Isolation of human testicular cells and co-culture with embryonic stem cells

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Abstract

Our overall goal is to create a three-dimensional human cell-based testicular model for toxicological and spermatogenesis studies. Methods to purify the major somatic testicular cells, namely Leydig cells (LCs), peritubular myoid cells (PCs) and Sertoli cells (SCs), from rats, mice and guinea pigs have been reported. In humans, the isolation of populations enriched for primary LCs, PCs or SCs also have described. One objective of this study was to determine if populations of cells enriched for all three of these cell types can be isolated from testes of single human donors, and we were successful in doing so from testes of three donors. Testes tissues were enzymatically digested, gravity sedimented and Percoll filtered to isolate populations enriched for LCs, PCs and SCs. LCs and PCs were identified by colorimetric detection of the expression of prototypical enzymes. Division of PCs and SCs in culture has been reported. We observed that primary human LCs could divide in culture by incorporation of 5-ethynyl-2'-deoxyuridine. SCs were identified and their functionality was demonstrated by the formation of tight junctions as shown by the expression of tight junction proteins, increased transepithelial electrical resistance, polarized secretion of biomolecules and inhibition of lucifer yellow penetration. Furthermore, we found that human SC feeder layers could facilitate germ cell progression of human embryonic stem cells (hESCs) by microarray analysis of gene expression.

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Introduction

The process of spermatogenesis whereby spermatogonial stem cells give rise to spermatozoa is a robust yet disruptable cellular transformation capable of producing about 1000 sperm per second in men. Human germ cells undergo mitosis (spermatogonial proliferation), meiosis (spermatocyte DNA recombination, reduction and division) and spermiogenesis (differentiation of spermatids) over a period averaging 64 days (Misell *et al.* 2006). The process is defined by intricate signaling and precise cell–cell interactions. Thus, we postulate that development of a robust *in vitro* spermatogenesis model requires a multicellular milieu to effectively represent the testicular stem cell niche (Oatley & Brinster 2012).

Here, we describe a protocol for the isolation of cell populations enriched for three major human testicular somatic cells, Leydig cells (LCs), peritubular myoid cells (PCs) and Sertoli cells (SCs) from testes of a single human donor. Previous studies have shown that human PCs proliferate *in vitro* (Schlatt *et al.* 1996,

Albrecht *et al.* 2006, Albrecht 2009). We have previously reported methodology that produces >95% pure populations of proliferative primary SCs from cadaveric testis (Chui *et al.* 2011, Xiao *et al.* 2014). Herein, we demonstrated the potential of LCs to divide in culture by evaluating 5-ethynyl-2'-deoxyuridine (EdU) incorporation into DNA along with immunochemical staining of LC markers. SCs can form tight junctions (TJs) that can be shown immunohistochemically and can be analyzed by measurement of transepithelial electrical resistance (TER) when the cells are grown on transwell (TW) inserts. Long-term culture of SCs *in vitro* could be necessary to achieve human spermatogenesis; therefore, we tested the functionality of the SCs as indicated by the maintenance of TER in a 3-dimensional TW model for an extended period of time. We cultured the human SCs in 24-well plates with TW inserts and observed the formation of TJs for more than a month.

There has been progress in generation of male germ cells from stem cells including pluripotent stem cells toward better understanding of male infertility

(Hou *et al.* 2014, Ishikura *et al.* 2016). In this study to further assess the functionality of the human SCs and the potential feasibility of *in vitro* human spermatogenesis, we compared germ cell differentiation of H1 human embryonic stem cells (hESCs) when cultured in primordial germ cell (PGC) medium supplemented with growth factors and hormones and growing adherently on either Matrigel (Corning) or on SC feeder layers. The feeder layers enhanced initial growth factor-induced germ cell differentiation, indicating that the SCs maintain functionality that may be essential for *in vitro* spermatogenesis (Huleihel *et al.* 2007). These data indicate that it could be feasible to develop a human multicellular *in vitro* model of spermatogenesis (Xiao *et al.* 2014) for reproductive toxicology.

Materials and methods

Procurement of cadaveric testes

Cadaveric testes from five deceased human males of 26–56 years of age were obtained within 36 h of death through protocols approved by the National Disease Research Interchange (www.ndriresource.org) in Philadelphia, PA, USA and the Donor Network West (www.donornetworkwest.org) in San Ramon, CA, USA. Donations were obtained with informed consent and were not accepted if there had been a history of testicular cancer. HIPAA regulations were followed for privacy protection.

Isolation of human SCs and PCs

The procedure was similar to that we previously reported for the isolation of SC-enriched populations of cells from testes of deceased human males between 12 and 36 years of age (Ketola *et al.* 2000, Chui *et al.* 2011) with modifications to enable simultaneous isolation of LCs and PCs (Lejeune *et al.* 1993, 1998, Cudicini *et al.* 1997). After washing each testis with ice-cold calcium/magnesium-free Hanks balanced salt

solution (HBSS; Cell Culture Facility, University of California, San Francisco, CA, USA) containing 100 U/mL penicillin and 100 µg/mL streptomycin (HBSS with P/S), the tunica albuginea was removed, the parenchyma was thoroughly minced, and then transferred to a beaker. Minced tissue was pooled, suspended in 100 mL HBSS with P/S and allowed to gravity sediment at room temperature (RT) for 15 min (Fig. 1). The supernatant was aspirated to remove germ cells and the gravity sedimentation step was repeated twice. After the final wash, HBSS with P/S was added in a 3:1 volume ratio (HBSS:tissue), and the tissue was shaken in a water bath incubator at 275–325 rpm at 37°C for 20 min. The supernatant was discarded, and 80 mL HBSS with P/S, 10 mL trypsin (2.5%), 10 mL collagenase Type IV (1%) and 2 mL DNase (1.0 mg/mL; all from Sigma-Aldrich) were added. The tissue was again shaken at 37°C but for only 5–15 min, after which the digested solution was passed through a metal sieve to remove connective and undigested tissue. Based on the total volume, 0.1% soybean trypsin inhibitor (stock: 0.34%; Sigma-Aldrich) was then added. The filtered solution was centrifuged at 200g for 5 min, and after discarding the supernatant, DMEM:F12 containing 100 U/mL penicillin and 100 µg/mL streptomycin (DMEM:F12 with P/S) was added in a 10:1 volume ratio (DMEM:tissue). The cell suspension was gravity sedimented at RT for 30 min, and the supernatant enriched for interstitial cells was set aside for further isolation of LCs using Percoll gradient centrifugation. Sedimented tubular fragments (~20–30 mL) were resuspended in 2 volumes of calcium and magnesium-free PBS (PBS-CMF) containing 1 M glycine and 2 mM EDTA (pH 7.4) and submitted to repeated pipetting for 10 min to remove PCs. Tubular fragments were isolated in two gravity sedimentations, and resulting supernatants enriched with PCs were pooled, centrifuged, plated and the cells were cultured in Minimum Essential Medium (MEM) with 10% fetal bovine serum (FBS) and P/S. The pellets enriched for tubular fragments and SCs were plated and the cells were cultured in DMEM:F12 with P/S and 10% FBS. Cell culture media were removed and fresh media was added every 2–3 days.

Figure 1 A schematic illustrating the methodology adapted from previous reports and used in isolation of cell populations (LCs, PCs and SCs) from human testis. Tissue was minced, shaken at 37°C and digested with enzymes. Several gravity sedimentation steps and a Percoll gradient were employed as illustrated.

Purification of human LCs on discontinuous Percoll gradient

The fraction enriched for LCs and interstitial cells was subjected to gravity sedimentation for 15 min. Then, LCs and interstitial cells were recovered from the supernatant by centrifugation (5 min, 200g), counted and a suspension of 10^8 cells in 5 mL of DMEM:F12 with P/S was applied to a 4-layer discontinuous Percoll (GE Healthcare) gradient of 21, 26, 34 and 60% in PBS-CMF. After centrifugation at 1500g for 30 min, the cell bands from the second and third Percoll fraction (F2; between 26 and 34%) and F3 (between 34 and 60%) were collected separately, diluted with 2 volumes of DMEM:F12 with P/S, centrifuged at 200g for 10 min and then plated and cultured in DMEM:F12 with P/S and 10% FBS. Cell culture media was removed and fresh media was added every 2–3 days.

Immunofluorescence staining for SCs

For expression marker staining, passage 1 or 2 testicular cells were plated post isolation after becoming confluent (1–2 weeks), and then cultured for 3–4 days in chamber slides or glass coverslips in 12-well plates. For all other analyses, cells of passage 2–3 were used. SCs (2.5×10^4) were plated in 8-well Nunc chamber slides (LabTek II, Nunc, Thermo Fisher Scientific) pre-coated with fibronectin (FN; 6.6 µg/mL; Life Technologies). At 72 h post plating, cells were fixed with 4% paraformaldehyde (PFA; Thermo Fisher Scientific), permeabilized using 0.5% Triton-X-100 (Sigma-Aldrich), blocked for 1 h in 10% normal goat serum and incubated with rabbit anti-GATA-4 (Table 1, Santa Cruz Biotechnology) followed by goat anti-rabbit Alexa Fluor 546 (Life Technologies). Images were acquired on a Nikon Eclipse E600 upright epifluorescence microscope (Histology and Light Microscopy Core Facility, Gladstone Institutes, San Francisco, CA, USA).

Alkaline phosphatase staining for PCs

The cells from PC-enriched supernatants were plated in a 12-well plate (3×10^5 cells/well) in MEM containing 10% FBS and P/S and stained using a Vector Red Alkaline phosphate substrate kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol.

Table 1 Antibodies used for experiments in this report.

Target	Source	Host	Catalog no.	Application (s)
GATA-4	Santa Cruz Biotechnology	rabbit	Sc-9053	ICC
calretinin	Novus Biologicals	rabbit	NBP2-12471	IHC, ICC
N-cadherin	Santa Cruz Biotechnology	rabbit	Sc-7939	IHC, WB
JAM-A (JAM1)	Zymed Technologies/ ThermoFisher	rabbit	36-1700	IHC, WB
ZO-1	Zymed Technologies/ ThermoFisher	mouse	33-9100	IHC, WB

3β-hydroxysteroid dehydrogenase staining of LCs

The enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) is an oxidoreductase that catalyzes the synthesis of progesterone from pregnenolone, 17-hydroxyprogesterone from 17-hydroxypregnenolone and androstenedione from dehydroepiandrosterone. Human LCs have been shown to express the type II 3β-HSD isoenzyme (Simpson *et al.* 1987, Ge *et al.* 2006). Staining for 3β-HSD activity was performed as previously described (Klinefelter *et al.* 1987). LCs from F3 (1×10^5 cells/well) were plated in 12-well plates. Dehydroepiandrosterone (0.5 µg; Sigma-Aldrich) was added to a mixture of nitroblue tetrazolium and nicotinamide adenine dinucleotide (both Sigma-Aldrich) solutions (1:1; 1 mg/mL) and mixed thoroughly. To each well, 500 µL of the solution was added and the plate was incubated at 37°C for 1.5–3 h.

Immunofluorescence staining of LCs

The LCs from F3 (2.5×10^4) were plated on 8-well Nunc chamber slides (Thermo Fisher Scientific). For staining, the cells were fixed with 4% PFA for 10 min and then permeabilized with 0.1% Triton-X-100 (Sigma-Aldrich). After blocking with 10% normal goat serum, cells were incubated with rabbit anti-calretinin (Table 1, Novus Biologicals, Littleton, CO, USA) followed by goat anti-rabbit Alexa Fluor 546 secondary antibody (Ab). Sections of frozen human testis tissue were used as a positive control.

Proliferation of SCs and LCs revealed by 5-ethynyl-2'-deoxyuridine (EdU) incorporation

SCs and LCs from F3 (1×10^4) were plated on 8-well Nunc chamber slides and incubated in 5% CO₂ at 37°C overnight. The next day, cells were stained using a Click-it EdU kit (Life Technologies/Thermo Fisher) per the manufacturer's recommendations. The cells were incubated in DMEM:F12 medium with EdU (5 µM) overnight. To identify proliferating LCs, after incorporation of EdU, cells were stained using rabbit anti-calretinin (Table 1, Novus Biologicals), followed by staining with goat anti-rabbit Alexa Fluor 546 secondary Ab.

TER measurements for evaluation of TJs of SCs

Transwell inserts (0.4 µm pore size, 0.33 cm² surface area, PET, Corning) for 24-well plates were coated with 100 µL of PBS containing human FN (6.6 µg/mL in PBS-CMF) by incubation at 37°C overnight. SCs ($0.6\text{--}2.5 \times 10^6/\text{cm}^2$) were plated into the apical chamber in DMEM:F12 medium in 300 µL DMEM:F12 medium with 10% FBS with 900 µL medium placed in the basolateral chamber. The medium was changed every 48 h. TER was measured using a Millicell-ERS Ohmmeter (EMD Millipore) equipped with an Ag/Ag electrode (STX2, World Precision Instruments, Sarasota, FL, USA). Readings were taken at three positions/well. At least three independent experiments were performed. TER values were calculated per cm², and average TER of FN-coated control wells without cells was subtracted from the average TER of wells with cells. To determine if TER values were altered by SC concentration, we plated 0.6×10^6

and 1.5×10^6 SCs/cm² in apical chambers of TW inserts in 24-well plates pre-coated with FN as described. To determine if TER values were altered by donor ages, populations enriched for SCs (MM-SC-1509, MM-SC-2305 and MM-SC-3608) that we had previously isolated from cadaveric tissue of deceased donors of 15, 23 and 36 years of age (Chui *et al.* 2011, Xiao *et al.* 2014) were plated in FN-coated TW inserts (0.5×10^5 cells/insert). We also tested a fibroblast cell line for use as a feeder layer that might promote TJ formation. HS-27 human foreskin fibroblasts (CRL-1634; ATCC) were plated (2.5×10^5) in the basolateral chambers of 24-well TW inserts. The next day, the plated HS-27 cells were treated with mitomycin to prevent overgrowth. After washing, SCs were plated (1×10^6 /cm²) in apical chambers pre-coated with FN. We determined whether HS-27 cells could form TJs when plated (2.5×10^6) on FN-coated TW inserts in which HS-27 cells were plated in the basolateral chamber. The HS-27 cells were treated with mitomycin as described earlier. To determine the effect of supplementation with FSH and testosterone, SCs were plated in TW inserts as described earlier in DMEM:F12 containing 10% FBS, 200 nM testosterone (Sigma-Aldrich) and 1.36 IU/mL (~100 ng/mL) human FSH (Ferring Pharmaceuticals). To assess the susceptibility of the TJs to cadmium exposure, SCs were plated (MM-SC-4012, 1.5×10^6 /cm²) in 24-well TW inserts in DMEM:F12 containing 10% FBS supplemented with testosterone and FSH as described earlier. Cells in three wells were exposed to cadmium chloride (CdCl₂) at 10 µM on day 12 and then to 30 µM on day 14. Cells in three other wells were exposed to 60 µM on day 14.

Immunofluorescence staining of SCs and immunoblots of TJ proteins

SCs (1×10^6 /cm²) were plated on chamber slides (4-well) or glass coverslips that were coated with FN solution (6.6 µg/mL) overnight. The coverslips were placed in 12-well plates and the cells were cultured in DMEM containing 10% FBS at 37°C in 5% CO₂. For staining, the cells were washed and then fixed in 4% PFA in PBS-CMF, permeabilized with 0.5% Triton-X 100 (Sigma-Aldrich) and stained with rabbit anti-N-cadherin (Table 1, Santa Cruz Biotechnology), rabbit anti-JAM-A and mouse anti-ZO-1 (Table 1, both from Zymed Life Technologies, Waltham, MA, USA) antibodies followed by corresponding goat anti-rabbit Alexa Abs 546 and goat anti-mouse Alexa 488 (Life Technologies). Imaging was performed on a Zeiss confocal laser-scanning LSM510 meta (Laboratory for Cell Analysis, Helen Diller Cancer Center Core, University of California) or on a Nikon Eclipse E600 upright epifluorescence microscope (Histology and Light Microscopy Core Facility, Gladstone Institutes). For immunoblots of TJ proteins, SCs (1.5×10^6 /cm²) were cultured on FN-coated TW inserts in 24-well plates for 8–12 days to ensure that TJ proteins were fully expressed. All reagents were from Thermo Fisher Scientific/Pierce unless indicated otherwise. The cells were rinsed with PBS-CMF and lysed using 1×RIPA buffer containing freshly added protease inhibitor cocktail and EDTA (0.5 mM). Total protein was quantified by a Bradford assay (Thermo Fisher Scientific). Protein (25 µg total) in Laemmli loading buffer (Bio-Rad) was loaded onto 5–20% SDS-PAGE gradient gels (Bio-Rad).

Proteins were electrophoresed at 200 volts and transferred using Midi transfer nitrocellulose membrane cassettes (Bio-Rad) using a Trans-blot Turbo Transfer System (Bio-Rad) at 2.5 A constant current for 10 min. Membranes were blocked with 5% Blotto solution (Santa Cruz Biotechnology) and stained with primary antibodies (Table 1) at 4°C overnight, followed by HRP-conjugated secondary antibodies at RT for 1 h, and development using Supersignal Femto substrate.

Lucifer yellow penetration assay

SCs in DMEM:F12 media containing 10% FBS were plated (1.5×10^6 /cm²) in human FN-coated TW inserts in 24-well plates. A stock solution (60 mM) was prepared by dissolving lucifer yellow (Sigma-Aldrich) in HBSS. Plates were incubated at 37°C in 5% CO₂ on an orbital shaker in a cell culture incubator. After 8–10 days, SCs were rinsed once with HBSS and then 300-µL of the lucifer yellow stock solution was added to the apical chamber and 900-µL of HBSS added to the basolateral compartment of each TW chamber. After 1-h incubation with shaking, media was collected from apical and basolateral compartments, and 100 µL of the collected media was transferred to a 96-well plate and fluorescence was measured with 485 nm excitation and 540 nm emission on a Polarstar Galaxy microtiter plate reader (BMG Labtech, Cary, NC, USA). Each condition was tested with three replicates. Lucifer yellow penetrance of FN-coated TW inserts without any cells was used as a control.

Analysis of secretion of inhibin B and estradiol-17β secretion by SCs and testosterone by LCs

Enzyme immunoassay (EIA) kits were used to quantify inhibin B and estradiol-17β (1,3,5-estratriene-3,17β-diol; estradiol, estrogen) secretion by SCs from donors of 23, 36 and 40 years of age growing in TW inserts by analysis of conditioned media. Media was harvested from the apical and basolateral chambers, and the supernatants were stored at –80°C after centrifugation at 2000g for 5 min. After thawing, aliquots of media from both chambers were analyzed by EIA. A competitive EIA kit for detection of human inhibin B was used according to the vendor's instructions (#MBS700779; MyBioSource, San Diego, CA, USA) with detection at 450 nm on a Thermomax (Molecular Devices, Sunnyvale, CA, USA) plate reader. A standard curve for the log of the concentration of human inhibin B (4–800 pg/mL) vs optical density was employed, and samples were analyzed in triplicate. For detection of estradiol levels in conditioned media from apical and basolateral chambers of the SCs growing in TW inserts, we used a competitive EIA kit according to the vendor's directions (#582251; Cayman Chemical). A standard curve derived from analysis of the estradiol standards (6.6–4000 pg/mL) as a logit B/B₀ was used and samples were analyzed in triplicate at 405 nm. To compare the total amount of inhibin B and estradiol secreted by the cells into the two chambers, the concentration detected in the apical media was divided by three since the volume was only 300 µL, whereas the volume of media in the basolateral chamber was 900 µL. Testosterone levels in media conditioned by LCs were measured by a

competitive EIA according to the manufacturer's instructions (EIA kit #582701; Cayman Chemical). This procedure was similar to that described earlier for the estradiol EIA kit, except testosterone standards (3.9–500 pg/mL) were used to create a standard curve. A standard curve as a logit B/B_0 was used to quantify testosterone levels in samples that were analyzed in triplicate at 405 nm.

Culture of human ESCs with SCs in vitro

The method for differentiating pluripotent stem cell lines was derived from Ramathal and coworkers (2014). The hESC H1 cell line initially was maintained in feeder-free conditions on Matrigel and grown in mTESR pluripotency maintenance medium (Stemcell Technologies, Vancouver, BC, Canada). H1 hESCs were lentivirally transduced with a mCherry reporter under the control of an EF1 α promoter (H1-mCH). Positively transduced cells were selected for by exposure to blasticidin (2 μ g/mL) for 7–10 days. For germline differentiation, embryoid bodies (EB) were formed from H1 cells in AggreWell plates (Stemcell Technologies) for standardization of EB size and quality. H1-mCH cells were exposed to BMP4 for 48 h during the EB formation period. Subsequently, EBs of approximately 500 cells each were plated on Matrigel-coated plates (adherent) or co-cultured in either small colonies (50–100 cells) or as single cells with SCs (MM-SC-1509) that were derived from the testes of a deceased donor of 15 years of age (Chui *et al.* 2011, Xiao *et al.* 2014). Individually cultured or co-cultured H1-mCH cells were differentiated in PGC medium (DMEM:F12 50:50 with 20% Knockout Serum Replacement (Life Technologies) with or without supplementation with BMP4 (50 ng/mL), 50 ng/mL EGF (both from R&D Biosystems), 2 ng/mL human recombinant leukemia inhibitory factor (hLIF; Millipore/EMD) and 10 nM retinoic acid (RA, Sigma-Aldrich). For controls, undifferentiated H1-mCH cells grown in mTESR 1 medium (Stemcell Technologies) were also collected for analyses. Following differentiation for 7 days, all co-cultures were enzymatically dissociated with Accutase (MP Biomedical, Santa Ana, CA, USA), filtered and H1-mCherry-positive cells enriched and separated from co-cultures using fluorescence-activated cell sorting (FACS) on a BD FACS ARIA instrument. Briefly, dead cells in the total population were gated out using forward and side scatter patterns as well DAPI staining intensity. Parental H1-mCherry hESC cells were used as a positive control to establish sorting gates for mCherry-positive and -negative cells. H1-mCherry-positive populations were directly sorted into single cell, 10-cell and 100-cell fractions in a 96-well PCR plate. All sorted cells were subjected to gene expression analysis.

Gene expression and single-cell analysis

After FACS of single cells into 96-well PCR plates, approximately 1 μ g total RNA from cells of each H1-mCherry-positive EB and colony of cells was reverse-transcribed using SuperScript III First-Strand Synthesis System (Life Technologies). RNA was isolated using 5 μ L/well of 2 $\times$ CellsDirect Reaction Buffer supplemented with 0.1 nM SUPERase-In (Life Technologies/ThermoFisher). For microfluidic PCR analysis of EBs and single

colony samples, cDNA was preamplified using a pooled mix of primer pairs (200 nM each) in a reaction containing 2.5 μ L TaqMan Pre-Amp master mix, 1.25 μ L pooled primer mix and 1.5 μ L cDNA. For single-cell RNA extracts, cDNA was preamplified in a reaction mix containing 5 μ L RNA extract, 0.2 μ L SuperScript III RT Platinum Taq Mix, 2.5 μ L pooled primer mix (200 nM) and 1.3 μ L DNA suspension buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA). Digital quantitative PCR analysis was performed on the Biomark 96.96 microfluidic qPCR chip (Fluidigm). For EBs and colony samples, transcription levels were determined in triplicate reactions. Relative expression was determined by normalization to the geometric mean of 5–6 housekeeping genes with the formula $2^{-(\Delta\Delta C_t)}$ as previously described (Ramathal *et al.* 2014). Single cells with failed or absent housekeeping gene expression and cells with C_t values greater than 25 cycles were excluded. For single cells, the relative expression of VASA across all the cells from each treatment condition was averaged. Finally, the fold-change of relative expression was determined by log conversion of normalized C_t values (power = 2) and plotted in bar charts.

Statistical analysis

Multiple group comparisons were analyzed by 1- or 2-way ANOVA followed by Bonferroni's or Tukey's *post hoc* tests. Within-group comparisons were done using two-tailed *t* tests. Data are expressed as mean $\pm$ s.d. Significance was defined at $P < 0.05$.

Results

Isolation of human SCs, LCs and PCs

We attempted to isolate SCs, LCs and PCs using a modification illustrated in Fig. 1 of methods described earlier (Lipshultz *et al.* 1982, Santiemma *et al.* 1992, Lejeune *et al.* 1993) from both cadaveric testes of five human donors ranging in age from 26 to 56 years. According to these previously described methods, the initial shaking step at 37°C at 275–325 rpm for 20 min facilitated removal of germ cells after gravity sedimentation. In the subsequent enzymatic digestion, we reduced exposure to 5–10 min and added DNase (final concentration 20 μ g/mL) to isolate fragments of intact seminiferous tubules. Samples of the digestion were removed periodically to check the progress by phase-contrast microscopy. Previously, Lejeune and coworkers (1993) purified human LCs and PCs from human testicular cells using Percoll gradient centrifugation. We modified this protocol and successfully isolated relatively pure SC fractions (>95%) and fractions enriched in LCs (40–60%) and PCs (30–60%) from testes of three of the human donors as shown by immunostaining (Figs 2 and 3). The composition of the testes from the donors appeared to be similar. We developed the methodology with the first two donor tissues and isolated SCs and PCs, but not LCs. Percentages of the cell types in the three populations were derived from

Figure 2 Images (A, B, C, D, E, F, G, H and I) of LCs, PCs and SCs isolated from a male donor of 56 years of age (MM-5613). All nuclei in the image of the SC-enriched population were immunofluorescently stained for expression of GATA-4 (A) as revealed by blue nuclear DAPI staining (B) and lack of red staining by secondary Ab alone (C). Alkaline phosphatase activity is clearly shown by red staining in PC-enriched cells (D), and in a single cell (F), whereas the low level of red staining in the LC-enriched population (E) reveals paucity of PC cells with alkaline phosphatase activity. Blue staining (G and I) revealed the activity of 3β -HSD in LCs in fraction 3 and very limited 3β -HD activity (H) in the SC-enriched fraction.

immunohistochemistry by determining the number of the phenotypically stained cell type from the 3 different donors compared to the number of DAPI-stained nuclei in 3 fields of view. Although some LCs (20–30%) were detected in F2, the population most enriched for LCs was recovered in F3 as reported previously (Lejeune *et al.* 1993). As we previously reported (Chui *et al.* 2011), immunocytochemistry showed that the majority (>95%) of cells in SC-enriched population expressed GATA-4 (Fig. 2A; 2B presents nuclear DAPI staining and 2C is the secondary Ab alone control).

Activity of alkaline phosphatase in PCs and 3β -HSD in LCs

Alkaline phosphatase is a known PC marker (Palombi & Di Carlo 1988), and the cell population enriched for PCs demonstrated high alkaline phosphatase activity (Fig. 2D and F), whereas the LC-enriched population did not (Fig. 2E). As expected, the isolated LCs stained positively for 3β -HSD (Fig. 1G and I; H is the SC control).

Human LCs and SCs proliferate in vitro

The proliferation of cultured human LCs was shown by dual staining with EdU and calretinin, a marker for LCs (Augusto *et al.* 2002). Calretinin has been found

to be expressed in the cytoplasm and in some of the nuclei of LCs (Augusto *et al.* 2002). We used staining of frozen sections of human testis (Fig. 3A and B) to confirm the specificity of the calretinin Ab for LCs (Fig. 3B). The human testis sections were immunostained for expression of zonula occludens-1 (ZO-1), a TJ adaptor that is expressed by SCs and not by LCs. As another positive control, we analyzed the incorporation of EdU by proliferating human SCs co-immunostained for nuclear GATA-4 expression, as we previously described (Fig. 3C and D) (Chui *et al.* 2011). Dividing cells in F2 and F3 were identified by nuclear incorporation of EdU over 16 h followed by staining for calretinin to positively identify proliferating LCs (Fig. 3E). Positivity for calretinin in nuclei is indicated by the co-stained nuclear color (yellow-green) of proliferating calretinin-expressing (red) cells compared to the nuclei (green) of proliferating cells not expressing calretinin (Fig. 3E and F). The proportion of the total population in F3 that was actively dividing was revealed by simultaneously staining all nuclei with EdU and DAPI (Fig. 3F and H). In Fig. 3F several non-proliferating cells can be observed as DAPI-stained (blue) nuclei that are calretinin negative and also EdU negative and, therefore, are not observable in Fig. 3E. In Fig. 3H the proliferating co-stained cells in F3 have blue-green nuclei, whereas the nuclei of non-dividing cells are blue due to only DAPI staining.

Figure 3 (A, B, C, D, E, F, G and H) Primary SCs and LCs from a male donor of 40 years of age (MM-4012) divide in culture. Sections of human testis show seminiferous tubules revealed by immunofluorescent staining of the TJ protein ZO-1 (A; green; arrows) and intrastitial LCs revealed by immunofluorescent staining for calretinin expression (B, red; arrowheads). Actively dividing Sertoli cells from MM-SC-4012 are labeled with nuclear Click-it EdU signal (green) that co-stained with GATA4 signals (red; C and D) to create yellow (C). Cultured dividing LCs from MM-4012 immunostained for calretinin (red; E and F) with nuclei stained green (E) with Click-It EdU or co-stained with Click-It EdU and DAPI (F). Cells from Fraction 3 of the Percoll gradient are shown stained for DAPI with (H) and without (G) Click-It EdU green co-staining.

SCs cultured on FN form and maintain TJs for more than 30 days

After culture of populations enriched for hSCs on FN-coated glass coverslips for 3–5 days, immunostaining was performed for junctional adhesion molecule A (JAM-A), N-cadherin (a basal ectoplasmic specialization protein of adherens junctions) and ZO-1 (Fig. 4). The presence of TJ proteins at intracellular interfaces was revealed by positive immunocytochemistry (Fig. 4A, B and E) compared to controls stained with secondary Ab alone (Fig. 4C and E) as shown in the photomicrographs. Specificity of the Abs was shown by immunoblots of hSC populations after lysis (Fig. 4F), and ZO-1 staining of TJs in human testis sections (Fig. 3A and B).

We used the Millicell-ERS (Electrical Resistance System, EMD Millipore) to measure membrane potential and resistance of SCs cultured on TW inserts using a protocol described previously (Chung *et al.* 1999, Chung & Cheng 2001, Chui *et al.* 2011). TER of 0.6×10^5 or 2.5×10^5 SCs was assessed in apical chambers on FN-coated TW inserts in 24-well plates with medium changes every 48 h (Chui *et al.* 2011). Elevated TER of SCs (Fig. 5A) was found to be between maintained at 50 and 90 ohms/cm² until the experiment was stopped on day 35.

TER of SCs from different donors on TW inserts is similar

We compared TER of 5×10^5 SCs per well that were previously isolated from 3 different donors of 15, 23 and 36 years of age and found that all created TJs. Interestingly, although the TER of the SCs was similar, cells from the oldest donor, MM-SC-3608 (Fig. 5B), produced significantly higher TER ($P < 0.001$) than the other two cell populations.

TER is reduced by cultures of SCs on HS-27 feeder layer in TW inserts

We plated a layer (2.5×10^5) of human foreskin fibroblast cells (HS-27; CRL-1634; ATCC) into the basolateral chamber to determine the effect of bioactive molecules secreted by the cells such as FN (Zhang *et al.* 2012) on maintenance of TER by SCs that were plated in the apical chamber (2.5×10^5) on FN-coated TW inserts. The TER of HS-27 cells (2.5×10^5) plated in the apical chamber with HS-27 fibroblasts (2.5×10^5) plated in the basolateral chamber also was analyzed to determine if the HS-27 cells can form TJs as do the SCs. Prior to plating, HS27 cells were treated with mitomycin to prevent cell proliferation. The results (Fig. 5C) showed TER of SCs was reduced when HS-27 cells were present

Figure 4 (A, B, C, D, E and F) SC-enriched populations from a male donor 40 years of age (MM-4012) cultured on FN-coated glass sides form TJs. Nuclei are blue stained with DAPI. Red immunostaining at intracellular interfaces shows expression of the TJ proteins JAM-A (A) and N-cadherin (B) by the SCs. Immunostaining by the secondary Ab alone reveals lack of JAMA-A and N-cadherin recognition (C). Green immunostaining reveals ZO-1 expression (D). Immunostaining by secondary Ab alone reveals lack of TJ recognition (E). Immunoblots (F) confirmed the specificity of the Abs to JAM-A, ZO-1 and N-cadherin TJ proteins.

Figure 5 TER of SC-enriched populations on 24-well plate FN-coated TW inserts with 0.4 μ m pores for 20–36 days (A, A, B, C, D and E). Variables are differing numbers of cells (A), SC-enriched populations from different donors (B), the addition of fibroblasts as feeders (C), FSH and testosterone treatment (D) and exposure of testosterone and FSH-treated SCs to 10 μ M CdCl₂ on day 12 and to 30 μ M CdCl₂ on day 14 and exposure of other SCs to 60 μ M CdCl₂ on day 14 (E). Averages shown are mean TER of cells in three TW insert chambers, and error bars are $\pm$ s.d. Statistical significance was analyzed by ANOVA with Bonferroni's *post hoc* analysis.

Figure 6 Percentage (A) of total apical lucifer yellow (LY, 60 μ M) penetrating SCs from donors of 23 and 36 years of age on FN-coated TW inserts (8- μ m pores) compared to the percentage penetration of inserts with and without cells, FSH (100 ng/mL) and testosterone (100 nM) or exposure to 30 μ M CdCl₂. Average penetration of FN-coated TW inserts was slightly >50% of the total, and penetration was ~30% in 3 wells with SCs (2.5 $\times$ 10⁵ per well). Levels (pg/mL) of (B) inhibin B in SC cells from donors of 23 and 30 years of age, and (C) estradiol in conditioned media from basal (basolateral) or apical chambers of TW inserts containing SCs from donors of 23, 30 and 36 years of age (500,000 cells/0.33 cm² insert) with or without treatment by FSH (100 ng/mL) and testosterone (100 nM) for 48 h or control media. Levels (pg/mL) of testosterone (D) were quantified in conditioned media collected from confluent cultures of cells enriched for LCs and SCs after 3–4 days. For analysis of testosterone secretion, the LC-enriched populations were from donors of 40 and 56 years of age and the SC-enriched populations were from donors of 23 and 36 years of age. The level of testosterone detected in the unconditioned culture media (MEM with 10% FBS) is shown as a control. All results are representative of 3 or more independent experiments and samples were analyzed in triplicate. Statistical significance of differences was analyzed by *T* test (**P* < 0.05, ** and ****P* < 0.01 and *****P* < 0.001).

in the basolateral chamber of the TW insert during the culture ($P < 0.01$). Also, low TER showed that TJs were not formed when only HS-27 cells were plated on TW inserts.

Supplementation of SCs with FSH and testosterone increases TER on TW inserts

Treatment of SCs with media containing 200 nM testosterone and 1.36 IU/mL (~100 ng/mL) human FSH (Ferring Pharmaceuticals) induced a statistically significant increase ($P < 0.001$) in TER (Fig. 5D).

CdCl₂ exposure reduces TER values

The cells in three wells were treated with 10 μ M CdCl₂ on day 12 and then subsequently with 30 μ M CdCl₂ on day 14. Cells in a different set of three wells were treated with 60 μ M CdCl₂ on day 14 (Fig. 5E). Exposure to 10 μ M CdCl₂ did not reduce TER significantly. However, a significant decrease in TER was observed after exposure of cells to either 30 μ M or 60 μ M CdCl₂ (both $P < 0.001$ compared to cells in unexposed wells) reflecting disruption of TJs.

Monolayer of SCs on TW inserts reduced penetration of lucifer yellow

The penetration of lucifer yellow in confluent SCs growing on FN-coated TW inserts was assessed (Fig. 6A). Penetration of 180- μ M lucifer yellow placed in the apical chamber of FN-coated and -uncoated TW inserts in 24-well plates with and without SCs (2.5×10^5) at 37°C for 1 h was found to be 25.5, 18.5 and 13.7% in uncoated, FN-coated and FN-coated inserts with cells, respectively. To increase the penetration we tested the following: (i) lucifer yellow at 30 and 60 μ M; (ii) 0.4 or 8.0 μ m pore size FN-coated inserts (without cells) and (iii) incubation with shaking at 37°C for 1 or 2.5 h. The greatest lucifer yellow penetration was in TW inserts of 8.0 μ m pores shaken for 1 h with 60 μ M lucifer yellow. Shaking for 2.5 h increased penetrance only slightly. Using these conditions, average penetration of TW inserts coated with FN alone was slightly more than 50% of the total. When SCs (2.5×10^5 per well) were present penetration was reduced to 30%. There was no change in penetration of the SCs when treated with FSH and testosterone, but lucifer yellow penetration significantly increased ($P < 0.01$) after exposure of SCs to 30 μ M CdCl₂.

Treatment of human SCs with FSH and testosterone induced secretion of inhibin B and estradiol

Treatment with FSH and testosterone (7 β -hydroxy-3-oxo-4-androstene) greatly increased the secretion of inhibin B and estradiol (estrogen) by cultured adult SCs (Fig. 6B).

Inhibin B was virtually undetectable in wells with cells not treated with FSH and testosterone ($P < 0.001$). Our data also show that the secretion of inhibin B was polarized with most occurring basolaterally and only 5 percent of the total secreted inhibin found in apical chambers of the TW inserts ($P < 0.001$). We observed that estradiol was secreted by human SCs cultured on TW inserts in the presence of exogenous FSH and testosterone (Fig. 6C) and that there was lower level of estradiol secreted by the SCs cultured in media in the absence of additional exogenous FSH and testosterone. More estradiol was secreted by hormone-treated SCs in the basolateral compared to the apical chambers.

Human LCs secrete testosterone in vitro

Our results show that cultures of testicular cells enriched for LCs in normal cell culture secreted testosterone (Fig. 6D), a hallmark of LCs.

Primordial germ cell-like differentiation of H1 hESCs induced by culture on SC feeder layers

SCs are somatic cells that make critical contributions to the testicular stem cell niche by the production of growth factors, nutrients, cytokines and immunosuppressive factors and maintenance of the blood–testis barrier (Petersen & Soder 2006, Svingen & Koopman 2013, Kaur *et al.* 2014). Primordial germ cell (PGC)-like differentiation of H1 hESCs was characterized by gene expression analysis after 7 days of culture as single cells, small colonies of 50–100 cells or as EBs with or without SC feeder layers and various growth factors (Fig. 7A). Supplements included in the media are critical for induction of mammalian germ cell differentiation; bone morphogenic protein (BMP)-4 (Kee *et al.* 2006), epidermal growth factor (EGF) (Yan *et al.* 1998), human leukemia inhibitory factor (hLIF) (Piquet-Pellorce *et al.* 2000, Dorval-Coiffec *et al.* 2005, Fukunaga *et al.* 2010) and RA (Gely-Pernot *et al.* 2012, Griswold *et al.* 2012). We also compared differentiation in PGC medium (DMEM:F12 with 20% Knockout Serum Replacement) to that in mTESR medium (Stemcell Technologies).

We assessed the expression levels of a classical germ cell-specific lineage protein, VASA (DDX4), in mCherry-positive H1 cells after purifying them from heterogeneous cell mixtures using FACS (Fig. 7B). We observed the highest expression levels of VASA in H1 hESCs differentiating as colonies on SC feeder layers treated with RA, hLIF and BMP4. Highlighting the importance of 3D architecture in germ cell progression, relative VASA expression levels differed between single cells or colonies and EBs.

Previous studies have shown that germ cell progression of hESC is induced by overexpression of *Dazl*, *Daz* and *Boule* genes encoding germ-cell-specific cytoplasmic RNA-binding proteins. Human

Figure 7 Gene expression analysis reveals co-culture of transduced H1 hESCs with human SCs from a donor 15 years of age increased PGC-like differentiation. (A) Microfluidic quantitative real-time PCR-based analysis of VASA gene expression in H1 hESCs differentiated on SC feeder layers as single cells, colonies of 50–100 cells or EBs, and supplemented with a variety of growth factors. Black ovals designate the presence of a factor or growth condition while white ovals indicate an absence of the factor/condition (A). Individual bar plots comparing the expression of genes indicative of germline formation in H1 hESCs grown either as colonies (50–100 cells) on Matrigel or as co-cultures with human SC feeder layers. All cultures were grown with and without RA, hLIF and BMP4 (RLB) supplementation as indicated in the plots (B). Error bars are $\pm$ s.d. Statistical significance was analyzed by ANOVA. * $P < 0.05$ and ** $P < 0.001$.

Dazl expression induced PGC formation, and *Daz* and *Boule* expression promoted later stages of meiosis and development of haploid gametes (Kee et al. 2009). VASA is an ATP-dependent RNA helicase expressed by human germ cells that is most abundant in spermatocytes and mature oocytes (Castrillon et al. 2000, Noce et al. 2001). Overexpression of *Vasa* in induced pluripotent stem cells and in hESCs promotes progression to PGCs and to meiosis (Medrano et al. 2012).

Based on our observations of VASA expression, we chose to differentiate H1 hESCs in a colony format with or without SC feeder layers and a cocktail of supplements that consisted of BMP4, hLIF and RA (Fig. 7B). Gene expression levels of *Dazl*, *Prdm1*, *Stella* (*Dppa3*), *Nanos3*, *Pum2* and *Vasa* were tested in FACS-purified H1 cells after differentiation. The highest expression levels of *Nanos3* and *Vasa* were found in H1 hESCs differentiated on SC feeders and receiving the cocktail of supplements. Interestingly, the highest expression of *Dazl*, *Prdm1*, *Pum2* and *Stella* was observed in H1 hESCs differentiated on SC feeder layers but without the supplementation of BMP4, hLIF and RA. *Dazl*, *Prdm1* and *Stella* are characteristically expressed in PGCs that are the first cells committed to the germ cell lineage (Payer et al. 2006, Saito & Lacham-Kaplan 2007).

PDRM1 (also known as BLIMP1) is a transcriptional repressor that plays a role in the specification of the PGC population (Ohinata et al. 2005, Chu et al. 2011, Schafer et al. 2011).

Discussion

There is a long history of attempts to replicate spermatogenesis *in vitro* (Reuter et al. 2012). Most strategies have focused on organ culture of testes or testicular fragments but others have explored cell and tissue culture approaches. Spermatogenesis *in vitro* had only been achieved with certain species of fish until Sato and coworkers reported in a ground-breaking study that spermatogenesis in neonatal mouse testes, which contain only primitive germ cells, could be initiated in an *in vitro* setting (Sato et al. 2011a,b). Fragments of testis tissue were maintained for over 2 months and the sperm produced was used in microinsemination to create healthy offspring. Shortly thereafter, complete spermatogenesis *in vitro* was achieved using cells isolated from enzymatically digested seminiferous tubules of 7-day-old mice cultured in a two-layer soft agar culture system (Abu Elhija et al. 2012, Mahmoud 2012). More recently, murine testicular cells including spermatozoa

were propagated using spontaneous outgrowths of small pieces of seminiferous tubules (Mäkelä *et al.* 2014). Despite these promising accomplishments, derivation of mammalian spermatozoa *in vitro* remains difficult to achieve.

We are focused on the creation of the human testicular stem cell niche *in vitro* for modeling spermatogenesis. Here, we successfully isolated populations enriched for the three major somatic cell types, LCs, PCs and SCs (Svingen & Koopman 2013) from testes of three human donor tissues. Immunocytochemistry showed that the majority (>95%) of cells in the SC-enriched populations expressed GATA-4. Although GATA-4 is expressed in both SCs and LCs in testes of human adults (Ketola *et al.* 2000), there was very little 3 β -HSD activity that is characteristic of LCs in the SC-enriched populations. Further evidence of the identity and phenotypical functionality of the SC-enriched populations *in vitro* was their expression of ZO-1, N-cadherin and JAM-A, and the polarized secretion of the biomolecules estradiol and inhibin B.

Contrary to the assumption that all LCs are essentially terminally differentiated cells, our data show that LCs could divide in culture. This result is consistent with the findings that adult rat LC regeneration occurs following destruction by ethane dimethane sulphonate (Teerds *et al.* 1999) and that proliferation of rodent LCs *in vitro* is enhanced by human chorionic gonadotropin exposure (Bi *et al.* 2011). Cultured LCs expressed both calretinin and the steroid-metabolizing enzyme, 3 β -HSD, and prototypically secreted testosterone. The ability to propagate the LCs in culture should facilitate the creation of a multicellular model of spermatogenesis. Primary PCs previously were shown to retain the ability to proliferate in culture (Albrecht *et al.* 2006, Schell *et al.* 2008). We further confirmed our previous work that a population of SCs isolated from humans can retain the ability to divide in culture (Chui *et al.* 2011). Future studies will focus on characterizing genotypic and phenotypic changes in these populations of testicular cells with *in vitro* passage.

We previously found by measuring TER that human SCs form TJs when grown on TW inserts coated with FN but not with any other extracellular matrix coatings (Chui *et al.* 2011). Interestingly, FN is also secreted by PCs (Tung *et al.* 1984). Previous studies using SCs from rodents and humans have shown intracellular TJ formation and maintenance for approximately one week. Our results showed that primary hSCs maintained TJs *in vitro* for 35 days (when the experiment was stopped), far longer than previously thought possible. These data suggest that it is likely that TJs can be maintained by SCs in culture for even longer periods of time such as the 64-day average required for human spermatogenesis (Misell *et al.* 2006).

Our observation of increased inhibin B and estradiol secretion by the human SCs in response to FSH and

testosterone treatment is supported by previous studies that have shown increased inhibin B secretion by FSH treatment of cultured rat SCs (Le Gac & de Kretser 1982, Bicsak *et al.* 1987) and human SCs (Carreau 1995). The highest levels of inhibin B secretion by human SCs were detected in the basolateral chambers of the TWs, reflecting greater secretion out of, rather than into, the seminiferous tubule lumen. Inhibin B secretion by adult SCs and LCs modulates FSH secretion by the pituitary in a negative feedback loop (Griswold 1998). Serum inhibin B levels have been proposed as a biomarker for testicular toxicity and in combination with serum FSH levels, increased the predictive power for the detection of testicular damage over levels of either alone (Stewart & Turner 2005). Polarized secretion of inhibin B by immature rat SCs *in vitro* has been reported but in these studies, apical levels were higher than the basolateral ones (Handelsman *et al.* 1989).

Supplementation with FSH and testosterone also induced secretion of estradiol by human SCs. Secretion of estradiol by cultured rat SCs has been induced by incubation with FSH in the presence of testosterone that is metabolized by SCs to estradiol (Dorrington & Armstrong 1975, Fritz *et al.* 1976, Jannini *et al.* 1994). Testosterone treatment of human SCs cultured with FSH has also been shown to increase estradiol secretion (Foucault *et al.* 1992, 1994).

In previous studies, testosterone secretion increased in cultures of primary human LCs up to 12 days post isolation (Lejeune *et al.* 1998). We also found testosterone secretion by cell populations dominated by SCs, an effect likely due to the presence of LCs (Lejeune *et al.* 1998). We observed enhanced germ cell-like differentiation of cocktail-supplemented H1 hESCs grown on SC feeder layers compared to that of H1 hESCs differentiated without SC feeders. This promising finding demonstrates that *in vitro* cultures of SCs retain the ability to support germ cell differentiation. Furthermore, the differential expression profiles of H1 hESCs grown on SCs with or without BMP4, hLIF and RA supplementation, respectively, suggest that temporal modulation of external paracrine signals is necessary to provide the appropriate milieu for germ cell formation. Thus, our data show a retention of phenotypic and functional characteristics by SCs that may be permissive for *in vitro* germ cell progression through meiosis and spermiogenesis. In the future, we intend to correlate expression levels of phenotypic genetic or proteomic markers by the human SCs, LCs and PCs and cellular functions essential in the progression of germ cells (Com *et al.* 2014).

We observed higher germ cell gene expression profiles in the cell colonies compared to the EBs. We surmise that this is because the total area of contact between the SCs and hESCs in cell colonies is greater than that in EBs. Thus, the physical interactions and paracrine effects of co-cultured SCs could be enhanced when hESCs are

grown as small colonies rather than as single cells or in larger, much denser EBs. It is possible that the cell relationship in colonies more closely recapitulates the *in vivo* relationship of SCs and germ cells and leads to more efficient induction of germ cell differentiation.

In the long term, we seek to create an *in vitro* model of spermatogenesis that can be used to assess the reproductive toxicology of chemicals and drugs. Most testicular toxicology is based on studies in animals, but an analysis of 150 pharmaceutical compounds in the development showed that the predictive value of studies using multiple mammalian species for revealing toxic effects in specific human organs was only 70%. When studies were conducted in a single mammalian species, the predictive value falls to 7 and 27% with rodents or non-rodent mammals, respectively (Olson *et al.* 2000). Thus, there is a need for novel and more accurate models of toxicity, and specifically for testicular toxicity. *In vitro* bioassays are cost-effective compared to animal testing, and ethical concerns surrounding animal experimentation increase the attractiveness of *in vitro* testing.

In conclusion, we have described a protocol to isolate populations enriched for the 3 major somatic cell populations, LC, PCs and SCs, from the testes of a single human cadaver, and have shown that the LCs can divide in culture and have demonstrated retention of phenotypical functionality in early passage number (1–3) cells. We also demonstrated that populations of primary hSCs maintained intact TJs *in vitro* for extended periods of time, and when used as feeder layers, induced germ cell lineage progression of hESCs. Overall, our results support the feasibility of creating a human testicular stem cell niche *in vitro* for application in modeling spermatogenesis.

Declaration of interest

M Gaur, P J Turek and C M John have equity in MandalMed that has pending U.S. patent (Application #20090028833) on proliferative SCs. R A Reijo-Pera and P J Turek are co-inventors on a U.S. patent for methodology to produce pluripotent human spermatogonial stem cells (US Patent #8,673,637). There are no other conflicting interests with the study.

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Author contribution statement

M Gaur, C Ramathal and C M John conducted laboratory experiments, generated and analyzed data and prepared the manuscript. R Reijo-Pera and P J Turek contributed materials.

C M John, P J Turek and R Reijo-Pera conceived of the study, participated in analysis and interpretation of data and writing and final approval of manuscript.

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