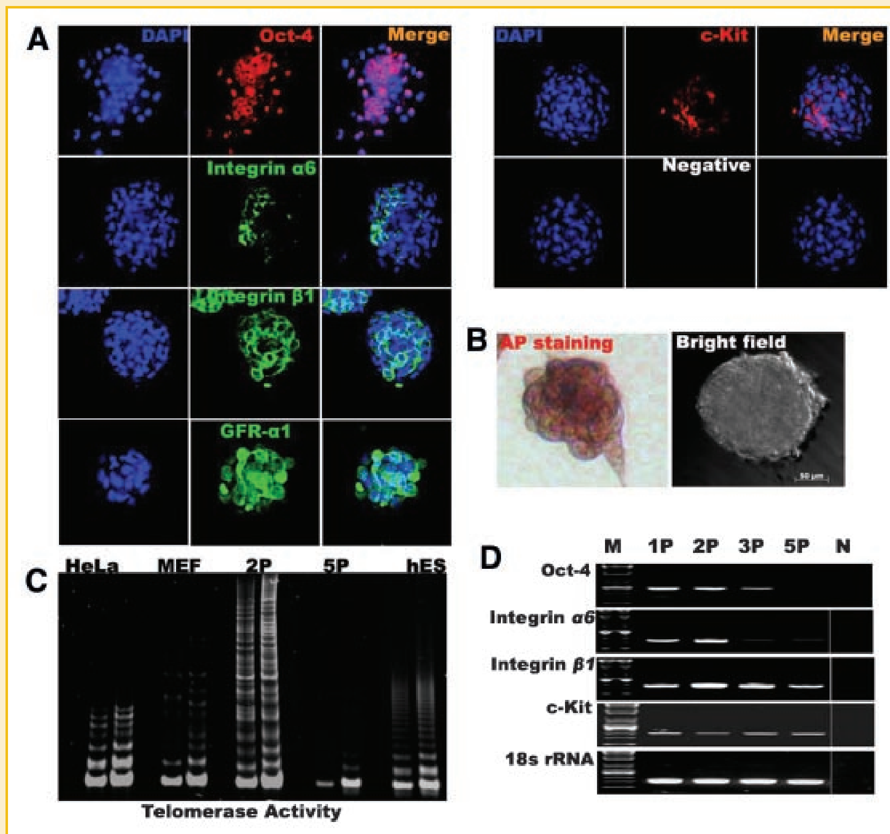

Fig. 2. The presence and characterization of spermatogonial stem cells in vitro cultured colonies obtained from testicular tissues of azoospermic patients. A: Immunocytochemistry of P-hSSC-like cells; general stem cell marker (Oct-4), spermatogonial stem cell markers (integrin α6 and integrin β1), differentiated spermatogonial stem cell marker (c-Kit). B: AP staining of hSSC-like cell colonies. C: Telomerase activity results show that early passage (2P) colonies expressed strong activity, but senescent colonies (5P) expressed weakly. D: RT-PCR results show that stem cell marker gene and SSC marker genes (Oct-4, integrin α6, and integrin β1) expressed in early (1–3) passaged colonies, but expressed weakly or not expressed in senescent colonies (more than 5 passaged).

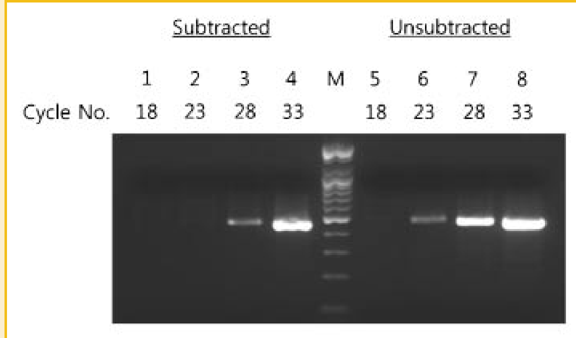

Fig. 3. Evaluation of SSH efficiency. The forward subtracted library was subjected to PCR amplification using G3PDH primers. The relative amount of G3PDH product is shown in lane 1–4 for subtracted clones 18, 23, 28, and 33, respectively, and in lane 5–8 for unsubtracted clones 18, 23, 28, and 33, respectively. M: 100-bp DNA ladder (Bioneer, Korea).

Tester and driver cDNAs were digested with *Rsa*I before adaptor ligation in the SSH step, so the length of clone sequences varied, ranging from 200 to 800 base pairs. Sequences were not obtained for 23 clones, which were thus omitted from this study. The remaining 128 clones were evaluated by BLAST analysis against sequences obtained from GenBank, EMBL, and human EST databases (Table I). We ultimately selected 62 unique clones, 23 of which corresponded to known genes and 39 of which corresponded to unknown genes (Fig. 4A and Tables II and III). The remaining 66 clones (52%) were redundant. Of the 23 known clones, 22% were associated with metabolism, 18% with transcription, 13% with development, 9% with proliferation, 9% with translation, 4% with tumor suppression, 4% with signal transduction, and 4% with cell cycle and growth, while 4% were cytokine-like and 13% uncharacterized proteins (Fig. 4B). The 39 unknown clones were divided into two groups in the NCBI databases: 7 were present in the human EST database, and 32 represented human genomic sequences.

EXPRESSION OF SUBTRACTED GENES IN hSSC-LIKE CELLS

Using RT-PCR to analyze the expression of the 23 known genes in P-hSSC-like cells and S-hSSC-like cells (Supplemental Table I), we found that 15 clones (65%) were expressed more highly in P-hSSC-like cells (Fig. 5A, Table II). Strikingly, *AKNA*, *NCOR1*, *MED1*, *ATP2B1*, *APPL1*, *SPP1*, *REV3L*, *eIF5A2*, *GYPE*, *TGDS*, and *CCDC109B* were detected only in P-hSSC-like cells. In addition, *SCGB3A2* and *FBN2* expression levels increased more than 40-fold in P-hSSC-like cells compared with S-hSSC-like cells (Fig. 5B).

TABLE I. Summary of Subtracted cDNA Category

Category of cDNA	N	%
Total cDNAs generated	157	
cDNAs excluded from sequence database	23	
Subtracted cDNAs	128	100%
Match with known genes	21	16.4%
Match with uncharacterized protein	2	1.6%
Only match with ESTs	7	5.5%
Only match with human genomic sequence	32	25.0%
Redundant results	66	51.6%

During in vitro culture to senescence, the expression levels of *TFB2M*, *SCGB3A2*, *MTUS1*, and *FBN2* were down-regulated. Interestingly, *eEF1A1* exhibited the highest redundancy in this dataset (Table II), but the expression level of this clone only changed by 1.14-fold (Fig. 5B). Of the 39 unknown genes, we selected two subtracted clones (CHA-S55 and -S212) that showed differential expression between P-hSSC-like cells and S-hSSC-like cells (data not shown). In both cases, the mRNAs for these genes were more abundant in P-hSSC-like cells (3.25- and 3.68-fold) compared with S-hSSC-like cells (Table III and Fig. 6).

DISCUSSION

Generally, azoospermia is divided into two categories, obstructive azoospermia (OA) and non-obstructive azoospermia (NOA) [Ezeh, 2000]. OA can be caused by genital tract obstruction, and the sperm of these patients are often surgically retrieved for infertility treatment by microepididymal sperm aspiration (MESA) or testicular sperm extraction (TESE). The sperm can fertilize ova by intracytoplasmic sperm injection (ICSI), and such fertilization is able to generate normal embryonic development and healthy offspring. In fact, it is believed that fertilization and pregnancy rates from testicular sperm obtained from OA are comparable to that using ejaculated sperm [Devroey et al., 1996]. However, NOA is caused by genetic and environmental factors and is defined as severely impaired or no spermatogenesis. In only about 30% of TESE cycles, a low count of spermatozoa or germ cells can be retrieved [Lee et al., 1998, 2006]. However, if sperm are retrieved from NOA, they can support normal embryonic development and produce healthy babies [Devroey et al., 1996]. Also, we have recently found that there was no difference in SSCs from OA and NOA patients and that they shared several other characteristics during long-term culture; the one exception was proliferating activity, which was lower in SSCs derived from NOA patients than in those derived from OA patients [Lim et al., in press]. However, the finding of Lim et al. was limited because direct comparisons with those of normal healthy males were not performed.

In the present study, the proliferation or senescence of hSSC-like cells was also confirmed by both telomerase activity and SA-β-gal staining. It was well known that most adult cells can divide 50–60 times before they reach senescence. Because of what is known as the Hayflick limit [Hayflick, 1965], partially reduced telomere lengths during cell division lead to the "end-replication problem" [Shore and Bianchi, 2009]. Senescent cells show enlarged and flattened morphology and senescence-associated β-galactosidase (SA-β-gal) staining [Beausejour, 2007]. In fact, senescent cells have higher lysosomal β-gal expression, so this method can be used as a biomarker of cellular senescence [Dimri et al., 1995; Campisi and d'Adda di Fagagna, 2007]. It has also been reported that MSCs that have progressed into cellular aging process show decreased telomerase activity [Singh et al., 2009]. In the present study, isolated hSSC-like cells from both types of azoospermic patients showed a high degree of proliferation (P-hSSC-like cells) during first two to three passages, but their activity was quickly reduced after five passages (S-hSSC-like cells). In fact, S-hSSC-like cells showed a

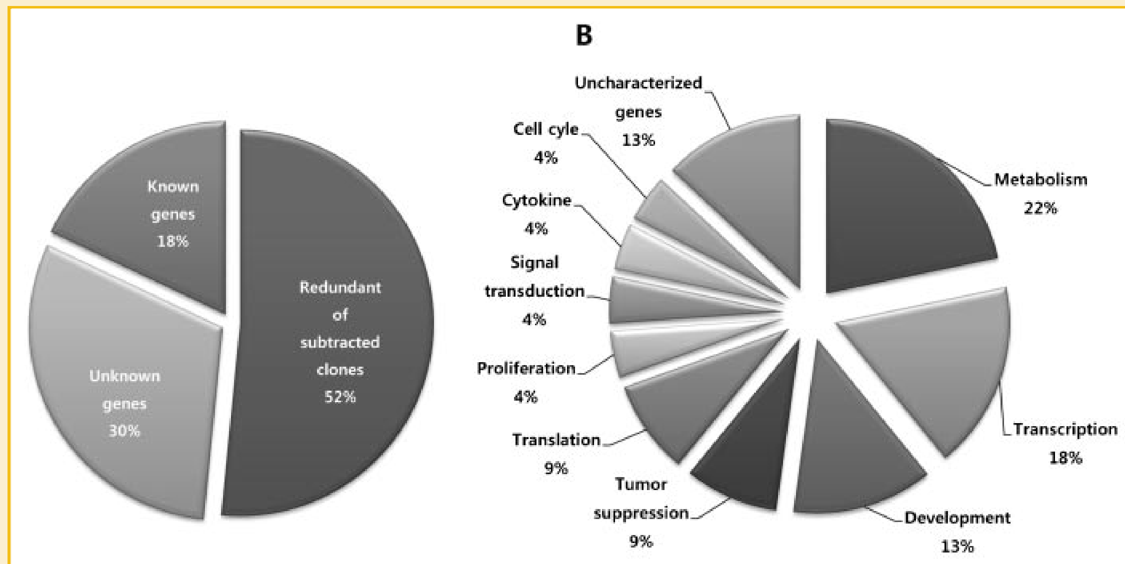

Fig. 4. Pie diagram showing functional classification of genes identified in subtracted clones. Randomly selected subtracted clones characterized by function using the NCBI database. A: cDNA sequences obtained in the subtracted P-hSSC-like cells library. B: Biological process information for known genes.

strong positive signal in the SA-β-gal assay and low telomerase activity (Figs. 1 and 2). Based on these results, we used suppression subtractive hybridization to identify proliferation/senescence-related genes differentially expressed between P-hSSC-like and S-hSSC-like cells.

Numerous approaches have been used to identify proliferation-specific genes and associated markers [Xu et al., 2001], including expressed sequence tag (EST) sequencing [Pennisi, 1997; Vasmatzis et al., 1998], serial analysis of gene expression (SAGE) [Velculescu et al., 1995], arbitrary primed-PCR [Peinado et al., 1992], representational difference analysis (RDA) [Lin et al., 2000], differential display polymerase chain reaction (DD-PCR) [Liang and Pardee, 1992] and suppression subtractive hybridization (SSH) [Diatchenko et al., 1996]. In the case of RDA, several rounds of subtraction are needed and it does not resolve the problem of the wide differences in abundance of individual RNA species. Although DD-PCR and arbitrary primed-PCR are potentially faster methods for identifying expression differences between two populations, both of

TABLE II. Characteristics of Differentially Expressed Known Genes in Subtracted Human SSC Clones

Biological process	Gene symbol	Definition	Accession no.	R*	Chromosomal localization
Transcription	AKNA	AT-hook transcription factor	NM_030767.4	1	9q32
	PQBP1	Polyglutamine binding protein 1, transcript variant 4	NM_001032383.1	1	Xp11.23
	NCOR1	Nuclear receptor co-repressor 1	NM_006311.2	1	17p11.2
	TFB2M	Transcription factor B2, mitochondrial, nuclear gene encoding mitochondrial protein	NM_022366	2	1q44
Development	MED1	Mediator complex subunit 1	NM_004774.3	1	17q12–q21.1
	FBN2	Fibrillin 2	NM_001999.3	1	5q23–q31
	SPP1	Secreted phosphoprotein 1, transcript variant 2	NM_000582.2	3	4q21–q25
Cell proliferation	APPL1	Adaptor protein, phosphotyrosine interaction, PH domain- and leucine zipper-containing 1	NM_012096	1	3p21.1–p14.3
	REV3L	REV3-like, catalytic subunit of DNA polymerase zeta (yeast) (REV3L)	NM_002912.3	3	6q21
Signal transduction	PPP4C	Protein phosphatase 4 (formerly X), catalytic subunit	NM_002720	1	16p12–p11
Cytokine	IL-8	Interleukin 8	NM_000584	2	4q13–q21
Tumor suppression	MTUS1	Mitochondrial tumor suppressor 1, nuclear gene encoding mitochondrial protein, transcript variant 1	NM_001001924.1	4	8p22
Cell cycle and growth	ANAPC4	Anaphase-promoting complex subunit 4	NM_013367	2	4p15.2
Translation	EEF1A1	Eukaryotic translation elongation factor 1 alpha 1	NM_001402	37	6q14.1
	EIF5A2	Eukaryotic translation initiation factor 5A2	NM_020390	1	3q26.2
Metabolism	ATP2B1	Plasma membrane calcium ATPase isoform 1, alternative splice products	L14561	2	12q21–q23
	TGDS	TDP-glucose 4,6-dehydratase (TGDS)	NM_014305	1	13q32.1
	CTSL2	Cathepsin L2 (CTSL2)	NM_001333.2	1	9q22.2
	SCGB3A2	Secretoglobin, family 3A, member 2	NM_054023	1	5q32
	DSCR8	Down syndrome critical region gene 8, transcript variant 3, non-coding RNA	NR_026839.1	1	21q22.2
Uncharacterized	GYPE	Glycophorin E, transcript variant 2	NM_198682.2	2	4q31.1
	CCDC109B	Coiled-coil domain-containing 109B	NM_017918	1	4q25
	FAM65A	Family with sequence similarity 65, member A	NM_024519.2	1	16q22.1

R*: redundant clones.

TABLE III. Summary of Differentially Expressed Unknown Genes in Subtracted Human SSC Clones

Biological process	Gene symbol	Partial size (bp)	Accession no.	R*	Chromosomal localization
Unknown genes	CHA-S55	480	GO581240	1	1q23
	CHA-S212	496	GO581241	1	1q23

R*: redundant clones.

these methods are associated with high levels of false-positives and are biased towards high-copy-number mRNAs. However, SSH has the distinct advantage over other PCR-based techniques in that it selectively amplifies target cDNA fragments (differentially expressed) while simultaneously suppressing non-target DNA amplification to generate a library of differentially expressed sequences. The normalization step equalizes the abundance of cDNAs within a target population, and the subtraction step excludes the common sequences between the driver and tester populations [Shridhar et al., 2002]. In addition, SSH has another advantages over microarrays because SSH can isolate novel differentially expressed genes [Jung et al., 2009]. In the present study, we obtained 128 positive clones using SSH, but 67 of these (52%) were redundant. This relatively high redundancy rate indicates that distinctive mammalian testis genes and gene products may have a high conservation rate among diverse organisms [Hwang et al.,

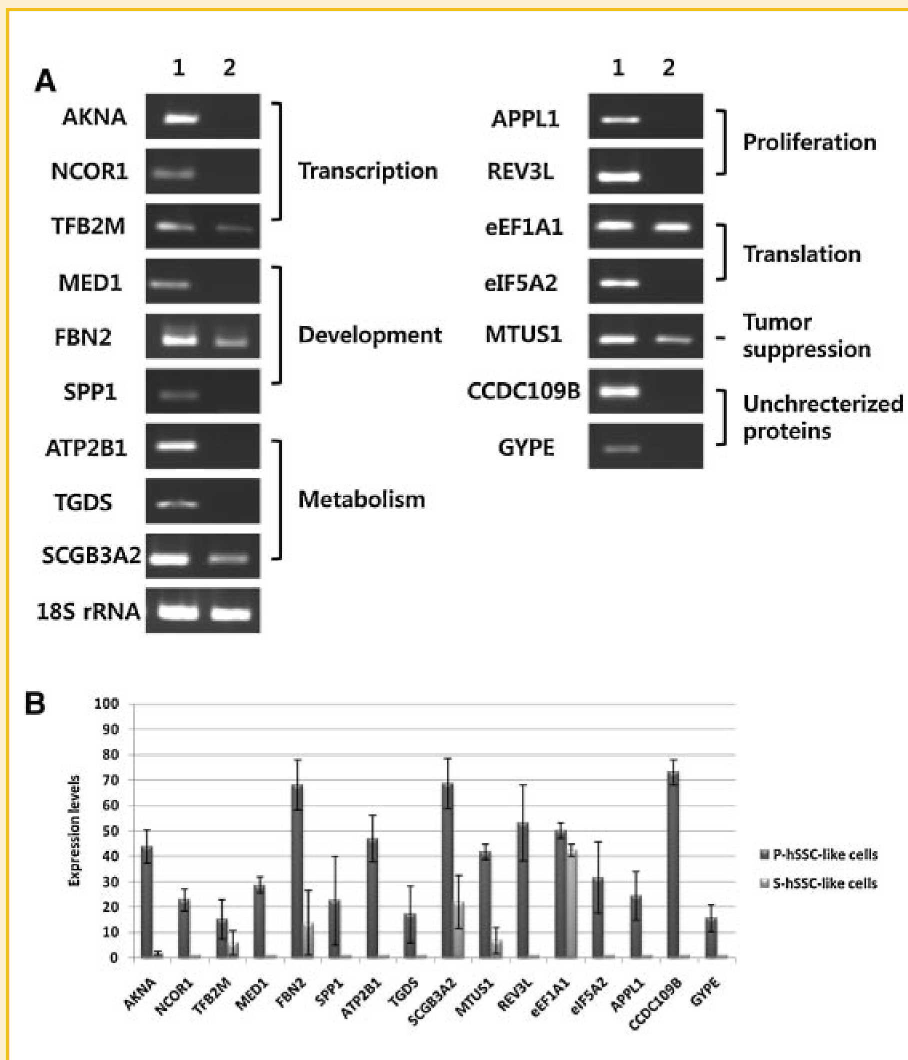

Fig. 5. Known genes differentially expressed in P-hSSC-like-cells and S-hSSC-like-cells. RT-PCR analysis showing the expression levels of known genes from subtracted clones in P-hSSC-like cell and S-hSSC-like cells. A: In vitro senescence, 18 known genes were down-regulated in hSSC-like cells. Lane 1: P-hSSC-like cells. Lane 2: S-hSSC-like cells. B: The calibrated density of 15 down-regulated known genes normalized to that of 18S rRNA.

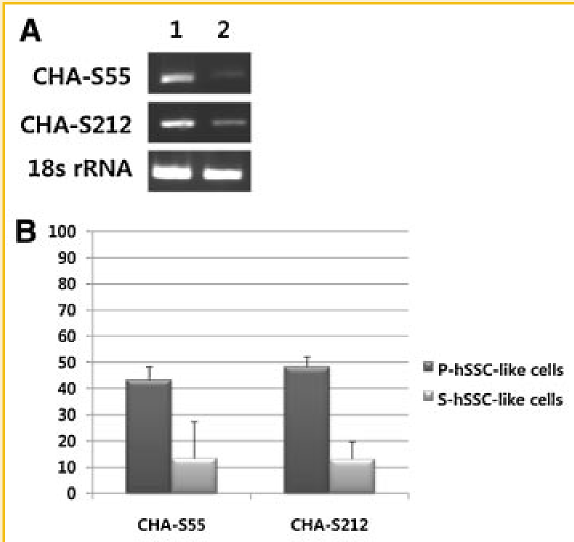

Fig. 6. Unknown/novel genes differentially expressed in P-hSSC-like-cells and S-hSSC-like-cells. RT-PCR analysis showing the expression levels of two unknown genes from subtracted clones in P-hSSC-like cell and S-hSSC-like cells. A: In vitro senescence, CHA-S55 and CHA-S212 corresponding to unknown genes were down-regulated in hSSC-like cells. Lane 1: P-hSSC-like cells. Lane 2: S-hSSC-like cells. B: The calibrated density of CHA-S55 and CHA-S212 normalized to that of 18S rRNA.

2001]. Despite this complication, we were able to identify 62 unique clones: 23 corresponding to known genes and 39 corresponding to unknown genes. By far the largest number of isolated clones corresponded to the translation elongation factor *eEF1A1* gene, which accounted for 37 of the 71 (52%) of the subtracted clones (Table II). Previous SSH experiments on several cell types have shown no redundant results such as *eEF1A1* [Yu et al., 2005; Choi et al., 2006; Noh et al., 2007; Jung et al., 2009]. We found similar *eEF1A1* expression levels in proliferating and senescent human SSC-like cells, suggesting it is required in both proliferating and senescent hSSC-like cells. Two known genes that may have germ-cell-related functions were also found in the present study. One of these genes was *SPP1*, which is expressed at both the mRNA and the protein levels in undifferentiated spermatogonia throughout the cycle of the seminiferous epithelium [de Rooij and Grootegoed, 1998]. These data indicate that Spp1 could function in the self-renewal of undifferentiated spermatogonia [Mizukami et al., 2008]. The other gene was *eIF5A*, which has an important role in eukaryotic cell survival [Guan et al., 2004]. *eIF5A2* has been identified in screens of populations of infertile men, including those with maturation arrest or Sertoli cell-only syndrome [Christensen et al., 2005]. Because testicular tissue from azoospermic patients was used for the current study, further investigation will be necessary to establish the functional significance of these genes.

Using RT-PCR, we analyzed the expression levels of differentially expressed genes identified in subtracted clones, showing that six of these genes—*AKNA*, *FBN2*, *ATP2B1*, *SCGB3A2*, *REV3L*, and *CCDC109B*—were down-regulated during senescence in hSSC-like cells. These genes have been related to proliferation in germ cells and thus might also be related to proliferation in hSSCs. *AKNA* (AT-hook transcription factor) coordinately regulates the expression of the CD40 receptor and its cognate CD40 ligand [Siddiqa et al., 2001]. *AKNA* related to transcription, most of transcription factors are often activated in young cell but down-regulated senescent cells, for example, c-myc and c-jun [Sikora, 1993]. The roles of myc and other transcription factors in oncogenesis have been reviewed by Meyer and Penn [2008]. *AKNA* also down-regulated senescence stage cells like c-myc. *FBN2* (fibrillin 2), which is homologous to *FBN1*, is highly expressed in embryonic tissues [Samuel et al., 2003]. Recently, *FBN1* has been shown to play a key role in lung development [Chen et al., 2005] and has been implicated in cell attachment, differentiation, and migration [Tseleni-Balafouta et al., 2006]. The well-known hSSC marker c-kit is essential for the maintenance of PGCs in both sexes. In males, it is expressed during embryogenesis and post-natal development [Mauduit et al., 1999] and is important for maintaining quiescent HSCs in hematopoietic systems [Thoren et al., 2008]. *FBN2* seems to have a similar function to c-kit in hSSC-like cells. *ATP2B1*, also known as *PMCA1* (plasma membrane calcium ATPase), is internalized during oocyte maturation. This process, which is representative of the endocytosis of several integral membrane proteins, is required for early embryogenesis [Marian et al., 2005; El-Jouni et al., 2008]. *SCGB3A2* (secretoglobin 3A2) was originally identified as a downstream target of the homeodomain transcription factor Nkx2-1 in the lung; ablation of *NKX2-1* results in severely hypoplastic lungs. *ATP2B1* and *SCGB3A2* are involved cellular metabolism and were down-regulated after cells entered senescence in our study. Previous studies have shown that in senescent MSCs, the expression of genes involved in cell cycle progression, DNA replication, mitosis, and DNA damage repair are down-regulated [Ksiazek, 2009]. A role for *hREV* in the cell cycle and cell proliferation has been reported [Xu and Li, 2008]. Most genes involved in proliferation are down-regulated in the senescent stage. Finally, CCDC109B is an uncharacterized protein.

CHA-S55 and CHA-S212, identified in the present study as genes down-regulated in senescent hSSC-like cells, are uncharacterized genes located on human chromosome 1. Additional research will be needed to understand the structures and functions of the proteins encoded by these genes.

In summary, we profiled differentially expressed genes in hSSC-like cells using the suppression subtractive hybridization technique. Fifteen of the known genes and two of the unknown genes were down-regulated in S-hSSC-like cells. Results of a literature review suggest that *SPP1* may be reliable marker of hSSC-like cells. Other known genes may also indirectly contribute to the specific characteristics of P-hSSC-like cells. Our findings thus identify several potential novel biomarkers of hSSCs. Although several other genes, including those of α6-integrin, β1-integrin, Oct-4 and Thy-1, have been used as SSC biomarkers [Shinohara et al., 1999; Ryu et al., 2004; Lee et al., 2006], these genes are usually expressed in both hSSCs and hESCs and thus represent the undifferentiated stem-cell state. Many testis-specific genes have been identified in mouse spermatogonia [Wang et al., 2001]. It will be beneficial to characterize the unknown hSSC-specific genes derived from this SSH study. The specific genes identified here, including those corresponding to two novel genes, may provide

important insights into the function of human SSCs and aid in their characterization.

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REFERENCES

Beausejour C. 2007. Bone marrow-derived cells: The influence of aging and cellular senescence. Handb Exp Pharmacol 180:67–88.

Campisi J, d'Adda di Fagagna F. 2007. Cellular senescence: When bad things happen to good cells. Nat Rev Mol Cell Biol 8:729–740.

Chen H, Suzuki M, Nakamura Y, Ohira M, Ando S, Iida T, Nakajima T, Nakagawara A, Kimura H. 2005. Aberrant methylation of FBN2 in human non-small cell lung cancer. Lung Cancer 50:43–49.

Choi HJ, Kim GI, Kim HJ, Jung HM, Noh HM, Kim KS, Kim JK. 2006. Discovery of differentially overexpressed genes in immortalized cells and human pulmonary non-small cell carcinomas. J Lung Cancer 5:96–101.

Christensen GL, Ivanov IP, Atkins JF, Mielnik A, Schlegel PN, Carrell DT. 2005. Screening the SPO11 and EIF5A2 genes in a population of infertile men. Fertil Steril 84:758–760.

de Rooij DG, Grootegoed JA. 1998. Spermatogonial stem cells. Curr Opin Cell Biol 10:694–701.

Devroey P, Nagy P, Tournaye H, Liu J, Silber S, Van Steirteghem A. 1996. Outcome of intracytoplasmic sperm injection with testicular spermatozoa in obstructive and non-obstructive azoospermia. Hum Reprod 11:1015–1018.

Diatchenko L, Lau YF, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED, Siebert PD. 1996. Suppression subtractive hybridization: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci USA 93:6025–6030.

Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, Peacocke M, Campisi J. 1995. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci USA 92:9363–9367.

El-Jouni W, Haun S, Machaca K. 2008. Internalization of plasma membrane Ca2+-ATPase during Xenopus oocyte maturation. Dev Biol 324:99–107.

Ezeh UI. 2000. Beyond the clinical classification of azoospermia: Opinion. Hum Reprod 15:2356–2359.

Guan XY, Fung JM, Ma NF, Lau SH, Tai LS, Xie D, Zhang Y, Hu L, Wu QL, Fang Y, Sham JS. 2004. Oncogenic role of eIF-5A2 in the development of ovarian cancer. Cancer Res 64:4197–4200.

Hayflick L. 1965. The limited in vitro lifetime of human diploid cell strains. Exp Cell Res 37:614–636.

Hwang SY, Oh B, Knowles BB, Solter D, Lee JS. 2001. Expression of genes involved in mammalian meiosis during the transition from egg to embryo. Mol Reprod Dev 59:144–158.

Jung HM, Choi SJ, Kim JK. 2009. Expression profiles of SV40-immortalization-associated genes upregulated in various human cancers. J Cell Biochem 106:703–713.

Kenyon J, Gerson SL. 2007. The role of DNA damage repair in aging of adult stem cells. Nucleic Acids Res 35:7557–7565.

Kondo T, Sakaguchi M, Namba M. 2001. Two-dimensional gel electrophoretic studies on the cellular aging: Accumulation of alpha-2-macroglobulin in human fibroblasts with aging. Exp Gerontol 36:487–495.

Ksiazek K. 2009. A comprehensive review on mesenchymal stem cell growth and senescence. Rejuvenation Res 12:105–116.

Lee JH, Lee DR, Yoon SJ, Chai YG, Roh SI, Yoon HS. 1998. Expression of DAZ (deleted in azoospermia), DAZL1 (DAZ-like) and protamine-2 in testis and its application for diagnosis of spermatogenesis in non-obstructive azoospermia. Mol Hum Reprod 4:827–834.

Lee DR, Kim KS, Yang YH, Oh HS, Lee SH, Chung TG, Cho JH, Kim HJ, Yoon TK, Cha KY. 2006. Isolation of male germ stem cell-like cells from testicular tissue of non-obstructive azoospermic patients and differentiation into haploid male germ cells in vitro. Hum Reprod 21:471–476.

Liang P, Pardee AB. 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257:967–971.

Lim J, Sung S, Kim H, Song S, Hong J, Yoon T, Kim J, Kim K, Lee D. 2010. Long-term proliferation and characterization of human spermatogonial stem cells obtained from obstructive and non-obstructive azoospermia under exogenous feeder-free culture condition. Cell Prolif. in press.

Lin H, Pizer ES, Morin PJ. 2000. A frequent deletion polymorphism on chromosome 22q13 identified by representational difference analysis of ovarian cancer. Genomics 69:391–394.

Marian MJ, Li H, Borchman D, Paterson CA. 2005. Plasma membrane Ca2+-ATPase expression in the human lens. Exp Eye Res 81:57–64.

Mauduit C, Hamamah S, Benahmed M. 1999. Stem cell factor/c-kit system in spermatogenesis. Hum Reprod Update 5:535–545.

Meyer N, Penn LZ. 2008. Reflecting on 25 years with MYC. Nat Rev Cancer 8:976–990.

Mizukami T, Kuramitsu M, Takizawa K, Momose H, Masumi A, Naito S, Iwama A, Ogawa T, Noce T, Hamaguchi I, Yamaguchi K. 2008. Identification of transcripts commonly expressed in both hematopoietic and germ-line stem cells. Stem Cells Dev 17:67–80.

Noh HM, Choi SJ, Kim SH, Kim KS, Kim JK. 2007. Differential expression of TPX2 upon differentiation of human embryonic stem cells. Reprod Dev Biol 31:211–226.

Oatley JM, Brinster RL. 2006. Spermatogonial stem cells. Methods Enzymol 419:259–282.

Peinado MA, Malkhosyan S, Velazquez A, Perucho M. 1992. Isolation and characterization of allelic losses and gains in colorectal tumors by arbitrarily primed polymerase chain reaction. Proc Natl Acad Sci USA 89:10065–10069.

Pennisi E. 1997. A catalog of cancer genes at the click of a mouse. Science 276:1023–1024.

Roobrouck VD, Ulloa-Montoya F, Verfaillie CM. 2008. Self-renewal and differentiation capacity of young and aged stem cells. Exp Cell Res 314:1937–1944.

Ryu BY, Orwig KE, Kubota H, Avarbock MR, Brinster RL. 2004. Phenotypic and functional characteristics of spermatogonial stem cells in rats. Dev Biol 274:158–170.

Samuel CS, Sakai LY, Amento EP. 2003. Relaxin regulates fibrillin 2, but not fibrillin 1, mRNA and protein expression by human dermal fibroblasts and murine fetal skin. Arch Biochem Biophys 411:47–55.

Shinohara T, Avarbock MR, Brinster RL. 1999. beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. Proc Natl Acad Sci USA 96:5504–5509.

Shore D, Bianchi A. 2009. Telomere length regulation: Coupling DNA end processing to feedback regulation of telomerase. EMBO J 28:2309–2322.

Shridhar V, Sen A, Chien J, Staub J, Avula R, Kovats S, Lee J, Lillie J, Smith DI. 2002. Identification of underexpressed genes in early- and late-stage primary ovarian tumors by suppression subtraction hybridization. Cancer Res 62:262–270.

Siddiqa A, Sims-Mourtada JC, Guzman-Rojas L, Rangel R, Guret C, Madrid-Marina V, Sun Y, Martinez-Valdez H. 2001. Regulation of CD40 and CD40 ligand by the AT-hook transcription factor AKNA. Nature 410:383–387.

Sikora E. 1993. Transcription factors in cellular senescence and death. Acta Biochim Pol 40:389–394.

Singh S, Dhaliwal N, Crawford R, Xiao Y. 2009. Cellular senescence and longevity of osteophyte-derived mesenchymal stem cells compared to patient matched bone marrow stromal cells. J Cell Biochem 1:839–850.

Thoren LA, Liuba K, Bryder D, Nygren JM, Jensen CT, Qian H, Antonchuk J, Jacobsen SE. 2008. Kit regulates maintenance of quiescent hematopoietic stem cells. J Immunol 180:2045–2053.

Tseleni-Balafouta S, Gakiopoulou H, Fanourakis G, Voutsinas G, Litsiou H, Sozopoulos E, Balafoutas D, Patsouris E. 2006. Fibrillin expression and localization in various types of carcinomas of the thyroid gland. Mod Pathol 19:695–700.

Vasmatzis G, Essand M, Brinkmann U, Lee B, Pastan I. 1998. Discovery of three genes specifically expressed in human prostate by expressed sequence tag database analysis. Proc Natl Acad Sci USA 95:300–304.

Velculescu VE, Zhang L, Vogelstein B, Kinzler KW. 1995. Serial analysis of gene expression. Science 270:484–487.

Wang PJ, McCarrey JR, Yang F, Page DC. 2001. An abundance of X-linked genes expressed in spermatogonia. Nat Genet 27:422–426.

Xu F, Li YJ. 2008. Role of hREV3 in cell cycle and proliferation. Yi Chuan 30:1003–1007.

Xu J, Kalos M, Stolk JA, Zasloff EJ, Zhang X, Houghton RL, Filho AM, Nolasco M, Badaro R, Reed SG. 2001. Identification and characterization of protein, a novel prostate-specific protein. Cancer Res 61:1563–1568.

Yu NA, Ahn JY, Chio EJ, Hong YK, Kim TG, Kim CH, Lee KS, Kim DS, Kim JK. 2005. Detection of differentially expressed genes in glioblastoma by suppression subtractive hybridization. J Korean Neurosurg Soc 37:433–448.