Pluripotent Stem Cells Derived From Adult Human Testes

Nady Golestaneh,^{1,2} Maria Kokkinaki,^{1,2} Disha Pant,^{1,2} Jiji Jiang,^{1,2} David DeStefano,³ Carlos Fernandez-Bueno,³ Janice D. Rone,^{2,4} Bassem R. Haddad,^{2,4} G. Ian Gallicano,^{1,2} and Martin Dym^{1,2}

Recent reports have demonstrated that adult tissue cells can be induced to pluripotency, the iPS cells, mostly with the addition of genes delivered using viruses. Also, several publications both in mouse and in human have demonstrated that spermatogonial stem cells (SSCs) from testes can convert back to embryonic stem (ES)-like cells without the addition of genes. Furthermore, these pluripotent ES-like cells can differentiate into all three germ layers and organ lineages. Thus, SSCs have great potential for cell-based, autologous organ regeneration therapy for various diseases. We obtained testes from organ donors and using 1 g pieces of tissue (biopsy size) we demonstrate that testis germ cells (putative SSCs and/or their progenitors) reprogram to pluripotency when removed from their stem cell niche and when appropriate growth factors and reagents in embryonic stem cell medium are added. In addition, our method of obtaining pluripotent ES-like cells from germ cells is simpler than the described methods and may be more suitable if this procedure is developed for the clinic to obtain pluripotent cells to cure disease.

Introduction

SPERMATOGENESIS IS THE PROCESS by which spermatogonial stem cells (SSCs) in the testis divide and differentiate to produce sperm. The SSCs are a type of “immortal” stem cell since the sperm transmit genetic information from generation to generation [1]. SSCs reside on the basement membrane of the seminiferous tubules and are surrounded by Sertoli cells that help form their niche. The Sertoli cells support and regulate the fate of SSCs to self-renew or to undergo differentiation by secreting growth factors and other extracellular signals. The differentiation process of spermatogenesis results in the production of sperm after ~35 days in the mouse and 64 days in the human [2]. In culture, primordial germ cells derived from embryonic tissues in mouse and human are able to form pluripotent embryonic germ (EG) cells [3–5]. Human EG cells are believed to have potential for treatment of human disease but access to human embryos on a regular basis for cell-based therapy is likely not to be feasible. SSCs from neonatal mouse testis can also form embryonic stem (ES)-like cells that are pluripotent similar to EG cells [6]. Long-term proliferation in culture and germ-line transmission of male SSCs have been established successfully in the mouse [7]. Due to the ethical problems

related to obtaining human ES cells, there is great interest in using human adult stem cells as a source of cell-based therapy.

Adult SSCs in their normal niche are unipotent, capable of only producing sperm. Recently, two separate laboratories have shown that the ES-like cells derived from SSCs and/or their progenitors from adult mouse testis are pluripotent and able to spontaneously differentiate into derivatives of the three EG layers in vitro [8,9]. The SSCs and/or their progenitors were removed from their niche prior to their reprogramming to ES-like cells in defined media. While the full potential of these ES-derived germ cells and gametes remains to be demonstrated, these discoveries provide a new approach for studying reproductive biology and medicine. Two recent papers demonstrated the generation of pluripotent stem cells from biopsies of human testes [10,11]. Within the past 2 years, induction of pluripotent stem (iPS) cells from mouse adult skin fibroblast cultures was established by introducing four transcription factors (*Oct3/4*, *Sox2*, *c-Myc*, and *Klf4*) with retroviral vectors [12,13]. This novel work with somatic skin cells was reproduced in humans independently by several laboratories [14–16]. iPS cells were also generated from adult mouse liver and

¹Department of Biochemistry and Molecular and Cellular Biology, ²Lombardi Comprehensive Cancer Center, and ⁴Departments of Oncology and Obstetrics and Gynecology, Georgetown University Medical Center, Washington, DC.

³Washington Regional Transplant Community, Annandale Virginia.

stomach epithelial cells, but again the same four transcription factors were introduced into the cells [17]. Indeed, more recent efforts have produced iPS cells from neural stem cells using only two factors, *Oct3/4* and either *c-Myc* or *Klf4* [18], and then finally only with one factor, *Oct3/4* [19]. The addition of genes, some potentially cancer-causing, and the use of retroviruses as a delivery mechanism preclude these pluripotent stem cells from use as therapeutic agents. Another recent report showed that pluripotent stem cells can be induced from fibroblasts and liver cells without the use of integrating viruses [20]; however, the fact that virus still had to be used to introduce the exogenous genes into the cells could still be problematic as it could not be ruled out that small pieces of adenoviral DNA may have inserted into the genome of adeno-iPS cells but remained undetected due to the detection limits of Southern blot analysis [20].

Using human organ donors as the source of testicular material, we now independently corroborate the two recently published reports showing pluripotent stem cells can arise from human testes [10,11], and we provide novel data that strengthens the argument for carrying out further research on pluripotent ES-like cells derived from adult human testes. Our work also emphasizes the importance of gaining a full understanding of the SSCs in man.

Materials and Methods

Isolation of germ cells from adult human testes

We obtained human testes from organ donors to the Washington Regional Transplant Consortium. Patients were brain dead with a beating heart and assisted ventilation. The abdomen was opened and the abdominal organs were carefully dissected away from the posterior abdominal wall. The aorta was then clamped and perfused immediately after clamping with Viaspan, a standard organ preservation solution. Viaspan entered all the abdominal organs as well as the testes. The testes and the other organs were retrieved after perfusion for 10 min and packed in cold Viaspan. The testes were sent to the Georgetown University Medical Center by courier within 3 h after death. The results presented in this article are based on 1 g pieces of tissue obtained from eight testes from four donors, ages 16, 34, 41, and 52 years.

The testes were collected aseptically in serum-free Dulbecco's modified Eagle's Medium (DMEM, high glucose formulation). After decapsulation of the testes, interstitial cells and blood vessels were removed by mechanical agitation and washing after the first enzymatic digestion with collagenase IV (Sigma, St. Louis, MO) and DNase I (Sigma) at the concentration of 1 mg/mL and 2 µg/µL, respectively. The isolated seminiferous tubules were further digested with collagenase IV, hyaluronidase (Sigma), and trypsin (Sigma) at the concentration of 1, 1.5, and 1 mg/mL, respectively, to obtain individual cells.

Culture of adult human germ cells: dedifferentiation to ES-like cells

Human germ cells were cultured in gelatin-coated six-well plate dishes in DMEM high glucose (Gibco, Grand Island, NY) medium containing 15% knockout serum replacement for ES cells (Gibco), 2 mM l-glutamine (Gibco), 0.1 µM β-mercaptoethanol (Sigma), 100× nonessential amino

acids (Gibco), 1× penicillin–streptomycin (Gibco), 10 ng/mL bFGF (BD Biosciences, Bedford, MA), and 0.12 ng/mL TGF-β (BD Biosciences). After 4 days in culture, small ES cell-like colonies formed. We mechanically removed the colonies, trypsinized them by trypsin/EDTA (Gibco), and cultured them in a new dish once a week. This was repeated weekly for up to 10 weeks and beyond. From ~1 g of testis tissue, we get ~5 × 10⁶ germ cells. After 4 weeks (one passage per week), we obtain about 500 ES cell-like colonies. Each colony has ~500 cells. Thus, we get about 250,000 ES-like cells from 1 g of tissue. We now freeze 1 g pieces of testis tissue and then thaw and prepare ES-like colonies. The yield of the ES-like colonies from the frozen testis pieces is ~30% lower than that from the fresh tissue. This is still a sufficient number of cells for experiments. In our experiments, early passage means passages 1 to 4; late passage means passages 5 and beyond.

In vitro differentiation of ES-like colonies: Hanging drop method

For differentiation into endodermal, mesodermal, and ectodermal lineages, typical protocols for human ES cells were used. Briefly, for initial differentiation procedures, ES-like cell colonies were trypsinized and cultured in hanging drops to form embryoid bodies (EBs) in DMEM high glucose supplemented with 20% fetal calf serum (FCS), 2 mM l-glutamine, 1× nonessential amino acids, 1× penicillin–streptomycin, and 0.1 µM β-mercaptoethanol as described for standard human ES cell differentiation.

Endodermal lineage islet-like cells. Following a slightly modified previously described protocol [21], EBs were transferred from hanging drops after 2 days to suspension culture in bacteriological Petri dishes containing differentiation media for 5 days (seven differentiation days total). EBs were then left in differentiation medium (controls) or transferred to islet cell-inducing medium by plating in a six-well dish containing DMEM/F12 supplemented with insulin–transferrin–selenium G, fibronectin (5 µg/mL), and glutamine (1 mM). After 7 days, the cells were dissociated into single cells and plated onto round coverslips in 24-well plates coated with gelatin (0.1%) in DMEM/F12 supplemented with N-2, B-27, glutamine (1 mM), and bFGF (10 ng/mL) for 7 days. Cell clusters that formed were then cultured in DMEM/F12 supplemented with glucose (90 mg/mL), N-2 supplement, B-27 serum-free supplement, glutamine (1 mM), and nicotinamide (10 mM) for 12 h. Coverslips were then processed for immunofluorescence using antibodies to insulin (Cell Signaling Inc. Danvers, MA) using an Olympus 500 confocal microscope.

Mesodermal cardiac cells. Following a modified protocol described previously [22], EBs were transferred from hanging drops after 2 days to suspension culture in bacteriological Petri dishes containing differentiation media for 5 days (seven differentiation days total). At day 7, EBs were transferred onto coverslips and placed into individual wells of a 24-well plate. The 250 nM cardiogenol-C was added for 10 days to assist differentiation down the cardiac pathway, followed by incubation for eight more days (25 differentiation days total) in DMEM + 20% FBS. EBs were then processed for confocal microscopy.

Ectodermal neural lineage. Following a modified protocol described previously [23], after 2 days in hanging drops,

EBs were placed in suspension culture in bacteriological Petri dishes containing DMEM/F12 and 15% FCS, 4 ng/mL human recombinant basic fibroblast growth factor (bFGF; Gibco-Invitrogen, Carlsbad, CA), 3.5 μ L/500 mL β -mercaptoethanol (Sigma-Aldrich, St. Louis, MO), and 10 μ M all-trans-retinoic acid (RA) (Sigma-Aldrich). The differentiating EBs were then placed in 24-well plates in the presence of DMEM/F12, supplemented with 15% of FCS, 4 ng/mL bFGF, 20 ng/mL EGF (Sigma-Aldrich), and the RA was omitted from the culture 2 days after plating. The cells were cultured for an additional 2 weeks and the neuronal differentiation was verified by RT-PCR and confocal microscopy.

Immunostaining

Alkaline phosphatase (AP) staining procedure. The undifferentiated state of ES cells is commonly characterized by high level of expression of alkaline phosphatase. We used the Chemicon Alkaline Phosphatase Detection Kit (Chemicon, Billerica, MA) and followed the manufacturer's instructions. Briefly, the ES-like colonies were cultured for 2 weeks prior to analyzing AP activity, the colonies were then fixed with 4% paraformaldehyde in PBS for 1–2 min, followed by washing three times with PBS and processing for AP, and then examined using a microscope equipped with phase or DIC optics. We did not see any AP staining in human germ cells, the starting material, and human foreskin fibroblasts used as negative controls (Supplementary Fig. 1). (supplementary figure is available online at <http://www.liebertpub.com>)

Immunofluorescence. Human germ cells isolated from testes were subjected to specific immunostaining with different anti-human antibodies. Anti-GFR α 1 (R&D Systems, Minneapolis, MN), anti-VASA (R&D Systems), and anti-GPR-125 (Abcam #ab51705, Cambridge, MA) were used to stain freshly isolated spermatogonia. Human ES-like colonies and differentiated EBs were subjected to specific immunostaining with different anti-human antibodies. POU5F1 (previously known as OCT3/4) (Santa Cruz Biotechnology, Santa Cruz, CA), Nanog (R&D Systems, Minneapolis, MN), SSEA-4 (R&D Systems, Minneapolis, MN), TRA-1-81 and TRA-1-60 (Abcam, Cambridge, MA) were used to immunostain the ES-like colonies. Cardiac α -actin (ACTC1, ARP, Belmont, MA), smooth muscle actin (ACTA2, Abcam), nestin (NES, BD Bioscience, Bedford, MA), cardiac troponin C (TNNC1, Santa Cruz Biotechnology, Santa Cruz, CA), and neurofilament polypeptide 160 kDa (NF-M, Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were used to immunostain the differentiated EBs. Cells were fixed with 4% paraformaldehyde for 5 min, permeabilized with PBS containing 2% BSA and 0.1% Triton X100, and then incubated with 1/100 of each antibody for 1 h at room temperature. Cells were washed and incubated with secondary FITC- or TRITC-conjugated antibodies (Jackson ImmunoResearch, West Grove, PA) for 1 h and washed four times with PBS/BSA1%. DAPI (4',6'-diamidino-2-phenylindole) was used to stain the cell nuclei. Cells were analyzed with Olympus Fluoview 500 Laser Scanning Microscope (Olympus, Melville, NY). Immunostaining of the human germ cells and human foreskin fibroblasts used as negative controls did not reveal any staining with these antibodies (data not shown). Omitting the first antibody and staining with the secondary antibody was also performed as control and did not show any staining (data not shown).

RT-PCR/semiquantitative RT-PCR analysis

Human ES-like colonies cultured in dedifferentiation media and human differentiating EBs were collected for total RNA isolation according to the manufacturer's protocol (Promega, Madison, WI). The 100 ng of DNase-treated RNA was used for first-strand cDNA synthesis. Two microliters of the cDNA reaction was used and amplified for 25–30 cycles. B-actin was used as an internal control. Three negative controls were performed for each RT-PCR to exclude the possibility of genomic DNA contamination: (1) no cDNA (water), (2) no RT enzyme was used, and (3) all primers were designed to anneal with the regions of mRNA that would span at least one intron in the genomic sequence.

Semiquantitative RT-PCR was performed by measuring the level of amplicon saturation with respect to the number of PCR cycles. For example, if 25, 30, 35, and 40 cycles were used to amplify *GAPDH* and the 35 and 40 cycle lanes were of equal intensities, then RT-PCR for each germ layer was amplified for 30 cycles. The intensity of each band could then be compared semiquantitatively as we have done previously [24].

Telomerase activity

Telomerase activity was measured by telomeric repeat amplification protocol (TRAP) assay using the Quantitative Detection Kit (QTD) (Enzyme, Gaithersburg, MD), especially designed for real-time PCR detection of telomerase activity. Telomerase from the cell extracts adds telomeric repeats onto a substrate oligonucleotide and the resulting extended products are subsequently amplified by PCR. The PCR products are then labelled using SYBR Green and measured by monitoring the increase in fluorescence. Cell lysates were prepared. Equal amounts of protein (0.35 μ g) were analyzed for each telomeric repeat amplification protocol (TRAP) assay. Reactions were done in triplicate with a negative control for each sample and consisted of heat inactivation for 10 min at 85°C prior to the telomerase activity assay. Control template standard curve was derived by using TSR, an oligonucleotide with a sequence identical to the telomere primers.

Chromosome analysis

The cells were grown in human ES cell media and chromosomes were harvested following an overnight incubation with colcemid (10 μ L/mL). Standard G-banding procedures were used to verify the chromosome identity of the ES-like cells isolated from the adult human testes [25,26].

Teratoma formation

SSC-derived human ES-like colonies or mouse ES (mES) colonies were grown in ES media for at least 14 days and dispersed into single cells by incubation in 0.25% trypsin for 2–5 min at 37°C. Nude mice were injected subcutaneously using a 30-gauge needle with 10,000, 750,000, 1×10^6 , and 2×10^6 ES-like cells, respectively, in triplicate and on separate days. The left hindquarter of each mouse received mES cells, while the right hindquarter received human ES-like cells. Tumor formation was identified by manually palpating the injected area beginning 7 days after injection. After 28 days of growth, tissue was removed, fixed in 4% formaldehyde, and processed for paraffin sectioning.

Results and Discussion

Dedifferentiation of spermatogonial stem cells and/or progenitors

We tested our hypothesis that under specific culture conditions human germ cells (putative SSCs and/or their progenitors) are pluripotent and are capable of dedifferentiating into ES-like cells. To this end, we established a protocol to isolate seminiferous tubules devoid of interstitial components from adult human testes by sequential enzyme dispersion, based on the literature [27,28] with some modifications. After further isolation, the male germ cells were obtained devoid of connective tissue cells and cultured in defined media designed for in vitro culture of human ES cells [29–33].

Culture systems for human ES cells have been investigated and a number of protocols including those in the absence of feeder layer cells have been established [31–33]. These systems present two advantages: first, the application of a well-defined protocol in order to maintain the human ES cells in an undifferentiated state, and second, the reduced exposure of human ES cells to animal pathogens, facilitating research practices and providing a safer alternative for future clinical applications [29,32]. Similarly, a well-defined culture medium is required for optimal derivation and maintenance of the SSCs and/or their progenitors in undifferentiated conditions and to induce their genetic reprogramming toward ES-like colonies.

After enzymatic dispersion, we obtained a germ-cell/somatic-cell mixture as shown in Figure 1A. In our culture conditions, the isolated germ cells first formed small colonies of cells (3–5 cells; Figure 1A inset). Differential plating removed most of the somatic cells and after a few days in culture many of the germ cells expressed GPR-125 (Fig. 1B) and GFR α 1 (Fig. 1C), markers found in SSCs. VASA, a germ-cell marker, was also expressed in the cells (Fig. 1D). After 7–10 days in culture, these colonies grew large enough (~100 cells; Fig. 2A, 2B) to isolate from the surrounding individual cells using 0.1–10 μ L pipette tips (cat# 53509132 VWR, Inc) attached to a 10 μ L pipetteman. Isolated colonies then formed packed ES cell-like colonies (Fig. 2C) after 4 weeks (one passage per week). We successfully expanded and passaged the colonies under human ES cell culture conditions for >20 passages (this is ongoing at one passage per week). The percent of cells in the human ES-like colonies after 4 weeks of culture was about 0.5 to 1% of the total germ cells in the starting material. The phenotype of these human ES-like colonies after 7–10 days (Fig. 2B) and 4 weeks (Fig. 2C) in human ES media resembled those of human ES cell colonies. Alkaline phosphatase (AP) was strongly expressed in the human ES-like colonies (Fig. 2D, 2E), whereas foreskin fibroblasts, used as a negative control, did not show any AP staining (Supplementary Fig. 1). (supplementary figure is available online at <http://www.liebertpub.com>) Human ES-like colonies also highly expressed the ES cell transcription factors, such as the POU domain containing class 5, transcription factor 1 (POU5F1) (Fig. 2F–2H) and NANOG (Fig. 2I–2K). The ES-like colonies were evaluated for the expression of known surface markers for undifferentiated human ES cells: stage-specific embryonic antigen-4 (SSEA-4), tumor rejection antigens *TRA-1-81* and *TRA-1-60* (Fig. 2L–2N) were detected in the human ES cell-like colonies. Except for low levels of SSEA-4, we did not find any staining for these markers in human germ

cells (the starting cell preparation) nor in human foreskin fibroblasts used as negative controls (Supplementary Figs. 2 and 3). (supplementary figure is available online at <http://www.liebertpub.com>) The ES-like undifferentiated colonies did not exhibit any staining for an irrelevant antibody, cardiac Troponin C (TNNC1) (Supplementary Fig. 4A and 4B), (supplementary figure is available online at <http://www.liebertpub.com>) whereas mouse-differentiated EBs stained positive for CtC (Supplementary Fig. 4C and 4D). (supplementary figure is available online at <http://www.liebertpub.com>) These data indicate that the human ES-like colonies express the surface markers and the transcription factors characteristic of undifferentiated human ES cells. To confirm our confocal data, we analyzed the human ES-like colonies using RT-PCR and showed expression of markers, such as *POU5F1*, *NANOG*, teratocarcinoma-derived growth factor-1 (*TDGF-1*), and *SOX2*, whereas human germ cells freshly isolated from the testes were negative for these markers (Fig. 3A). Furthermore, the expression of ES cell markers *ESG-1* and *THY-1* was up-regulated in the human ES-like colonies compared to the human-isolated germ cells (Fig. 3A). The negative control, human foreskin fibroblasts did not express any of these markers (Fig. 3A). Thus, the human ES-like colonies derived from testis germ cells present relatively high levels of human ES cell markers [34,35]. VASA, a germ-cell specific marker, was strongly expressed in human freshly isolated germ cells, but not in the human ES-like cells, demonstrating a change in gene expression in the human ES-like cells during dedifferentiation (Fig. 3B). VASA was not expressed in foreskin fibroblasts used as a negative control (Fig. 2B). *S100A4* (S100 calcium binding protein A4) was not expressed in human ES-like cells nor in human freshly isolated germ cells, but was present in human foreskin fibroblasts, excluding the possibility of fibroblast contamination in our isolated ES-like colonies (Fig. 3C). Since we used TGF- β in our culture system for the germ cells and putative humans SSCs (and/or their progenitors) to induce their reprogramming into pluripotency, the cells were examined for TGF- β R expression. Figure 3D shows the expression of TGF- β R1 in both human ES-like cells and human germ cells. β -Actin (*ACTB*) was used as an internal control, as shown in Figure 2E.

Telomerase activity

Telomerase activity was measured by the telomeric repeat amplification protocol (TRAP) assay [36]. Telomerase activity in the human ES-like colonies was comparable to mouse ES cells. We did not find significant differences ($P = 0.1178$) in telomerase activity between passages 4 and 10 (Fig. 3F), whereas the activity was absent in human foreskin fibroblasts (negative control, $P = 0.0006$). Interestingly, telomerase activity was significantly higher in the mouse CCE ES cell line compared to the mouse E14 ES cell line showing the variability of telomerase activity between two ES cell lines. These telomerase observations show that human ES-like cells present distinct characteristics of human and mouse ES cells.

Chromosome analysis

In order to verify the chromosome integrity of the spermatogonia/progenitor-derived ES-like cells isolated from the adult human testes, we conducted a karyotype analysis of the cells at passage 3. The ES-like cells were cultured and

chromosomes were prepared and G-banded using standard protocol. Chromosomes were identified and classified in accordance with the nomenclature proposed by the International System for Human Cytogenetic Nomenclature (ISCN) [25]. Analysis revealed cells with a 46,XY normal karyotype (Fig. 3G).

Differentiation of ES-like colonies

To induce the in vitro differentiation of human ES-like colonies, we applied the "hanging drop" method followed by suspension culture of EBs and culture plating used for ES-like cell differentiation [35]. Human ES-like colonies were able to aggregate and form EBs both in hanging drops and in suspension culture. In order to verify the induction of differentiation, gene expression was analyzed in EBs at day 20 of differentiation; there was an overall decrease in the expression of *POU5F1*, *NANOG*, *ESG-1*, *TDGF-1*, *SOX2* and *THY-1* as measured by semiquantitative RT-PCR (Fig. 4A). To verify the ability of the EBs to differentiate into derivatives of the three germ layers, we analyzed the expression of a series of specific genes and proteins during in vitro differentiation. The mesodermal lineage differentiation was confirmed by the expression of bone morphogenetic protein 4 (*BMP4*), myoglobin (*MB*), cardiac muscle-, skeletal muscle-, and vascular tissue-specific genes. Specifically, human-

FIG. 1. Stem cell characterization of the starting material. (A) Germ cells in human embryonic stem cell medium at Day 1 after isolation. Inset is a high magnification view showing the different cell types obtained. (B, C, D) A significant number of the cells stained for GPR-125, GFR α 1, and VASA. GPR-125 and GFR α 1 are spermatogonial stem cell markers. Human vasa protein is specifically expressed in the germ-cell lineage. Scale bar = 40 μ m for B–D and inset.

FIG. 2. Cellular characterization of human embryonic stem (ES)-like colonies. (A, B, C) Typical ES-like colonies isolated from the germ-cell mixture. (A, B) ES-like colonies after 7–10 days of culture in human ES medium. (C) ES-like colony at passage 4 (one passage per week). (D, E) Alkaline phosphatase (AP) staining at passages 1 and 4, respectively. (F–K) Immunostaining of the ES-like colonies with antibodies against POU5F1 (F–H) and NANOG (I–K). Arrows point to nuclei (DAPI) positive for POU5F1 (FITC). Arrowheads point to nuclei (DAPI) positive for NANOG (Texas Red). (L–N) Expression of human ES cell surface markers SSEA-4 (arrows in L and L'), TRA-1-81 (M), and TRA-1-60 (N) by immunostaining. The strands in M and N are typical for membrane staining in ES colonies (arrows). Scale bars in L to N = 20 μ m.

FIG. 3. Molecular characterization of human embryonic stem (ES)-like colonies. (A) RT-PCR analyses of transcription factors essential for undifferentiated cells in human ES-like colonies under dedifferentiation conditions compared to freshly isolated germ cells and human foreskin fibroblasts. Human ES-like colonies express *POU5F1*, *NANOG*, *ESG-1*, *TDGF-1*, *SOX2* and *THY-1*. (B) RT-PCR analyses of VASA (germ-cell marker), (C) *S100A4* (S100 calcium binding protein A4) and (D) *TGF-βR1* (TGF-β receptor 1) in human ES-like colonies, freshly isolated human germ cells and human foreskin fibroblasts. (E) β-Actin is used as an internal control. (F) Telomerase activity of human ES-like colonies. 1. Human foreskin fibroblasts. 2. Human ES-like colonies at passage 4. 3. Human ES cell-like colonies at passage 10. 4. Mouse E14 ES cells. 5. Mouse CCE ES cells. Telomerase activity was measured by the telomeric repeat amplification protocol assay using the Quantitative Detection Kit. Telomerase activity in ES-like colonies was comparable to mouse embryonic stem cells. There is no statistical difference between human ES-like colonies at passages 4 and 10. There is a large statistical difference between the mouse E14 ES cell line and the mouse CCE ES cell line. (G) This figure shows a representative cell with a normal 46,XY karyotype.

differentiated EBs at day 20 of differentiation expressed *BMP4* and myoglobin, which were totally absent in human germ cells and human foreskin fibroblasts (Fig. 4B, Panel 1). The cardiac transcription factors *GATA4*, *NKX2.5*, and *MEF2C* were expressed at day 20 of differentiation (Fig. 4B, Panel 2). We also found *MEF2C* in the initial germ-cell isolation; however, the isolated human ES-like colonies were all negative for *MEF2C*.

The skeletal muscle-specific transcription factor *MYOD1* was also expressed at day 20 of differentiation (Fig. 4B, Panel 3). The differentiation to smooth muscle and vascular endothelial cells was confirmed by the expression of smooth muscle α-actin (*ACTA2*), platelet-endothelial-cell adhesion molecule 1 (*PECAM1*), and Von Willebrand factor (*VWF*) (Fig. 4B, Panel 3). Again, isolated human ES-like colonies were negative for endodermally derived gene expression

demonstrating that removing colonies from the initial germ-cell isolation mixture is an important step for ensuring that the human ES-like colonies are devoid of contaminating cells from the enzymatically processed seminiferous tubules.

Neuroectodermal differentiation of the EBs was characterized by the expression of nestin (*NES*) (a marker of neural precursor), synaptophysin (*SYP*), and dopamine receptor 2 (*DRD2*), the marker of differentiated neurons (Fig. 4B, Panel 4). In addition, the expression of cytokeratin 10 (*KRT-10*) (expressed in keratinizing stratified epithelia) and the expression of *KRT-18* (a distinct marker of early endoderm) were observed in differentiating EBs (Fig. 4B, Panel 5).

Differentiation of the EBs into cells from each germ layer was readily observed 25 days after initiation of differentiation. Pancreatic endodermal sheets of cells derived from the EBs efficiently produce insulin in 28 out of 30 EBs tested

FIG. 4. Differentiation of human embryoid bodies (EBs) in vitro. **(A)** RT-PCR analyses were performed at Day 20 of human EB differentiation. Except for THY-1, all other tested transcription factors were absent in the EBs, confirming that during differentiation the embryonic stem-like cells have gradually lost their stemness. Compare with Figure 3A. **(B)** RT-PCR analyses were performed at Day 20 of human EB differentiation. Freshly isolated human germ cells and human foreskin fibroblasts were used as the negative control. Mesodermal Differentiation: **Panel 1.** Bone morphogenetic protein 4 (*BMP4*), myoglobin (*MB*). **Panel 2.** Cardiac transcription factors—*GATA4*, *NKX2.5*, and *MEF2C*. **Panel 3.** Skeletal muscle-specific transcription factor—*MYOD1*, Smooth muscle α -actin (*ACTA2*), platelet-endothelial-cell adhesion molecule 1 (*PECAM1*), and Von Willebrand factor (*VWF*). Ectodermal Differentiation: **Panel 4.** Neuroectodermal differentiation—nestin (*NES*, a marker of neural precursor); synaptophysin (*SYP*), and dopamine receptor 2 (*DRD2*), the marker of differentiated neurons. **Panel 5.** Cytokeratin 10 (*KRT-10*)—expressed in keratinizing stratified epithelia. *ACTB* served as control (right image). Endodermal Differentiation: **Panel 2.** *GATA4*. **Panel 5.** Cytokeratin 18 (*KRT-18*) is a marker of early endodermal differentiation. *ACTB* served as control (right image).

FIG. 5. Immunostaining of human embryoid bodies (EBs) and differentiated cells. (A, B, C) Immunostaining of human EBs at Day 15 of differentiation with an antibody against insulin. (A) Cytoplasmic insulin is noted in a rectangular sheet of cells (arrows). Inset shows a phase contract image of the sheet of cells (*). (B) Merged image with DAPI staining shows insulin in the cytoplasm and DAPI in the nucleus. (C) Control showing only DAPI staining. (D) Nestin immunostaining in differentiating cells at Day 20 of differentiation. (E) Neurofilament (NF-M) immunostaining in differentiating cells at Day 25 of differentiation. (F–H) Cardiac α -actin immunostaining in differentiating cells at Day 25 of differentiation. Arrowheads point to the striations typically noted in cultured cardiac muscle cells.

(Fig. 5A–5C). Neuroectoderm differentiation was demonstrated by immunostaining using antibodies against nestin (Fig. 5D) and neurofilament-M (Fig. 5E). Distinct areas of staining were observed in 10 out of 24 EBs. Cardiomyocyte differentiation within EBs was also readily detected using antibodies against cardiac α -actin in 100% of the EBs tested—striations typically noted in cultured cardiac muscle cells were readily observed (Fig. 5F–5H). We did not find any staining for these markers in human freshly isolated germ cells, nor in human foreskin fibroblasts used as negative controls (Supplementary Figs. 5 and 6). (supplementary figure is available online at <http://www.liebertpub.com>)

Human ES-like cells form teratomas

Nude mice were injected subcutaneously with 10,000, 750,000, 1×10^6 , and 2×10^6 ES-like cells, respectively, in triplicate. Only when we injected 2×10^6 cells, did small (5 mm $\times$ 3 mm $\times$ 3 mm) teratomas form after 1 month of growth (Fig. 6). In contrast, injection of 7.5×10^5 mouse ES cells resulted in large teratomas within 3 weeks. Interestingly, the teratomas reported [11] were only slightly larger than ours even though they injected 10 times more cells. The same group [11] also showed that for optimal teratoma growth, human ES-like cells should be grown in culture on specific substrates. It is enticing to speculate that while human ES-like cells are capable of forming each germ layer, in vitro, the risk of teratoma may be minimal. Kossack and colleagues also did not find large teratomas and speculated that human ES-like cells may not have reprogrammed sufficiently to produce teratomas [10]. This

finding is in contrast to human ES cells and iPS cells and could be important with respect to grafting differentiated cells in vivo.

Identity of the human germ cells that convert to ES-like cells

Very little information is known about human spermatogonial renewal mechanisms. Clermont and colleagues first characterized and identified the A_{dark} and the A_{pale} spermatogonia, and both of them are considered undifferentiated stem cells. They suggested that the A_{dark} spermatogonia were the reserve stem cells, only to be called upon after injury, while the A_{pale} spermatogonia were the normally renewing stem cells (Fig. 7) [2,37–39]. In this pattern of human spermatogonial renewal, the A_{pale} spermatogonia divide to give rise to new A_{pale} or to form the differentiated type B spermatogonia that further divide and differentiate to give rise to the primary spermatocytes and spermatids. This classification for human spermatogonia has been adopted by most researchers. Other classifications have been proposed by certain investigators including Ehmcke and colleagues [40]; nevertheless in 2009, >40 years after Clermont presented his initial scheme for human spermatogonial renewal, the identity of the true human SSC and the mechanisms of renewal still remain unknown. It is likely that the human SSC is a subpopulation of the A_{dark} and/or the A_{pale} spermatogonia and thus only a few SSCs will be found in each cross section of a seminiferous tubule.

It is tempting to speculate that SSCs and/or their progenitors are the cell type that dedifferentiates to pluripotency;

FIG. 6. Teratoma formation from the embryonic stem (ES)-like cells. (A) Low magnification view of an hematoxylin and eosin-stained, paraffin-embedded tumor section. (B) Higher magnification view clearly shows epithelial structures (asterisks) embedded within the tumor. (C) Small areas of striated muscle (asterisks) are evident representing mesodermal structures. (D) Areas containing smooth muscle are also present (asterisks). (E) Gland-like structures representing endoderm are highlighted by brackets. (F) Stratified epithelial tissue highlighted by brackets is also present representing ectodermal structures. (G) High magnification of a tumor section further reveals stratified epithelial tissue. Dotted line shows the basal layer of cells at right.

FIG. 7. A proposed scheme of stem cell renewal in the human testis. The A_{dark} spermatogonia are believed to be a reserve type of stem cells in the testis likely not involved in normal spermatogenesis, while the A_{pale} are the renewing stem cells. The A_{pale} can divide and give rise either to new A_{pale} or to the more differentiated type B spermatogonia, which then form the spermatocytes and the spermatids by further division and differentiation. Adapted from Clermont [39].

indeed in the previously published articles on this topic in mouse [8,9], each group suggested that it was the SSC that converted to pluripotency. However, definite evidence was not presented. In the first publication on mouse by Guan and colleagues [8], STRA-8 cells were isolated and presumed to be SSCs, but STRA-8 is not a specific marker for the SSC. In the second article [9], the Seandel and colleagues used isolated GPR-125 cells and converted these cells to pluripotency. GPR-125 appeared to be present in all spermatogonia in the mouse (A_{isolated} , A_{paired} , A_1 to A_4 , intermediate, and type B spermatogonia) and possibly early spermatocytes as well. In a review describing the mouse work, de Rooij and Mizrak concluded that various approaches appear to be suitable to obtain ES-like cells derived from SSCs [41]. They also noted that the purity of the SSCs may not be very important, nor the precise culture medium. In the human, testis cells with surface markers such as GFR α 1, CD90 (Thy-1), CD133, and CD49f (α 6-integrin) were used for derivation of the human ES-like colonies [11]; however, it is unlikely that all these products are specific stem cell markers or even specific spermatogonial markers. Indeed, CD49f staining in a seminiferous tubule shown in the Conrad article [11] indicates that this is not a marker for SSCs. The CD49f appears to be present in all cells along the basement membrane including all A_{dark} , A_{pale} , B spermatogonia, and possibly even spermatocytes and Sertoli cells. There are far too many stained cells for them to be classified as spermatogonia or SSCs (germline stem cells). Kossack and colleagues [10] used biopsies of testis tissue without purifying the SSCs by MACS. It is still not possible to state with certainty that it was the SSCs that converted to pluripotency. In our human work, we started with a mixture of the germ cells, but the cells that appeared to develop into the ES-like cells resembled spermatogonia morphologically and not spermatocytes or spermatids. Also

many of the cells in our starting material possessed the GFR α 1 receptor and GPR-125, putative human SSC markers. Further work is required to isolate and characterize human SSCs with specific cell surface markers and then attempt to dedifferentiate this specific population. It will also be necessary to prove that the cells in question that converted to ES-like cells are true SSCs and this can only be accomplished with the transplant assay as developed by Brinster and colleagues [42].

The terminology in the published reports on the conversion of SSCs to ES-like cells remains confusing. In their mouse work, Guan and colleagues named the cells multipotent adult germ-line stem cells (maGSCs), even though in their title they used the term "pluripotency," while Seandel and colleagues [9] adopted the term multipotent adult spermatogonial-derived stem cells (MASCs). In the Conrad article [11], the term "pluripotent human adult germ-line stem cells" was used to describe the ES-like cells that were derived from human spermatogonia. The Kossack group [10] used the term ES-like cells for the adult human pluripotent cells, as we did. In a review article on the topic De Rooij and Mizrak [41] used ES-like cells to describe the SSC/progenitor-derived pluripotent cells.

If this technique of obtaining ES-like cells from testis is to be used in the clinic for patient therapy, it is unlikely that SSC isolation will occur. After biopsy resection, isolated germ cells may be dedifferentiated directly from the biopsy material, as described here and in the report by Kossack [10]. Although normal biopsies are usually tiny in size, ~100 to 200 mg, when patient specific stem cells are prepared from testes it should be possible to take a 1 g biopsy. We are consistently using 1 g of testis tissue for our dedifferentiation experiments. We have established human ES-like cell lines after freezing the cells at passage 5 in liquid nitrogen followed by thawing and culturing them for an additional 20 passages; we continue to expand these lines.

Conclusions

The data from our studies show that human spermatogonia (SSCs and/or their progenitors) can be isolated and cultured in vitro in defined medium for several passages without losing their pluripotent characteristics. The human SSCs and/or their progenitors maintained under these conditions formed ES-like colonies that expressed AP, *POU5F1*, *NANOG*, *SSEA-4*, *TRA-1-81*, and *TRA-1-60* and showed high telomerase activity. Additionally, the formed EBs were able to differentiate into derivatives of various somatic cell lineages.

These observations are of paramount importance since the SSCs and/or their progenitors from human testicular biopsies could be used to establish pluripotent cells to create tissues for regenerative medicine, and for studying genetic diseases without the ethical problems associated with human ES cells. Furthermore, there is no requirement for the random incorporation of exogenous genes into the genome to reprogram the SSCs/progenitor cells to pluripotency, as is the case for iPS cells [12–15]. Also enticing is the fact that human ES-like cells may not form large teratomas as do human ES cells and iPS cells. Additional studies are required to delineate the growth factors indispensable for the long-term maintenance of human SSCs/progenitor cells in the undifferentiated state. In conclusion, there are now at least three methods to produce human pluripotent stem cells: the traditional method

using donated eggs, the reprogramming of adult somatic and/or stem cells, and the reprogramming of SSCs and/or their progenitors. Further research is required to assure the optimum value of the human ES-like cells derived from testes. But it is possible that in the future, male patients will be cured of disease with a biopsy of their own testis.

Acknowledgments

Supported in part by NIH grant HD033728 to Dr. Dym and an Intramural Research Grant awarded to Drs. Dym and Gallicano.

References

1. Dym M. (1994). Spermatogonial stem cells of the testis. *Proc Natl Acad Sci USA* 91:11287–11289.
2. Clermont Y. (1972). Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiol Rev* 52:198–236.
3. Shim SW, DW Han, JH Yang, BY Lee, SB Kim, H Shim and HT Lee. (2008). Derivation of embryonic germ cells from post migratory primordial germ cells, and methylation analysis of their imprinted genes by bisulfite genomic sequencing. *Mol Cells* 25:358–367.
4. Shambloot MJ, J Axelman, S Wang, EM Bugg, JW Littlefield, PJ Donovan, PD Blumenthal, GR Huggins and JD Gearhart. (1998). Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc Natl Acad Sci USA* 95:13726–13731.
5. Donovan PJ and MP de Miguel. (2003). Turning germ cells into stem cells. *Curr Opin Genet Dev* 13:463–471.
6. Kanatsu-Shinohara M, K Inoue, J Lee, M Yoshimoto, N Ogonuki, H Miki, S Baba, T Kato, Y Kazuki, S Toyokuni, M Toyoshima, O Niwa, M Oshimura, T Heike, T Nakahata, F Ishino, A Ogura and T Shinohara. (2004). Generation of pluripotent stem cells from neonatal mouse testis. *Cell* 119:1001–1012.
7. Kanatsu-Shinohara M and T Shinohara. (2007). Culture and genetic modification of mouse germline stem cells. *Ann NY Acad Sci* 1120:59–71.
8. Guan K, K Nayernia, LS Maier, S Wagner, R Dressel, JH Lee, J Nolte, F Wolf, M Li, W Engel and G Hasenfuss. (2006). Pluripotency of spermatogonial stem cells from adult mouse testis. *Nature* 440:1199–1203.
9. Seandel M, D James, SV Shmelkov, I Falciatori, J Kim, S Chavala, DS Scherr, F Zhang, R Torres, NW Gale, GD Yancopoulos, A Murphy, DM Valenzuela, RM Hobbs, PP Pandolfi and S Rafii. (2007). Generation of functional multipotent adult stem cells from GPR125+ germline progenitors. *Nature* 449:346–350.
10. Kossack N, J Meneses, S Shefi, HN Nguyen, S Chavez, C Nicholas, J Gromoll, PJ Turek and RA Reijo-Pera. (2009). Isolation and characterization of pluripotent human spermatogonial stem cell-derived cells. *Stem Cells* 27:138–149.
11. Conrad S, M Renninger, J Hennenlotter, T Wiesner, L Just, M Bonin, W Aicher, HJ Buhning, U Mattheus, A Mack, HJ Wagner, S Minger, M Matzkies, M Reppel, J Hescheler, KD Sievert, A Stenzl and T Skutella. (2008). Generation of pluripotent stem cells from adult human testis. *Nature* 456:344–349.
12. Takahashi K and S Yamanaka. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126:663–676.
13. Wernig M, A Meissner, R Foreman, T Brambrink, M Ku, K Hochedlinger, BE Bernstein and R Jaenisch. (2007). In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* 448:318–324.
14. Takahashi K, K Tanabe, M Ohnuki, M Narita, T Ichisaka, K Tomoda and S Yamanaka. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131:861–872.

15. Yu J, MA Vodyanik, K Smuga-Otto, J Antosiewicz-Bourget, JL Frane, S Tian, J Nie, GA Jonsdottir, V Ruotti, R Stewart, Slukvin, II and JA Thomson. (2007). Induced pluripotent stem cell lines derived from human somatic cells. *Science* 318:1917–1920.
16. Park IH, R Zhao, JA West, A Yabuuchi, H Huo, TA Ince, PH Lerou, MW Lensch and GQ Daley. (2008). Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* 451:141–146.
17. Aoi T, K Yae, M Nakagawa, T Ichisaka, K Okita, K Takahashi, T Chiba and S Yamanaka. (2008). Generation of pluripotent stem cells from adult mouse liver and stomach cells. *Science* 321:699–702.
18. Kim JB, H Zaehres, G Wu, L Gentile, K Ko, V Sebastiano, MJ Arauzo-Bravo, D Ruau, DW Han, M Zenke and HR Scholer. (2008). Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors. *Nature* 454:646–650.
19. Kim JK, V Sebastiano, G Wu, MJ Arauzo-Bravo, P Sasse, L Gentile, K Ko, D Ruau, M Ehrlich, D van den Boom, J Meyer, K Hübner, C Bernemann, C Ortmeir, M Zenke, BK Fleischmann, H Zaehres and HR Schöler. (2009). Oct4-induced pluripotency in adult neural stem cells. *Cell* 136:411–419.
20. Stadtfeld M, M Nagaya, J Utikal, G Weir and K Hochedlinger. (2008). Induced pluripotent stem cells generated without viral integration. *Science* 322:945–949.
21. Segev H, B Fishman, A Ziskind, M Shulman and J Itskovitz-Eldor. (2004). Differentiation of human embryonic stem cells into insulin-producing clusters. *Stem Cells* 22:265–274.
22. Wu X, S Ding, Q Ding, NS Gray and PG Schultz. (2004). Small molecules that induce cardiomyogenesis in embryonic stem cells. *J Am Chem Soc* 126:1590–1591.
23. Glaser T and O Brustle. (2005). Retinoic acid induction of ES-cell-derived neurons: the radial glia connection. *Trends Neurosci* 28:397–400.
24. Foshay K, G Rodriguez, B Hoel, J Narayan and GI Gallicano. (2005). JAK2/STAT3 directs cardiomyogenesis within murine embryonic stem cells in vitro. *Stem Cells* 23:530–543.
25. (2005). ICSN. *An international system for human cytogenetic nomenclature*. Mitelman, F (ed); S. Karger, Basel.
26. Barch MJ, T Knutsen, and JL Spurbeck. (1997). *The AGT Cytogenetics Laboratory Manual* (1998), 3rd ed, edited by Margaret J. Barch, Turid Knutsen, and Jack L. Spurbeck, 666 pp, Philadelphia, Lippincott-Raven Publishers.
27. Bellvé AR, JC Cavicchia, CF Millette, DA O'Brien, YM Bhatnagar and M Dym. (1977). Spermatogenic cells of the prepubertal mouse. Isolation and morphological characterization. *J Cell Biol* 74:68–85.
28. Dym M, MC Jia, G Dirami, JM Price, SJ Rabin, I Mocchetti and N Ravindranath. (1995). Expression of c-kit receptor and its autophosphorylation in immature rat type A spermatogonia. *Biol Reprod* 52:8–19.
29. Amit M, C Shariki, V Margulets and J Itskovitz-Eldor. (2004). Feeder layer- and serum-free culture of human embryonic stem cells. *Biol Reprod* 70:837–845.
30. Hong-mei P and C Gui-an. (2006). Serum-free medium cultivation to improve efficacy in establishment of human embryonic stem cell lines. *Hum Reprod* 21:217–222.
31. Levenstein ME, TELudwig, RHXu, RA Llanas, K VanDenHeuvel-Kramer, D Manning and JA Thomson. (2006). Basic fibroblast growth factor support of human embryonic stem cell self-renewal. *Stem Cells* 24:568–574.
32. Ludwig TE, V Bergendahl, ME Levenstein, J Yu, MD Probasco and JA Thomson. (2006). Feeder-independent culture of human embryonic stem cells. *Nat Methods* 3:637–646.
33. Ludwig TE, ME Levenstein, JM Jones, WT Berggren, ER Mitchen, JL Frane, LJ Crandall, CA Daigh, KR Conard, MS Piekarczyk, RA Llanas and JA Thomson. (2006). Derivation of human embryonic stem cells in defined conditions. *Nat Biotechnol* 24:185–187.
34. Thomson JA, J Itskovitz-Eldor, SS Shapiro, MA Waknitz, JJ Swiergiel, VS Marshall and JM Jones. (1998). Embryonic stem cell lines derived from human blastocysts. *Science* 282:1145–1147.
35. Reubinoff BE, MF Pera, CY Fong, A Trounson and A Bongso. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat Biotechnol* 18:399–404.
36. Kim NW, MA Piatyszek, KR Prowse, CB Harley, MD West, PL Ho, GM Coviello, WE Wright, SL Weinrich and JW Shay. (1994). Specific association of human telomerase activity with immortal cells and cancer. *Science* 266:2011–2015.
37. Clermont Y. (1963). The cycle of the seminiferous epithelium in man. *Am J Anat* 112:35–51.
38. Clermont Y. (1966). Spermatogenesis in man. A study of the spermatogonial population. *Fertil Steril* 17:705–721.
39. Clermont Y. (1966). Renewal of spermatogonia in man. *Am J Anat* 118:509–524.
40. Ehmcke J and S Schlatt. (2006). A revised model for spermatogonial expansion in man: lessons from non-human primates. *Reproduction* 132:673–680.
41. de Rooij DG and SC Mizrak. (2008). Deriving multipotent stem cells from mouse spermatogonial stem cells: a new tool for developmental and clinical research. *Development* 135:2207–2213.
42. Brinster RL. (2007). Male germline stem cells: from mice to men. *Science* 316:404–405.

Address correspondence to:

Dr. G. Ian Gallicano

Department of Biochemistry and Molecular and Cellular Biology

Georgetown University Medical Center

3900 Reservoir Road NW

Med/Dent NE203

Washington DC, 20057

E-mail: gig@georgetown.edu

Dr. Martin Dym

Department of Biochemistry and Molecular and Cellular Biology

Georgetown University Medical Center

3900 Reservoir Road, NW

Med/Dent NE209

Washington DC, 20057

E-mail: dymm@georgetown.edu

Received for publication November 5, 2008

Accepted after revision March 12, 2009

Prepublished on Liebert Instant Online March 12, 2009