Isolation and Characterization of Novel, Highly Proliferative Human CD34/CD73-Double-Positive Testis-Derived Stem Cells for Cell Therapy

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Human adult stem cells are a readily available multipotent cell source that can be used in regenerative medicine. Despite many advantages, including low tumorigenicity, their rapid senescence and limited plasticity have curtailed their use in cell-based therapies. In this study, we isolated CD34/CD73-double-positive (CD34⁺/CD73⁺) testicular stromal cells (HTSCs) and found that the expression of CD34 was closely related to the cells' stemness and proliferation. The CD34⁺/CD73⁺ cells grew in vitro for an extended period of time, yielding a multitude of cells (5.6×10^{16} cells) without forming tumors in vivo. They also differentiated into all three germ layer lineages both in vitro and in vivo, produced cartilage more efficiently compared to bone marrow stem cells and, importantly, restored erectile function in a cavernous nerve crush injury rat model. Thus, these HTSCs may represent a promising new autologous cell source for clinical use.

Introduction

HUMAN ADULT TISSUE-SPECIFIC stem cells have clinical utility due to their ability to repair and/or replace damaged tissue [1]. However, identification of adult stem cells has proven to be difficult, mainly due to the lack of appropriate tissue-specific stem cell markers. Further limiting their clinical application, these stem cells have a finite lifespan in culture and demonstrate restricted differentiation capacity, particularly when compared to human embryonic stem cells (ESCs) [2]. Among the adult stem cells that have been isolated thus far, bone marrow-derived mesenchymal stem cells (BM-MSCs) are most well characterized. These stem cells were identified over 10 years ago and give rise to various differentiated cell types of mesodermal origin [3,4]. However, isolation of BM-MSCs is quite painful for patients, and once isolated, they are difficult to maintain in culture due to their rapid senescence (usually by 8 passages). Moreover, these stem cells rapidly lose their differentiation capacity after ex-

tended in vitro culture. Other sources of stem cells include dental pulp [5], Wharton's jelly [6], amniotic membrane [7], and adipose tissue [8]; however, stem cells obtained from these sources also have a limited lifespan and differentiation capabilities.

Among the specific stem cell markers, CD34 is found in early hematopoietic and vascular-associated tissues [9]. CD34 is a 116-kD type I transmembrane glycoprophosphoprotein; however, little is known about its precise function [10]. In the hematopoietic system, upon cytokine or growth factor stimulation, cells expressing CD34 on the cell surface can expand and differentiate into all the lymphohematopoietic lineages. Thus, CD34 has been used as a marker to identify and isolate lymphohematopoietic stem/progenitor cell populations. More recently, CD34 has been employed as a marker to help identify other tissue-specific stem cells, including muscle satellite cells and epidermal precursors [11,12]. Recently, it was found that CD34-positive (CD34⁺) stromal cells are distributed in various organs, including the

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breast, fallopian tubes, thyroid gland, colon, pancreas, uterine cervix, and testis [13]. In adipose-derived stromal cell populations, CD34⁺ cells are resident pericytes that play a role in vascular stabilization by mutual structural and functional interactions with endothelial cells [14]. Furthermore, other studies have shown that CD34⁺ cells demonstrated a higher proliferative and colony-forming capacity and a lower differentiating capability compared to CD34-negative (CD34⁻) cells. Taken together, these studies suggested that CD34 expression was inversely correlated to the physiological process of differentiation from an immature status into specific lineages [15]. Furthermore, CD73 is a glycosyl phosphatidylinositol-linked, membrane-bound glycoprotein that hydrolyzes extracellular nucleoside monophosphates into bioactive nucleoside intermediates [16]. This antigen is found in most cell types, including MSCs [17], subsets of B-cells and T-cells [18–20], and endothelial cells [20–22]. In addition, this molecule has been used as a marker to identify MSCs originating from several different tissues [23], although with conflicting results. Interestingly, almost none of the MSCs isolated thus far have shown both CD73 and CD34 expression; thus, we sought to determine if testis stromal cells coexpressing these two cell surface markers represent a new type of adult stem cell.

Mammalian testis consists of germ cells and various types of somatic cells. Although the lack of specific markers has made it difficult to identify and localize potential stem cells in tissues, several studies have isolated and propagated unipotent stem cells such as spermatogonial stem cells (SSCs) and Leydig stem cells [24,25]. In addition, germ cell-derived ESC-like cells have been previously generated using testis biopsies from both human and mouse [26–29]. These cells differentiated into cells of all three germ layers and formed tumors when they were injected into NOD-SCID mice [26]. However, studies on testis somatic stem cells are limited. Only recently has an MSC-like population been isolated from the adult human testes and partially characterized by differentiating the cells into mesodermal-lineage cells [30]. These cells were mostly positive for CD90 and negative for CD34, suggesting that they were testis-derived MSCs with limited lifespans *in vitro*. In the mouse, CD34-positive stromal cells efficiently supported the proliferation of adult spermatogonial progenitor cells [28]. However, no study has investigated whether CD34/CD73-double-positive (CD34⁺/CD73⁺) testis stromal cells are a somatic stem cell source or defined their differentiation and proliferation capabilities. Thus, the aims of this study were to determine and isolate a novel testis-derived somatic stem cell. To accomplish this, we characterized CD34⁺/CD73⁺ testis stromal cells, determined whether the CD34⁺/CD73⁺ stromal cells were true multipotent stem cells, and investigated the importance of CD34 gene expression in the testis stromal cells with regard to cell proliferation, differentiation capability, and stemness gene expression.

In this study, CD34⁺/CD73⁺ cells exhibited a significantly longer proliferative capacity and a greater differentiation potential in cells of adipogenic, osteogenic, neuronal, and pancreatic lineages compared to CD34⁻ cells. The cells did not form teratomas in NOD-SCID mice and retained a high genetic stability as indicated by their normal karyotype after 30 passages. These cells also promoted the functional recovery of penile erection in a bilateral cavernous nerve

crush injury (BCNCI) model in rats. In addition, the percentage of cells expressing CD34 correspondingly decreased as the cell passage number increased *in vitro* and as the cells became terminally differentiated. Thus, our results indicated that CD34 and CD73 are effective markers for the initial identification of histocompatible, multipotent, somatic stem cells, which are easily obtainable from simple testis biopsies. Utilization of these cells would permit patient-specific cell-based therapy without the potential immunological incompetence associated with allogenic adult stem cells or the ethical controversy associated with human ESCs.

Materials and Methods

Isolation and culture of human highly proliferative and potent testis-derived stem cells

All consenting donors were fully informed and understood the scope of this study, and there was no monetary compensation for the donations. Twenty-five samples of testis tissue, remaining after clinical requirements, were obtained from patients undergoing testicular sperm extraction (TESE)—intracytoplasmic sperm injection treatment in our IVF laboratory. Ten samples were discarded during passage 1–2 because the cells were positive for mycoplasma contamination (MycoAlert[®], mycoplasma detection kit; Lonza). Finally, 15 testicular tissue samples were obtained from obstructive (OA, *n*=2) or nonobstructive azoospermic (NOA, *n*=13) patients and enrolled in the study (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/scd). This study was approved by the Institutional Review Board of the CHA Gangnam Medical Center, Seoul, South Korea.

Testis tissues were washed in the RBC lysis buffer (Roche Diagnostics) and interstitial cells containing testicular somatic cells and putative somatic stem cells among intact seminiferous tubules were dissociated enzymatically with agitation at 37°C for 30 min in the enzyme solution [the Hanks' balanced salt solution (HBSS; Gibco) containing 0.5 mg/mL collagenase (type IV; Gibco) and 0.25 mg/mL dispase II (neutral protease, grade III; Roche)]. To isolate the interstitial cells, only outer surrounding cells of seminiferous tubule were dissociated by enzyme digestion plus mechanical separation, avoiding dissociation of tubule as much as possible. The suspension of interstitial cells was collected, washed, and filtered through a 40-μm mesh (BD). Then, they were plated (5.0×10^5 /mL) in culture flasks coated with 0.1 gelatin (Gibco) and cultured in a basic culture medium (50:50 mixture of DMEM-F12:StemPro-34 SFM [Invitrogen Corporation] supplemented with 10% fetal bovine serum [FBS; Gibco], and penicillin/streptomycin solution [1X; Gibco]). After 7–8 days, cells at 80% confluency were detached using 0.5% Trypsin-EDTA (Gibco) and passaged. After 2–3 passages, to isolate somatic stem cells in testis, primary cultured cells were detached, and then sorted with a Dynabeads Flowcomp (Invitrogen) to obtain CD34/CD73 cells with a modified method from Xu et al. (2009) [31]. In brief, the detached cells were incubated with the CD73 antibody (Santa Cruz Biotechnology) and with biotin-conjugated goat anti-mouse IgM, and then mixed with the streptavidin-bound magnetic Dynabead. After sorting, cells were incubated in a releasing buffer for 15 min and transferred to the

Dynal MPC-1 magnet. The released CD73⁺ cells were collected and plated for 4–7 days, and then were detached and subjected to a second isolation with a biotinylated CD34 antibody (Santa Cruz Biotechnology) using the procedures described above. Sorted CD34⁺/CD73⁺ cells are referred to as testis-derived stem cells (TSCs) and were replated (5.0×10^5 /mL/25 cm²) and cultured in culture flasks coated with 0.1% gelatin (Gibco) in the basic culture medium. Sorted CD34⁺/CD73⁺ cells are referred to high proliferative TSCs (HTSCs) and were replated (1,000 cells/25 cm²) and cultured in a basic culture medium for 10 to 14 days. The medium was changed every other day. The cumulative population doubling with each subculture was calculated with the formula $2^X = N_H/N_I$, where N_I is the seeded cell number, N_H is the cell harvest number at confluence (>80%), and X is population doubling [32]. The calculated population doubling was then added to the previous population doubling level to yield the cumulative population doubling level.

Flow cytometric analysis

Sorted cells by magnetic beads were cultured and re-analyzed by flow cytometric analysis for the more accurate characteristics. After p2, HTSCs at 80% confluency were passaged every 3–4 days. At p5, some HTSCs were re-suspended, and then incubated with antibodies for 20 min at 4°C in the dark to detect CD34, CD73, HLA-ABC, CD166, CD44, CD29, CD105, CD90, CD31, CD45, HLA-DR, TRA-1-60, SSEA3, SSEA4, TRA-1-81, CD14 (BD), CD133 (eBioscience, Inc.), c-Kit (Santa Cruz Biotechnology), and Stro-1 (Biolegend). Cells were washed, suspended in 500 μ L PBS, and immediately analyzed in a flow cytometer (FACS Vantage SE System; BD). To identify dead cells, we incubated HTSCs with propidium iodide (Sigma-Aldrich). The percentage of cells that were positive for each specific antibody was calculated by comparison with the appropriate isotype control.

Immunocytochemistry

Paraffin sections were deparaffinized for localization of CD34 and CD73 in human testis. After washing and blocking, a primary antibody diluted in the blocking buffer was applied overnight at 4°C. Tissues were incubated with a secondary antibody in the blocking buffer for 1 h at RT, and then counterstained with DAPI and mounted. The primary antibodies used were CD34 (Santa Cruz Biotechnology), CD73 (Santa Cruz Biotechnology), CD31 (Abcam), and α SMA. Secondary antibodies conjugated to FITC (1:200; Jackson ImmunoResearch) or Cy3 (1:200; Jackson ImmunoResearch) were used.

HTSCs were fixed in 4% paraformaldehyde (PFA) and stored at 4°C. After permeabilization in 0.1% Triton X-100 (Sigma) and blocking in a blocking solution (Dako Cytomation, Inc.), the primary antibody diluted in the blocking buffer was applied overnight at 4°C. Cells were incubated with the secondary antibody in the blocking buffer for 1 h at RT, and then counterstained with 4',6-diamidino-2-phenylindole (DAPI) (1:500; Jackson ImmunoResearch) and mounted (Dako Cytomation, Inc.). The primary antibodies used were CD34 (Santa Cruz Biotechnology), CD73 (Santa Cruz Biotechnology), GFR α 1 (Chemicon), VASA (anti-DDX4/MVH; Abcam), GATA4 (Abcam), β 3-HSD (Abcam),

Desmine (Abcam), α SMC (Abcam), C-peptide (Monosan), insulin (#4590; Cell signaling), nestin (Millipore), neuron-specific β -tubulin class III (TuJ1; Santa Cruz Biotechnology), GFAP (Millipore), GABA (γ aminobutyric acid; Sigma-Aldrich), 5'-HT (Serotonin, Immunostar), ChAT (choline acetyltransferase; Millipore), and Glutamate (Sigma-Aldrich). Secondary antibodies conjugated to FITC (1:200; Jackson ImmunoResearch), Cy3 (1:200; Jackson ImmunoResearch), and/or TRITC (1:200; Jackson ImmunoResearch) were used.

Karyotyping

For the cytogenetic analysis of HTSCs, cells at p5, 13, 20, and 30 were incubated for 3 h in a basic culture medium containing 0.1 μ g/mL colcemid (KaryoMax Colcemid Solution; Gibco). Then, they were treated with a hypotonic solution (1% sodium citrate buffer) for 30 min, and fixed with methanol and acetic acid (3:1, vol/vol). Cells were spread onto glass slides and dried, and chromosomes were identified by G banding. To karyotype each cell line, more than 20 metaphase chromosomes were counted by a cytogenetic expert.

Real-time reverse transcription–polymerase chain reaction

RNA was isolated using the Tri-reagent (Sigma-Aldrich) according to the manufacturer's instructions. The purity of RNA was assessed using a spectrophotometer (ND-1000, NanoDrop; Thermo Scientific). Total RNA (1 μ g) was first incubated with 1 IU of DNase I with 5 mmol MgCl₂ at 37°C for 30 min, and then reverse transcription–polymerase chain reaction (RT-PCR) was performed using a Prime script 1st strand cDNA Synthesis Kit (TaKaRa Bio, Inc.). For negative control of the RT sample, no reverse transcriptase was added to the reaction. Subsequent PCR reactions were performed using cDNA, primer pairs (Supplementary Table S2), and RNAs from TSCs at p5, HTSCs at p5, hESCs (CHA-hES4 line at p72) [33], BM-MSCs at p3 (PT-2501; Lonza), and adipocyte-derived hMSCs (AD-MS) at p3 [34]. Target mRNAs were quantified relative to β -actin. Amplification products were quantified on a DNA Engine 2 fluorescence detection system (MJ research) using the DyNamo SYBR Green qPCR kit (Finnzymes). Reactions were performed in a reaction mix containing 4 μ L DEPC-treated water, 2 μ L forward primer (5 pmol), 2 μ L reverse primer (2 pmol), 10 μ L premix with SYBR Green, and 2 μ L cDNA template in a total volume of 20 μ L. Fluorescence was measured at the end of each cycle during the 72°C extension step. In the final step of the real-time PCR, a melting curve was generated by raising the temperature from 65°C to 95°C at a rate of 0.1°C/s, with constant measurement of fluorescence, followed by cooling at 40°C for 30 s. Relative gene expression was quantified using the $2^{-\Delta\Delta CT}$ method [35].

Colony forming unit assay

For colonies, three different concentrations (2×10^5 , 1×10^5 , and 0.5×10^5 cells/mL) of HTSCs and BM-MSCs were placed into culture flasks containing the NH CFU-F medium (Miltenyi Biotec). On day 14, cells were fixed with methanol and dried. The cells were then stained using the Giemsa staining solution (Sigma-Aldrich) and incubated for 5 min at

RT. After washing and drying, colonies between 1 and 8 mm in diameter (more than 20 cells) were counted.

Teratoma formation in immunodeficient mice

To analyze tumor growth, undifferentiated HTSCs were resuspended in PBS (1×10^6 cells/20 μ L) and injected into testicles of immunodeficient SCID mice. As a positive control for teratoma formation, human ESCs (1×10^6 cells/20 μ L, CHA-hESC35: hES12012006; Korea Stem Cell Registry, KNIH) were injected into the testicles of immunodeficient SCID mouse. After 12 to 16 weeks to allow tumor formation, mice were euthanized. Teratoma tissues were placed in 4% PFA, and then embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (Sigma) for histological examination. To identify human cells in the mouse tissue, we performed immunohistochemistry using a human-specific antibody (Stem-121™; Stem Cells, Inc.) in SCID mouse testis with or without HTSC injection.

In vitro differentiation into adipogenic, osteogenic, and chondrogenic cells

HTSCs were collected at p5, p13, and p20 and resorted HTSCs were also collected at p20. As controls, hESCs at p72 and BM-MSCs at p3 were also collected to analyze their differentiation potential into the 3 germ cell layers. All types of cells were resuspended and replated into 6-well culture dishes. After 24 h, the nonadherent cells were removed by replacing the medium, and the attached cells were cultured until confluence. The cells were then grown for 21 days in the adipogenic, osteogenic, and chondrogenic medium (Invitrogen). Adipogenic differentiation was visualized using Oil Red O (Sigma-Aldrich) staining. The expression of adipogenesis-specific genes (PPAR γ and C/EBP α) was analyzed by real-time RT-PCR [36]. Osteogenic differentiation was visualized by Alizarin Red S (Sigma-Aldrich) staining and analyzed by osteogenesis-specific gene expression (COL I and CBFA I) [36]. Chondrogenic differentiation was visualized by Alcian blue (Sigma-Aldrich) staining and analyzed by chondrogenesis-specific gene expression (COMP and SOX9) [36].

Differentiation potential of single-cell-derived HTSCs

Whether HTSCs with multipotency were derived from a population of cells or not, a single HTSC had been isolated by serial dilution and propagated by single-cell clonogenic culture methods [37]. In brief, isolated HTSCs were suspended in a 1 mL culture medium and conducted a serial dilution. This process was repeated to achieve a final dilution of 10 cells in a 1 mL medium. For single-cell culture, 100 μ L of the diluted cell suspension was transferred into each well of a 96-well plate containing 200 μ L of the culture medium. The resultant serial dilution was examined under an inverted microscope. Wells with more than one cell or no cell were marked and exempted from the culture selection. Differentiation potential into adipogenic, osteogenic, and chondrogenic cells of repopulated HTSCs from a single cell was also analyzed by in vitro differentiation methods as previously mentioned.

In vitro differentiation into neurogenic cells

Neurogenic differentiation of HTSCs and BM-MSCs was induced in DMEM-F12 media (Gibco) with N2 supplement (Gibco), 2 mM L-glutamine (Gibco), and penicillin/streptomycin solution (1X; Gibco) [38]. After 3 days, cells were fixed and processed for immunocytochemistry. After the cell clumps formed floating spherical cells resembling neurospheres, cells (1×10^6 cells/mL) were detached, re-plated on fibronectin (10 μ g/mL; Sigma)-coated dishes, cultured in the Neural Progenitor Basal Medium (NPBM; Cambrex) supplemented with 2 mM L-glutamine, 10 ng/mL epidermal growth factor (EGF; Invitrogen), 10 ng/mL bFGF, and penicillin/streptomycin solution, and cultured for 3 days [39]. Growth factors were added every day. Induction of terminal neural differentiation was initiated by plating cells in the Neurobasal Medium (Gibco) supplemented with 0.5 μ M all-trans-retinoic acid (Sigma), 1% FBS (Gibco), 5% horse serum (Gibco), 1% N2 supplement, and penicillin/streptomycin solution [40]. Cells were differentiated for 10–14 days. Neurogenic differentiation was observed using microscopy and confirmed by RT-PCR using neuronal markers.

Electrophysiology

To analyze the function of differentiated cells into neuronal lineage from HTSCs, action potential and Na⁺ current were recorded at room temperature (23°C $\pm$ 1°C) by the whole-cell patch-clamp technique using an EPC-10 amplifier (HEKA Elektronik) and their results were compared with undifferentiated HTSCs. About 4–6 M Ω when filled with intracellular solutions contained (in mM) 130 K-gluconate, 8 NaCl, 0.5 EGTA, 10 HEPES, 4Mg-ATP, 0.3 Na-GTP, adjusted to pH 7.3 with KOH, and 280 mOsm. The extracellular solution consisted of (in mM) 140NaCl, 5.4 KCl, 0.5 MgCl₂, 2.5 CaCl₂, 5.5 HEPES, and 10 glucose, adjusted to pH 7.4 with NaOH. Action potential was elicited by a current injection of 0–300 pA in a 0.5 or 1 s current clamp. To confirm the expression of Na⁺ currents in the same cell, Na⁺ currents were evoked from a holding potential of –60 mV by stepping to voltages between –80 and +20 mV in 10 mV increment for 100 ms. Data were filtered at 3 kHz, digitized at 10 kHz, and analyzed using Pulse program ver.8.67 (HEKA Elektronik).

In vitro differentiation of HTSCs into insulin-secreting cells

Differentiation into insulin-secreting cells was induced according to the manufacturer's instructions for the specific medium (Bcell Bio) [32]. Differentiation efficiency was analyzed by measuring insulin and C-peptide secretion into the culture medium. In brief, cells were treated with low-glucose (5.5 mM) DMEM containing 0.5% bovine serum albumin for 12 h, and then stimulated by high-glucose (25 mM)-DMEM for 2 h at 37°C. The amounts of insulin and C-peptide released into the medium were measured with human insulin and C-peptide enzyme-linked immunosorbent assay (ELISA) kits (Mercodia) according to the manufacturer's instructions. Synthesis of insulin and C-peptide mRNAs in differentiated cells was confirmed by RT-PCR. Also, immunocytochemistry of C-peptide and insulin was performed in undifferentiated and differentiated cells.

In vivo differentiation into adipogenic, osteogenic, and chondrogenic cells

The animal experiments concerning mouse handling were approved by the Institutional Animal Care and Use Committee of CHA University. BALB/c female mice (6 weeks old) were divided into 4 groups. The dextran (DEX)-loaded microspheres (30 mg) harboring HTSCs were implanted subcutaneously into the backs of 12 nude mice. In group I, the control group ($n=3$), DEX-loaded microspheres were injected into the back subcutis of female mice. In group II ($n=3$), 100 ng/mL TGF β 3-coated microspheres were injected into the back subcutis of female mice. In group III ($n=3$), 100 ng/mL BMP2-coated microspheres were injected into the back subcutis of female mice. In group IV ($n=3$), 50 ng/mL IGF- and bFGF (Invitrogen)-coated microspheres were injected into the back subcutis of female mice. At 4 weeks post-treatment, the female mice were euthanized via an overdose injection of anesthetic (ketamin), and the skin surrounding the injection site ($2 \times 2 \text{ cm}^2$) was carefully excised for subsequent biological examination. Tissues were harvested and processed for RT-PCR, immunoblotting, immunocytochemistry, and immunohistochemistry to confirm *in vivo* differentiation [36]. We performed immunoblotting and immunocytochemistry using adipogenesis-specific antibodies [PPAR γ (SC-7273, Santa Cruz Biotechnology) and C/EBP α (SC-61, Santa Cruz Biotechnology)], osteogenesis-specific antibodies [CBFA I (SC-10758, Santa Cruz Biotechnology) and COL I (MAB3391, Chemicon)], and chondrogenesis-specific antibodies [SOX9 (AB5535, Chemicon) and COL II (MAB1330, Chemicon)] in the differentiated tissues.

Transplanted HTSCs into BCNCI rat model

Thirty-two 12-week-old male Sprague-Dawley rats were randomly divided into four groups (eight rats per group) as follows: (1) only laparotomy (sham group); (2) BCNCI and 0.1 mol/L phosphate-buffered saline instillation (injury group); (3) BCNCI and periprosthetic BM-MSCs instillation, 1×10^7 stem cells suspended in 100 μ L sterile PBS (BM group); and (4) BCNCI and periprosthetic HTSC instillation, 1×10^7 stem cells suspended in 100 μ L sterile PBS (HTSC group). Before injection, the stem cells were labeled with CellTrackerTM CM-DiI (C7000; Invitrogen) for stem cell tracking.

Four weeks following implantation, the erectile function was assessed by cavernous nerve electrostimulation (3V, 0.2 ms, 20 Hz) proximal to the site of injury (Fig. 6C). Systemic mean arterial pressure (MAP) and intracavernosal pressure (ICP) were recorded and analyzed electronically (Powerlab; AD Instruments, Fig. 6D). The ICP/MAP ratio was compared among the groups. After the functional evaluation, the prostate lobes of each rat were harvested. We performed immunohistochemistry using a human-specific antibody (Stem-121TM) and the neuron-specific β -tubulin class III (TuJ1; Santa Cruz Biotechnology) in the crush injury area of each rat.

Statistical analysis

All experiments were replicated at least 3 times, and data are presented as the means $\pm$ SEMs. The differences among groups were analyzed with ANOVA (multiple comparison using

bonferroni correction). A P -value < 0.05 was considered statistically significant. Also, correlations between CD34 expression and genes specific to the three germ layer lineages were analyzed using the Pearson's coefficient of correlation.

Results

Isolation and expansion of human HTSCs

Fifteen donors enrolled in the study with a mean age of 36.7 ± 6.7 years (between 29 and 55 years). The clinical profiles and testicular pathology of the OA and NOA patients showed high individual variability, although the statistical significance between the patient groups was not observed because only two OA patients were enrolled (Supplementary Table S1). However, the level of testosterone was not different between the two types of patients, and thus, we expected similar interstitial and peritubular cell compositions between the OA and NOA patients. Histological observation of human testis tissues revealed that CD34-positive cells were distributed in and between the seminiferous tubules. Furthermore, CD31 and α SMA expression were localized in the blood vessel-like structures and tubules, respectively (Supplementary Fig. S1). Moreover, the expression patterns of CD34, CD73, CD31, and α SMA were very similar in the testis between OA and NOA patients. To localize CD34⁺/CD73⁺ cells in the human testis, paraffin sections of biopsied seminiferous tubules were prepared and stained using CD34 and CD73 antibodies. There were few CD73⁺ cells and their expression was not observed beneath the basal lamina of the seminiferous tubules. Thus, the CD34⁺/CD73⁺ cells localized outside of the tubules (in the peritubular or interstitial cells) were broadly distributed, although there were only a small number of cells (Supplementary Fig. S1). However, CD34⁺/CD31⁺ cells were not observed in the testes.

The collected interstitial cells containing various germ cells, testicular somatic cells, and putative somatic stem cells were primarily cultured for 2–3 passages. A small number of CD34⁺ cells were present among the primary cultured cells, which included a small number of Sertoli cells (GATA4⁺), Leydig cells (β HSD⁺), germ cells (GFR α 1⁺), and peritubular myoid cell markers (Desmin and α SMC) (Fig. 1A). However, VASA-positive cells were very rare and were not found in this cell population. Upon culturing *in vitro*, adherent cells isolated from human testicular interstitial cells were sorted by CD73 expression. These cells exhibited an MSC-like morphology and constitutively expressed the CD73 antigen; thus, herein we referred to these cells as testis-derived stem cells (TSCs). TSCs consisted of two subpopulations of cells (CD34⁺ and CD34⁻). TSCs (CD34⁻) were reisolated using magnetic-activated cell sorting (MACS), and then subsequently replated onto separate culture dishes. Due to the very small number of CD73⁺ cells (Fig. 1A), the number of CD34⁺/CD73⁺ cells was very small (approximately 1,000 cells [0.03%] from 100 mg of biopsied tissue, Fig. 1B). In addition, due to their initially small number, the CD34⁺/CD73⁺ cells only reached 80% confluency after 14 days in culture, which represented passage 1 (p1) of these cells. Yet, once the cells were further expanded, the CD34⁺/CD73⁺ cells exhibited a high proliferative activity and were thus referred to as HTSCs. Furthermore, immunocytochemical analysis confirmed that HTSCs coexpressed CD34 and CD73. In contrast, TSCs and BM-MSCs expressed only

FIG. 1. (A) Specific marker expression in primary testis interstitial cells. Immunocytochemistry analysis reveals the expression of CD34 (red, white arrowheads) and GATA4 (green, open arrowheads) in the first row, 3 β -HSD (green, open arrowheads) in the second row, GFR α 1 (red, white arrowheads) and VASA (green, empty arrowheads) in the third row, and α SMA (red) and Desmin (green) in the fourth row. (B) Separated cell numbers in each step per 100 mg of testis tissue. The starting materials were quantified at day 0, attached cells at day 3, CD73-sorted cells at p2, and CD34-sorted cells at p3. (C) Expression of CD34 and CD73 in BM-MSCs, TSCs, and HTSCs. DAPI nuclear staining is shown in blue. FITC staining is shown in green; Cy3 and TRITC staining is shown in red. (D–F) Specific marker expression in highly proliferative testis-derived stem cells (HTSCs) at p3 and p8. Immunocytochemistry analysis revealed expression of CD34 (red) and no expression of GFR α 1 or VASA (green) (D), expression of CD34 (red) and no expression of 3 β -HSD or GATA4 (green) (E), and expression of CD34 (red) and Desmin or α SMA (green) (F). The negative controls were not treated with a primary antibody (IgG only). Scale bars indicate 10 μ m. Color images available online at www.liebertpub.com/scd

CD73 and not CD34 (Fig. 1C). To analyze the contamination of germ cells and other somatic cells, the HTSCs were reanalyzed by immunocytochemistry using germ cell markers (GFR α 1 and VASA), a Leydig cell marker (3 β -HSD), a Sertoli cell marker (GATA4), and peritubular myoid cell markers (Desmin and α SMC). After sorting by CD73 and CD34, most cells expressed CD34 at p3 and did not express markers for germ cells or testicular somatic cells. After sorting at P8, the number of CD34⁺ cells had decreased significantly. Markers for the germ cells, Leydig cells, and Sertoli cells were still not detected, although signals for Desmin and α SMC reappeared in a small number of CD34⁺ cells (Fig. 1D–F).

The cells were fed every 3–4 days with a fresh medium and had maintained their MSC-like morphology until p26 or p27 (Fig. 2A, B). In contrast, BM-MSCs and TSCs maintained an MSC-like morphology until p6 and p13, and then showed a senescent morphology (gross enlargement and flattening of

the cells, Fig. 2A). Throughout the culturing, TSCs (CD34[−] / CD73⁺) underwent an average of 34.3 ± 2.1 population doublings, and the total number of the cells recovered per patient was 2.2×10^{13} cells. In contrast, HTSCs (CD34⁺ / CD73⁺) underwent an average of 67.3 ± 2.1 population doublings, and the total number of cells recovered was 5.6×10^{16} per patient (Fig. 2B). At p8, the HTSCs were resorted using MACS for CD34 (resorted HTSCs) were maintained up to p36 (79.8 ± 2.4 population doublings and 1.3×10^{19} cells in resorted HTSCs).

Changes in CD34 expression during long-term culture and population recovery by resorting

We analyzed the population of CD34⁺ cells during long-term culture of HTSCs. The proportion of CD34⁺ cells steadily decreased over time (Fig. 2C). The population of

FIG. 2. Morphological and proliferative characteristics of various types of testis-derived stem cells (TSCs). **(A)** Phase-contrast images demonstrate differences in the morphology of cells maintained under the same culture conditions (MSC-like *vs.* senescent-like morphology). Bone marrow-derived mesenchymal stem cells (BM-MSCs) at p1 and p6, human TSCs at p1 and p13, and highly proliferative testis-derived stem cells (HTSCs) at p5, p13, p20, and p27. **(B)** Comparison of the cumulative doubling number of BM-MSCs, TSCs, HTSCs, and resorted HTSCs. **(C)** After further culturing of HTSCs, the population of CD34⁺ cells was reduced, but the CD73⁺ cells remained abundant. During culture, the HTSCs steadily lost CD34 expression, which was not regained. However, proliferation of HTSCs was further extended when the subpopulation of CD34⁺ cells was isolated by MACS and replated. Scale bars indicate 50 μ m. Color images available online at www.liebertpub.com/scd

CD34⁺ cells was barely detectable when the HTSCs reached passages 15–20. After this point, the cell doubling time increased and their morphology had also changed (Fig. 2A, B). At p8, the HTSCs were resorted using MACS for CD34 (resorted HTSC) and their proliferation was analyzed. The population of CD34⁺ cells in the resorted HTSCs was maintained for an additional 8 passages, but decreased thereafter and where they proliferated until p30 (Fig. 2C).

Characterization of HTSCs (CD34⁺/CD73⁺ HTSCs)

To characterize the HTSCs, we performed flow cytometry, immunofluorescence, and RT-PCR. At p3, after sorting,

multicolor flow cytometry was performed using various markers. The HTSCs were positive for CD34 (96.5% $\pm$ 3.5%), CD73 (95.6% $\pm$ 1.5%), class I major histocompatibility (MHC) antigens (HLA ABC), CD29, CD44, CD90, CD105, and CD166 and were negative for CD31, CD45, HLA DR, TRA-1-60, SSEA3, SSEA4, TRA-1-81, c-Kit, CD133, CD140, STRO-1, and CD14 (Fig. 3).

To examine their tumorigenic potential, three HTSC lines were injected into the testicles of SCID mice. At 12–16 weeks after the injection, none of the HTSC lines had formed tumors in any of the 10 recipient SCID mice (Supplementary Fig. S2A), although a small number of injected human HTSCs (human-specific antibody Stem-121TM-positive cells)

FIG. 3. Flow cytometric analyses of HTSCs. HTSCs were strongly positive for HLA-ABC, CD73, CD166, CD44, CD29, CD90, CD105, and CD34; weakly positive for CD14, CD133, and Stro1; and negative for SSEA3, TRA-1-81, c-Kit, CD31, TRA-1-60, CD140, HLA-DR, CD45, and SSEA4 antigens. APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin. Color images available online at www.liebertpub.com/scd

had remained in the testis (Supplementary Fig. S2C). To determine if these cells displayed other typical characteristics of stem cells, the isolated HTSCs were plated for colony-forming assays, and the results were compared with those obtained from BM-MSCs. The HTSCs formed stem cell colonies, and their efficiency of colony formation was significantly higher compared to BM-MSCs ($15.2\% \pm 2.1\%$ vs. $1.0\% \pm 0.1\%$, Supplementary Fig. S2D). However, the hematopoietic cell colonies were not observed for either type of stem cells. In addition, to analyze the genetic stability of HTSCs during long-term propagation in vitro, we performed karyotyping analysis at p5, p13, p20, and p30, which consistently showed normal diploid karyotypes (46, XY) without chromosomal aberrations (Supplementary Fig. S2E). In addition, RT-PCR analysis demonstrated that HTSCs expressed only low levels of pluripotency-related *OCT4*, *SOX2*, and *NANOG* genes, and did not express germ cell-specific *VASA* mRNA at p5, p13, and p20 (Supplementary Fig. S2F).

In vitro differentiation into chondrogenic, adipogenic, and osteogenic cells

HTSCs were collected at p5, p13, and p20, and then resorted (p20; at p12 after sorting). The cells were differentiated in the chondrogenic differentiation medium for 3 weeks. BM-MSCs at p3 and TSCs at p3 were used as experimental controls. Derivatives from all of the HTSCs, TSCs and BM-

MSCs showed Alcian blue staining, which indicated polysaccharide production. However, the mRNA levels of chondrogenic genes, *COMP* and *SOX9*, were significantly higher in HTSC-derived chondrogenic cells compared to BM-MSC-derived chondrogenic cells (Fig. 4A). In HTSCs, *COMP* and *SOX9* expression decreased in culture, but were maintained in the resorted HTSCs.

To test their adipogenic capacity, the HTSCs were cultured in the adipogenic medium for 3 weeks. BM-MSCs at p3 and TSCs at p3 were used as experimental controls. Under these conditions, derivatives from the HTSC-derived adipocytes showed a typical morphology of lipid-laden cells containing intracellular lipid droplets and stained positive for Oil Red O. Adipogenically differentiated cells obtained from BM-MSCs and early passaged HTSCs exhibited similar expression levels of adipogenic genes encoding *PPAR γ* and *C/EBP α* (Fig. 4B). However, these gene levels were low in TSCs. The gene expression levels of *PPAR γ* and *C/EBP α* in HTSCs steadily decreased with continued culture, but were maintained when the resorted HTSCs were differentiated.

HTSCs, TSCs, and BM-MSCs were also placed in the osteogenic medium for 3 weeks. Under these conditions, derivatives from all of the HTSCs and BM-MSCs stained positively for Alizarin Red S, an indicator of calcium production. Both types of cells showed similar expression levels of osteocyte marker genes, *COL 1* and *CBFA 1* (Fig. 4C).

FIG. 4. In vitro differentiation potential into mesodermal-lineage (chondrogenic, adipogenic, and osteogenic) cells of BM-MSCs, testis-derived stem cells (TSCs), and HTSCs. **(A)** Alcian blue staining for sulfated proteoglycans (left upper panels) in BM-MSCs at p5, TSCs at p3, and HTSCs at p5, p13, p20, and resorted p20 after chondrogenic differentiation for 3 weeks. The gene expression of SOX9 and COMP (left lower panels) before and after chondrogenic differentiation was also analyzed in the same groups. **(B)** Staining of lipid droplets with Oil Red O (center upper panels) and the gene expression of PPAR γ and C/EBP α (center lower panels) was determined to evaluate adipogenic differentiation. **(C)** Calcium deposits were visualized using Alizarin Red S (right upper panels) and the gene expression of CBFA I and COL I (right lower panels) was determined to evaluate osteogenic differentiation. Scale bars indicate 100 μ m. **(D)** In vitro differentiation potentials of embryonic stem cells (ESCs), BM-MSCs, adipose-derived (AD)-MSCs, TSCs, and HTSCs into mesodermal-lineage cells (comparison of the gene expression of SOX9, COMP, PPAR γ , C/EBP α , CBFA I, and COL I). Color images available online at www.liebertpub.com/scd

However, the intensity of the staining and the level of osteocyte gene expression were low in TSCs.

Gene expression profiles of various stem cells after differentiation into chondrogenic, adipogenic, and osteogenic cells

Comparative analyses of the differentiation potential among ESCs (at p72), BM-MSCs (at p3), adipocyte-derived hMSCs (AD-MSC at p3), TSCs (at p5), and HTSCs (at p5) were performed by examining the relative expression levels of specific genes involved in chondrogenesis, adipogenesis, and osteogenesis (Fig. 4D). The chondrogenic potential of the HTSCs was significantly higher ($P < 0.001$) compared to BM-MSCs, AD-MSCs, and TSCs, but not as good as that of ESCs. The adipogenic potential of the HTSCs was comparable to that of ESCs and BM-MSCs, but superior to those of AD-MSCs and TSCs.

In vivo differentiation of adipogenic, osteogenic, and chondrogenic cells

HTSCs loaded in growth factor-containing microspheres were divided into 4 groups, and then subcutaneously transplanted into the backs of 10 nude BALB/c female mice. The expression of the differentiation-associated genes, including PPAR γ , C/EBP α , CBFA I, COL I, SOX 9, and COL II,

was detected using RT-PCR. Genes that were characteristic of adipogenic, osteogenic, and chondrogenic cells were highly expressed in differentiating HTSCs transplanted with specific growth factor-containing microspheres (Supplementary Fig. S3A).

Western blotting analyses showed the presence of corresponding proteins in the sera of these mice (Supplementary Fig. S3B). Further, specific staining or immunocytochemical analysis using specific markers demonstrated the adipogenic, osteogenic, and chondrogenic differentiation potential of HTSCs in vivo and in vitro (Supplementary Fig. S4A, B).

In vitro differentiation into chondrogenic, adipogenic, and osteogenic cells of cloned HTSCs

A single HTSC can expand and form cloned HTSCs with high population numbers after 3 weeks in culture. Cloned HTSCs have shown a similar differentiation potential into chondrogenic, adipogenic, and osteogenic cells when compared with HTSCs (Supplementary Fig. S5).

In vitro differentiation into insulin-secreting cells

The endodermal lineage multipotencies of HTSCs, resorted HTSCs, and BM-MSCs were examined by differentiating the cells into insulin-secreting cells in vitro. After culture in insulinogenic media, the insulin-secreting activity,

particularly the glucose-dependent secretion of the cells, was analyzed by stimulating the cells with low (5.5 mM) or high (25 mM) concentrations of glucose. When the cells were not induced to differentiate, the amount of insulin and C-peptide released into the medium in response to either glucose stimulation were similar for all of the HTSCs (at p5, p13, p20, and resorted p20) and BM-MSCs (p3). After culturing the same cells in the differentiation medium, insulin and C-peptide release by the cells were greatly increased by high glucose stimulation. When stimulated with high glucose, the HTSCs at p5 and p13 secreted similar amounts of insulin and C-peptide as the BM-MSCs at p3. By p20, the HTSCs showed a decline in their capacity to secrete insulin and C-peptide, but cells that were resorted for CD34 at p8 showed a rescue of this decline and the cells maintained high insulin and C-peptide secretion at p20 (Fig. 5A, B). The pancreatic beta-cell marker genes of *insulin* and *NGN3* were distinctly expressed in all of the HTSCs and BM-MSCs cultured in insulinogenic differentiation media, but not in undifferentiated cells (Fig. 5C). In addition, the pancreatic markers *PDX1*, *GLUT1*, and *ISL-1* were highly expressed in the differentiated compared to the undifferentiated HTSCs (Supplementary Fig. S6A). Furthermore, the expression of c-peptide and insulin was colocalized only in the differentiated, not undifferentiated, HTSCs (control, Fig. 5D).

In vitro differentiation into neurogenic cells

HTSCs at p5, 13, and 20, resorted HTSCs at p20, and BM-MSCs at p3 were cultured in the neurogenic step I medium for 3 days. At the end of this step, most of the derived cells from the HTSCs and BM-MSCs were positively stained with anti-nestin (Fig. 6A). When these nestin⁺ cells were cultured in the neurogenic step II medium for an additional 3 days, the morphology of the derived cells from the HTSCs and BM-MSCs changed into a bipolar form, which is characteristic of neurons. The expression of neural cell-specific genes, such as *GFAP* and *β -tubulin 3*, was observed in the bipolar cells from both HTSCs and BM-MSCs (Fig. 6B). Moreover, neurogenic markers (*GFAP2*, *TuJ1*, *TH*, and *Nestin*) were highly expressed in the differentiated compared to the undifferentiated HTSCs (Supplementary Fig. S6B), and neuronal cells were also positively stained for specific antibodies (anti-TuJ1, anti-GFAP, anti-GABA, anti-5'-HT, anti-ChAT, and anti-Glutamate antibody) in the differentiated cells from HTSCs (Supplementary Fig. S7). An incomplete action potential-like depolarization was observed in some neuronal lineage from HTSCs, but not in all undifferentiated HTSCs. Also, Na⁺ currents, which contributed to action potential-like depolarization, were recorded at only neuronal lineage from HTSCs, indicating that the Na channel was expressed in neuronal lineage from HTSCs, although their amplitudes were small (~20 pA) compared to the amplitude of neuronal Na⁺ currents (Supplementary Fig. S8).

Functional recovery of BCNCI using transplanted HTSCs

We used a bilateral cavernous nerve crush injury model in rats to examine the potential of using HTSCs in a clinically relevant cell-based therapy. HTSCs or BM-MSCs were periprostatically injected into rats where the cavernous nerve had

been damaged on both sides. Functional recovery of the crush injury area, systemic MAP and ICP were evaluated to compare the therapeutic effects of HTSCs to those of BM-MSCs (Fig. 6C, D). The mean ICP/MAP ratios were significantly lower in the injury group (0.20 ± 0.01) compared to the sham group (0.70 ± 0.02) ($P < 0.001$) (Supplementary Table S3 and Fig. 6E). Periprostatic injection of either BM-MSCs or HTSCs resulted in a significant increase in the ICP/MAP ratio compared to the injury group ($P < 0.001$). Upon stimulation of the distal portion of the cavernous nerve, partial recovery of the erectile response was observed in both the BM-MSC (0.44 ± 0.05) and HTSC (0.44 ± 0.07) groups without any significant difference between the two groups. In the prostates of the treated animals, Stem-121TM (FITC, green color, arrow-head) and TuJ1 (Cy5, purple color, arrow) double-positive cells were found in the crush injury area and some Cell-TrackerTM CM-DiI (red color, open arrow heads) was observed in the area of double-positive cells. Thus, the injected HTSCs could differentiate and integrate into neurons (Fig. 6F).

Discussion

In this study, we isolated and characterized HTSCs from human testis tissue. While Kanatsu-Shinohara et al. (2004) clearly showed that SSCs can be used as a source of culture-derived multipotent stem cells [29], no other studies have convincingly reproduced these results, particularly in human counterparts [26,41–43]. Moreover, most of these isolated human cells were thought to originate from SSCs, although their exact origins have been controversial [26,44–46]. Taken together, all of the designated testicular stem cells used in previous studies appear to have originated from testicular germ cells and not from somatic cells. In addition, putative novel mesenchymal and pluripotent stem cells, which form clusters and demonstrate differentiation potential into mesenchymal or neuronal lineages, have been recently isolated in the testis of NOA patients [47]. These cells exhibit ESC-like colony morphology and have different characteristics from human HTSCs in terms of stemness gene expression. Although these HTSCs exhibit a similar morphology and characteristics to human testicular MSC-like cells [30] and BM-MSCs [48], these HTSCs are different from other known MSCs or MSC-like cells in that they initially coexpress both CD34 and CD73. This is in contrast to the classical definition of MSCs, which are characterized by the presence of CD73, CD90, and CD105 membrane antigens, but lack the expression of other cell marker genes (CD34 and CD45) [49]. Thus, these HTSCs are novel stem/progenitor cells from human testis somatic cells. Indeed, the CD34⁺/CD73⁺ cells were rarely found in vivo (Supplementary Fig. S1) because they constituted only 0.03% of the initially CD73-sorted TSC population (Fig. 1B). In addition, the lack of CD31⁺/CD34⁺ cells in the testis and the lack of any CD73 expression in the basal lamina of the seminiferous tubules (Supplementary Fig. S1) suggests that CD34⁺/CD73⁺ cells (HTSCs) existed in peritubular or interstitial cells and could be mesenchymal or precursor cells for the peritubular or interstitial cells. However, further investigation is required to avoid the potential for culture artifact due to the low number of CD34⁺/CD73⁺ cells in the testis histology.

Although it is considered a hallmark of hematopoietic stem cells, CD34 is expressed in a wide variety of

FIG. 5. In vitro differentiation potential of BM-MSCs and highly proliferative testis-derived stem cells (HTSCs) into insulin-secreting cells. **(A, B)** After culture in various differentiation conditions, an enzyme-linked immunosorbent assay was performed to measure the secretion of insulin and C-peptide. **(C)** Insulin and NGN-3 gene expression were compared between insulinogenic cells derived from HTSCs and BM-MSCs. **(D)** Immunocytochemistry for C-peptide (FITC) and insulin (Cy3) in differentiated and undifferentiated cells. DAPI nuclear staining is shown in blue. FITC staining is shown in green. Scale bars indicate 10 μ m. Color images available online at www.liebertpub.com/scd

FIG. 6. (A) In vitro differentiation potential of bone marrow-derived mesenchymal stem cells (BM-MSCs) and highly proliferative testis-derived stem cells (HTSCs) into neuronal-lineage cells. (A) Nestin was expressed in differentiated neuronal cells from BM-MSCs and HTSCs ($\times 200$). (B) GFAP and β -Tubulin 3 gene expression in cells subjected to neurogenic differentiation in induced HTSCs compared to BM-MSCs. (C) Assessment of blood pressure by cavernous nerve electrostimulation proximal to the site of injury in a rat. The corpus cavernosum was cannulated using a 24-gauge needle. (D) Measurement of erectile function. The red, blue, and green curves represent the mean arterial pressure (MAP), intracavernous pressure (IAP), and ICP/MAP ratio, respectively, in response to nerve stimulation. (E) Injection of BM-MSCs or HTSCs increased the ICP/MAP ratio compared with injury alone. (F) Localization of TuJ1 (red, arrow) and human cell-specific antibody Stem 121 (green, white arrowhead). DAPI nuclear staining is shown in blue; CellTracker is also shown in red (open arrowheads). Scale bars indicate 100 μ m. Color images available online at www.liebertpub.com/scd

nonhematopoietic tissues and cells, such as vascular endothelial cells and soft tissue neoplasms [50,51]. In human adipose-derived stem cells, CD34 expression is detected, but decreases over time in culture, which may be related to their replicative capacity, differentiation potential, and immaturity or stemness of the cells [15]. To isolate stem cells with regenerative potential from human testicular biopsied tissue, we used CD73 as an additional selection marker combined with CD34 because CD73 is constitutively expressed in various MSCs [23].

CD34⁺/CD73⁺ HTSCs displayed a higher proliferative capacity compared to CD34⁻/CD73⁺ TSCs. An extremely small number of HTSCs were able to proliferate and expand into a remarkably large population after culturing for more than 25–36 passages. The capability of these cells to proliferate and differentiate was strongly related to their CD34 level. As shown in Figs. 2 and 7, CD34 expression was inversely related to the differentiation state of these cells. As the cells differentiated toward a specific cell type, CD34 expression decreased, indicating that CD34 is a stemness/juvenility marker of this type of stem cell. In addition, similar to the adipose-derived stem cells previously described, the proportion of HTSCs that were CD34⁺ cells and their proliferative potential also declined with successive passages. In contrast, the expression of CD73 was not affected by the duration of culture; it showed consistently high expression over more than 30 passages (Fig. 2C). CD34 resorting helped to extend the proliferative capacity of the culture and also improved the differentiation potential. There was no clear explanation for this; however, one potential explanation could be CD73 induced differentiation during expansion of the cell colonies. Although the cell number was initially small, the number of cells and the sizes of their colonies had increased as the culture progressed, which potentially caused the cells located in the middle of the colonies to encounter hypoxia. Hypoxia is a major regulator of CD73 [52–54], and hypoxic conditions can stimulate the CD73 kinase activity, thereby initiating a cascade of events that eventually results in cell differentiation. This process occurs during the differentiation of human MSCs and adipose-derived stem cells into osteogenic and adipogenic lineages [55,56]. Because signal transduction occurs in a cell-autonomous manner, only cells located in the middle of colonies may initially differentiate. However, as the culture period continues, CD73 signaling predominates, and a growing number of cells begin to differentiate and lose CD34 expression. Resorting for CD34 eliminates CD34⁻/CD73⁺ cells, and only CD34⁺/CD73⁺ cells remain. Because the differentiation of the HTSCs into specific cell types causes the downregulation of CD34, and long-term cultures usually cause senescence and differentiation, the extent of CD34 expression on the testis stromal cells may be a useful selection marker for young and healthy stem cells. Furthermore, because of the close relationships between CD34 expression and cell stemness and longevity, it would be of great interest to determine if overexpression or prolonged expression of CD34 in testis stromal cells can increase the lifespan and plasticity of the cells.

The HTSCs demonstrated common characteristics of MSCs, but not of ESCs (Fig. 3). Although a small subset of genes was analyzed, we directly compared the differentiation potential of various types of stem cells: ESCs, BM-MSCs

at p3, TSCs at p5, and HTSCs at p5. In *in vitro* and *in vivo* differentiation studies, HTSCs differentiated into adipogenic, osteogenic, and chondrogenic cells in a manner comparable to that of BM-MSCs (Fig. 4). The HTSCs also differentiated into neurogenic cells and insulin-secreting cells *in vitro* when specific protocols were applied [32,40,57] (Figs. 5 and 6). Although the expression of neuronal-specific genes and antibodies has been shown in neurogenic cells derived from HTSCs *in vitro*, the action potentials of a functional neuron have not been detected in these cells (Supplementary Figs. S7 and S8). However, in the present study, some of the differentiated cells showed expression of the Na channel, which is a marker of an early neuron, and suggest that HTSCs could differentiate into early neuronal lineages. Thus, their differentiation potential into functional neurons should be confirmed in future studies using more effective differentiation conditions.

In the *in vivo* cell transplantation study, undifferentiated HTSCs supported recovery from a bilateral cavernous nerve crush injury and recovery of blood flow in an injured rat model after 4 weeks of cell injections (Fig. 6C–E). Radical prostatectomy for prostate cancer often results in erectile dysfunction caused by damage to the neurovascular bundle (cavernous nerve), which resides along the posterolateral region of the prostate. We found that periprostatic injection of HTSCs or BM-MSCs around the crush injury area improved the erectile function. In addition, we found exogenous HTSC-derived neurons inside of the cavernous nerve in these rats (Fig. 6E, F) and observed functional improvement of blood pressure. This improvement might be mediated by an elevation of adenosine induced by CD73 ecto-5'-nucleotidase activity, as revealed in the studies of CD73-knockout mice and adenosine A (2B) receptor-knockout mice [58,59]. Moreover, BM-MSCs, which were used as controls for HTSCs, have beneficial effects on the erectile function in diabetic rats and increased the content of the endothelium and smooth muscle in the corpus cavernosum after an intracavernous transplantation [60]. In the present study, after HTSC injection, some neurons (TuJ1-positive cells) around the injury site were positive for a human cell prestained marker (CellTracker™ CM-Dil) and a human-specific antibody, indicating that the injected HTSCs have the potential to differentiate into neuronal cells after transplantation (Fig. 6F). Thus, this method may be useful to obtain, expand, and store autologous stem cells (HTSCs) from testis biopsies for the treatment of erectile dysfunction in patients diagnosed with prostate cancer. Furthermore, a small volume of testicular material may also be easily biopsied under local anesthesia in these patients. Recently, a number of fertility clinics have routinely used TESE or testicular biopsy to achieve a pregnancy from azoospermic or severe male factor-infertile patients. After fertility treatment, the remaining testicular materials were discarded. This technology may be very useful for these patients and does not require an unnecessary operation.

The testes contain not only germ cells, but also different types of culture-induced pluripotent stem cells, as revealed by the presence of multipotent SSCs [27–29]. This latter type of stem cell can be used for patient-specific cell therapy without significant ethical issues [26]. However, the efficiency of establishing stem cell lines is still extremely low, and cell lines that have been isolated have not been well

characterized in humans [46]. Moreover, the stem cell lines established using spermatogonial germ cells developed into tumors after injection into NOD-SCID mice [28], casting doubt on their clinical use in human medicine. In the present study, we have efficiently established multipotent stem cell lines (100% isolation rate) using defined culture conditions and a simple MACS system from all donors with normal (2 donors with obstructive azoospermia) and abnormal testis physiology (13 donors with nonobstructive azoospermia without germ cells). One drawback may be that magnetic bead separation is messy and does not always provide a clean separation of positive and negative cells because the cells can be separated on the basis of differences in the surface antigen composition. In addition, it is not easily compatible with multiparameter sorting. In contrast, flow cytometric analysis can separate the cells despite the lack of difference in the composition of their surface antigens, and support a more accurate and efficient tool for cell sorting [61]. However, the cells should be fluorescently labeled before FACS analysis, which may affect long-term culture due to toxicity via fluorescence bleaching. In addition, MACS instruments are not expensive compared to a FACS instrument, and thus, the magnetic bead separation technology was selected to sort the CD34⁺/CD73⁺ cells. However, for more accurate characteristics of HTSCs, the sorted cells were reanalyzed using flow cytometric analysis in the present study.

Typically, human adult stem cells can only replicate a limited number of times and enter the senescence phase shortly after isolation and in vitro expansion. In addition, human adult stem cells have a high incidence of chromosomal abnormalities during in vitro expansion. However, it is still controversial whether chromosomal aneuploidy is associated with neoplastic proliferation [62,63], although genetic instability in human tumors usually involves chromosomal alteration. In the present study, none of the tested cell lines developed tumors in immunodeficient mice, and the chromosomal integrity was maintained up to passage 30, indicating that these cell lines were safe alternatives to human ESCs. Moreover, because there was no difference between the isolated HTSCs from these two types of male donors with regard to the differentiation efficiency, the methods we described may be applicable to all male patients.

In summary, we found that CD34⁺/CD73⁺ expression in human testis stromal cells was positively correlated with its proliferative capacity, differentiation potential, and juvenescence or stemness. Thus, CD34 and CD73 can be used as initial selection markers to obtain highly proliferative adult stem cells from a simple testis biopsy. Utilization of these cells may permit patient-specific cell-based therapies without concern for tumor development or ethical controversy, which are current issues plaguing the use of human ESCs. In addition, because of their high proliferation capacity, CD34⁺/CD73⁺ HTSCs may be particularly useful in therapies requiring a large number of cells.

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No competing financial interests exist.

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