Human Reproduction, Vol.25, No.1 pp. 158–167, 2010
Advanced Access publication on October 8, 2009 doi:10.1093/humrep/dep354

human reproduction
ORIGINAL ARTICLE *Reproductive biology*

Embryonic stem cell-like cells derived from adult human testis

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BACKGROUND: Given the significant drawbacks of using human embryonic stem (hES) cells for regenerative medicine, the search for alternative sources of multipotent cells is ongoing. Studies in mice have shown that multipotent ES-like cells can be derived from neonatal and adult testis. Here we report the derivation of ES-like cells from adult human testis.

METHODS: Testis material was donated for research by four men undergoing bilateral castration as part of prostate cancer treatment. Testicular cells were cultured using StemPro medium. Colonies that appeared sharp edged and compact were collected and subcultured under hES-specific conditions. Molecular characterization of these colonies was performed using RT–PCR and immunohistochemistry. (Epi)-genetic stability was tested using bisulphite sequencing and karyotype analysis. Directed differentiation protocols *in vitro* were performed to investigate the potency of these cells and the cells were injected into immunocompromised mice to investigate their tumorigenicity.

RESULTS: In testicular cell cultures from all four men, sharp-edged and compact colonies appeared between 3 and 8 weeks. Subcultured cells from these colonies showed alkaline phosphatase activity and expressed hES cell-specific genes (*Pou5f1*, *Sox2*, *Cripto1*, *Dnmt3b*), proteins and carbohydrate antigens (POU5F1, NANOG, SOX2 and TRA-1-60, TRA-1-81, SSEA4). These ES-like cells were able to differentiate *in vitro* into derivatives of all three germ layers including neural, epithelial, osteogenic, myogenic, adipocyte and pancreatic lineages. The pancreatic beta cells were able to produce insulin in response to glucose and osteogenic-differentiated cells showed deposition of phosphate and calcium, demonstrating their functional capacity. Although we observed small areas with differentiated cell types of human origin, we never observed extensive teratomas upon injection of testis-derived ES-like cells into immunocompromised mice.

CONCLUSIONS: Multipotent cells can be established from adult human testis. Their easy accessibility and ethical acceptability as well as their non-tumorigenic and autogenic nature make these cells an attractive alternative to human ES cells for future stem cell therapies.

Key words: embryonic stem cells / adult human testis / pluripotency / *in vitro* differentiation

Introduction

Since their discovery, human embryonic stem (hES) cells have had the prospect of being the savior for patients suffering from diseases that result from specific cell loss or damage such as Alzheimer's disease, Parkinson's disease, spinal cord injury, diabetes and cancer (Cowan *et al*., 2004). Two major drawbacks have precluded their use in clinical settings thus far. First, hES cells are derived from human embryos and are therefore not genetically identical to the person for whom they are destined to be used. As a consequence, transplantation of such cells will invoke immune rejection and constituent immune suppression will be necessary to allow the transplanted cells to survive.

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Second, the generation of hES cells entails the destruction of human preimplantation embryos, which is considered ethically unacceptable by many members of society.

As a result of these drawbacks, researchers have focused on finding alternative sources of multipotent cells in the adult human body. Recently, induced pluripotent stem cells (iPS cells) have successfully been derived from adult and fetal human fibroblasts (Takahashi *et al*., 2007; Lowry *et al*., 2008; Mali *et al*., 2008; Nakagawa *et al*., 2008; Park *et al*., 2008). However, their generation currently requires viral transduction with transcription factors, sometimes including c-myc, a well-known oncogene (Hyun *et al*., 2007; Nakagawa *et al*., 2008; Wernigr *et al*., 2008). As a consequence, extensive studies will be needed to ascertain the actual potency of iPS cells and to control their elevated tumorigenic potential (Gearhart *et al*., 2007; Hyun *et al*., 2007).

Spermatogonial stem cells from neonatal and adult mice are able to develop into multipotent cells when cultured under specific conditions *in vitro* (Kanatsu-Shinohara *et al*., 2004; Guan *et al*., 2006; Seandel *et al*., 2007; de Rooij and Mizrak, 2008). In addition, efficient and long-term culture of spermatogonial stem cells is now possible allowing sufficient cells for regenerative therapy to be generated (Kanatsu-Shinohara *et al*., 2003).

Very recently, three groups have independently found formation of ES-like cells by exposing human testicular cells to specific ES conditions *in vitro* (Conrad *et al*., 2008; Golestaneh *et al*., 2009; Kossack *et al*., 2009). Here, we show that ES-like cells can also be generated under culture conditions known to support long-term proliferation of spermatogonial stem cells (SSCs).

Materials and Methods

Testicular cell isolation and culture

After written informed consent, testis material was donated for research by four men undergoing bilateral castration as part of prostate cancer treatment. Testicular cells were isolated by enzymatic digestion (trypsine, collagenase and hyaluronidase in MEM-containing DNase) and cultured in supplemented StemPro-34 medium as previously described for mouse testicular cells (Kanatsu-Shinohara *et al*., 2003), but, importantly, with the omission of feeder cells. When somatic cells, originated from the testicular cell isolation, tended to overgrow the culture, differential passaging was performed. After their initial appearance, sharp-edged, compact colonies, which morphologically resembled ES colonies, were picked and subcultured as described earlier (Xu *et al*., 2001) with some modifications. Briefly, individual colonies were subcultured, after collection from the main cultures, again without feeder cells, in ES cell medium (DMEM-KO medium), 20% ES qualified FCS, 2-mM L-glutamine, 1% non-essential amino acids, 1× Insulin-Transferrin-Selenium (all Gibco, Carlsbad, CA), 25-ng/ml bFGF and 0.1-mM β-mercaptoethanol (both, Sigma, St. Louis, MO) on Matrigel (Becton Dickinson, Bedford, MA) coated culture dishes. Cells were split each week 1:3 by scraping and transferring them to new matrigel-coated dishes and the cells were successfully frozen-thawed multiple times. Human ES cells used as controls (line ES[4]) were cultured as described elsewhere (Raya *et al*., 2008).

To study the potency of these testis-derived ES-like cells, *in vitro* directed differentiation was applied to obtain derivatives of all three germ layers. Differentiation into epidermal (Bagutti *et al*., 1996), neural (Strubing *et al*., 1995) and osteogenic cells (Sottile *et al*., 2003) was performed following the hanging drop method. Differentiation into myogenic cells was induced with the addition of 1% DMSO in the medium (Zheng *et al*., 2006). Pancreatic cell lineage differentiation was performed as described previously (Baharvand *et al*., 2006), but with omission of nestin-positive cell selection.

Characterization of undifferentiated and differentiated testis-derived ES-like cells

RT–PCR

RNA was extracted from testis-derived ES-like cells, testis tissue and ES[4] human ES cells. A quantity of 1 μg of total RNA from hES cells or germline stem cells or 0.03 μg of mRNA from testis tissue was used in a reverse transcriptase reaction with random primers and M-MLV reverse transcriptase (Invitrogen Carlsbad, CA). PCR amplification was performed using gene-specific forward and reverse primers (Supplementary Table S1) as follows: 3 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at a specific annealing temperature for each primer (*Pou5f1* [touchdown 70, 69, 68, 66, 64, 62, 60, 58, 57 (28x)°C], *sox2* 55°C, *Cripto* [touchdown 64, 60 (2×), 56 (2×), 52 (4×), 48 (26×)°C], *Dnmt3b* 58°C, *Plzf* 55°C and *Tbp* 59°C), 1 min 72°C and a final elongation of 5 min at 72°C.

Immunofluorescence

For characterization, testis-derived ES-like cells were fixed in 80% methanol or 4% paraformaldehyde. The following antibodies were applied: anti-POU5F1 (sc-8629, Santa Cruz, CA), anti-NANOG (MAB1997, R&D Systems, Minneapolis, MN), anti-SOX2 (Y-17, sc-17 320, Santa Cruz), anti-SSEA4 (MAB4304, Chemicon, Billerica, MA), anti-SSEA1 (ab-16 285, Abcam, Cambridge, UK), anti-TRA-1-60 (ab-16288, Abcam) and anti-TRA-1-81 (NB100-1833, Novus Biologicals, Littleton, CO). Prior to antibody incubation for POU5F1, NANOG and SOX2, cells

Table I Patient information

Patient	Age (years)	Testis histology	Number of 10^3 cells initial culture	Number of ES-like clones	Intervention before castration
URO.0003	88	Normal spermatogenesis	75	1	Local radiotherapy to the prostate 10 years before orchidectomy
URO.0008	61	Focal full spermatogenesis	Low/ND	8	None
URO.0012	95	Focal full spermatogenesis	1.3	2	None
URO.0021	67	Poor spermatogenesis	270	∼90	None

ND = not determined. The number of human testis derived ES-like colonies that developed in each culture experiment varied between patients.

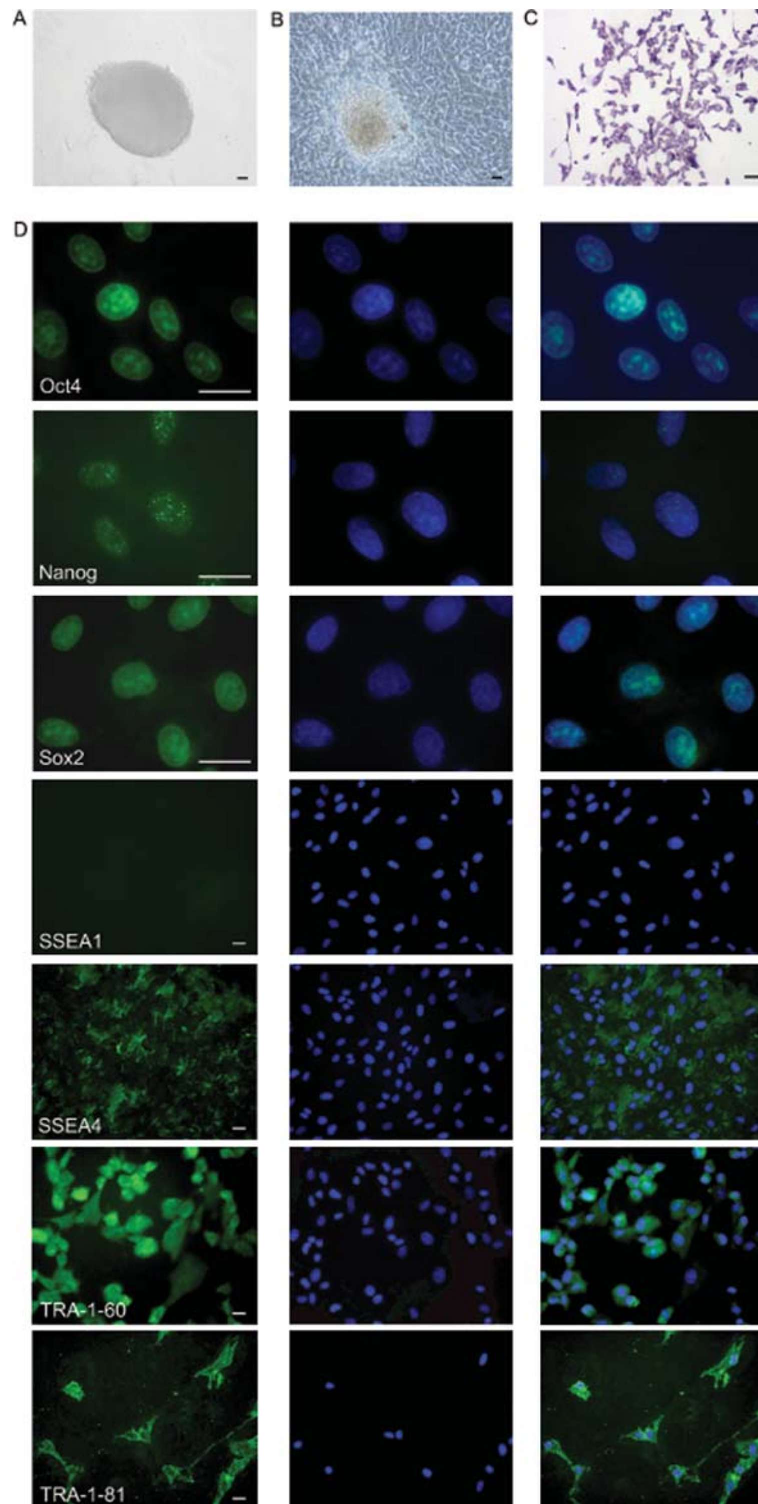

Figure 1 Characterization of ES-like cells. (**A**) Digital interference contrast image showing a sharp-edged and compact ES-like colony. (**B**) Phase contrast image depicting outgrowth of ES-like colony on matrigel. (**C**) Bright field image showing alkaline phosphatase activity of ES-like cells grown on matrigel as a monolayer. (**D**) Immunofluorescence staining of human adult ES-like cells for POU5F1 (OCT-4), NANOG, SOX2, SSEA1, SSEA4, TRA-1-60, TRA-1-81 (left panel). The middle panel shows DAPI staining and the right panel shows the merged images. Bars represent 60 μm (a,b,c) and 20 μm (d).

were pretreated with 0.1% Triton X-100. After incubation of primary antibodies and washing, cells were incubated with rabbit anti-mouse FITC (F 7506, Sigma) for SSEA4, SSEA1, Tra-1-60, TRA-1-81, donkey anti-goat FITC (Jackson, Maine) for POU5F1 and SOX2 and goat anti-rat Alexa 488 (A11 006, Molecular Probes, Carlsbad, CA) for NANOG. As negative control isotype, IgG or blocking peptide was used. Cells were examined using a fluorescence microscope (Leica DMRA).

Immunocytochemistry

To detect the differentiation of testis-derived ES-like cells into particular lineages, immunocytochemistry was applied with the following antibodies: for neural derivatives, anti-neurofilament (NF, RT97, HybridomaBank, The University of Iowa, Iowa) and anti-glial fibrillary acidic protein (GFAP, Z 0334, DakoCytomation, Glosturp, Denmark); for epidermal cell differentiation, anti-cytokeratin 14 (CK14, B429) (sc-58 723, Santa Cruz); for osteogenic differentiation, anti-osteocalcin (OC-30, ab-134180, Abcam); for myogenic differentiation, anti-α-smooth muscle actin antibody (MU128-UC, BioGenex, San Ramon, CA); and for insulin-producing cells, anti-c-peptide (RE11 247C100, BioVendor, Candler, North Carolina). Formalin-fixed cells were treated with Triton X-100 and endogenous peroxidase was blocked with 0.35% peroxidase. After blocking with 5% goat serum and 1% BSA, and overnight incubation with the primary antibody at 4°C, cells were incubated with PowerVision poly HRP-anti-mouse IgG, (#DPVM-110HRP, Immunologic, ImmunoVision Tech. Co., Brisbane, CA) for 30 min at room temperature, and signal was visualized with 3,3′-diaminobenzidinetetrahydrochloride and counterstained with hematoxylin. The following reference tissues were used as positive controls: brain tissue for NF and GFAP stainings, skin tissue for CK14 staining, testis tissue for α-SMA staining, placenta tissue for OC-30 staining and pancreas tissue for c-peptide staining. The corresponding IgGs were applied to differentiated ES-like cells as a negative control.

Alkaline phosphatase activity

Alkaline phosphatase activity as a marker for undifferentiated ES-like cells was demonstrated with NBT/BCIP (Roche) according to the manufacturer's instructions.

Histochemical stainings

To detect insoluble calcium and phosphate deposition, Alizarin Red S and von Kossa staining were performed, respectively (Sottile *et al*., 2003; Huitema *et al*., 2006; Zheng *et al*., 2006).

To show the lipid droplet accumulations within the cells, Oil Red O staining was performed (Pittenger *et al*., 1999).

Quantification of insulin secretion

Secreted insulin in culture medium was detected with the IMMULITE@ 2000 Insulin kit (L2KIN2, Siemens). Medium was collected from differentiated cells cultured for 8 h without N-2 supplement (Invitrogen) and serum to check the basal insulin secretion. To detect glucose-induced insulin release, 10-mM glucose in Krebs-Ringer bicarbonate solution was added to the differentiated cells for 2.5 h as described previously (Sagev *et al*., 2004) after an initial culture of 5.5 h in medium without N-2 supplement and serum.

Teratoma induction

Testis-derived ES-like cells in matrigel were injected into NMRI nu/nu mice (Charles River, The Netherlands) or severe combined immunodeficient (SCID) beige mice (Charles River Laboratories, France) (Supplementary Table S2). The procedures were approved and performed according to the regulations provided by the animal ethics committee of the Academic Medical Center of the University of Amsterdam and the Barcelona Biomedical Research Park. Mice were sacrificed 9–20 weeks post-injection and the injection sites were dissected and microscopically assessed for the presence of teratomas using standard

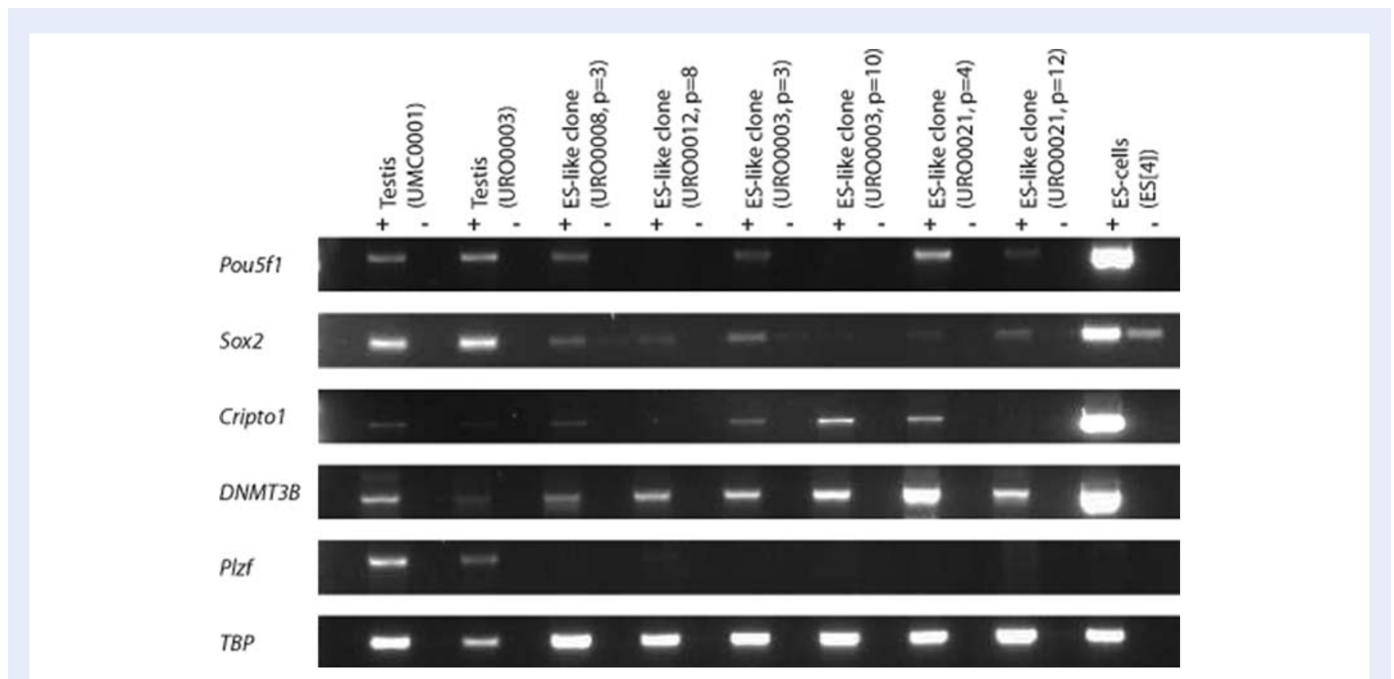

Figure 2 Characterization of ES-like cells at the genomic level. Gel electrophoresis of RT–PCR products of ES cell-specific genes (*Pou5f1, Sox2, Cripto1, Dnmt3b*), a spermatogonial-specific gene (*Plzf*) and a ubiquitously expressed gene *Tata box-binding protein (TBP*) in normal human testis, ES-like cells from all four men at several passage numbers and a human ES cell line (ES[4]).

hematoxylin/eosin staining. To detect the presence of remaining cells of human origin at the transplanted site, we performed HLA immunostaining on cryosections, which were treated with 0.1% Triton X-100, 5% goat serum and 1% BSA in PBS for 30 min at RT and subsequently with HLA-ABC:fITC antibody for an hour at RT. For the secondary antibody, the sections were submerged into biotin conjugated goat anti-mouse antibody and finally the signal was detected with the AEC system (Sigma-Aldrich).

Karyotyping

To study the chromosomal stability, ES-like cells were synchronized in the cell cycle by starvation for 48 h using serum deprivation culture conditions.

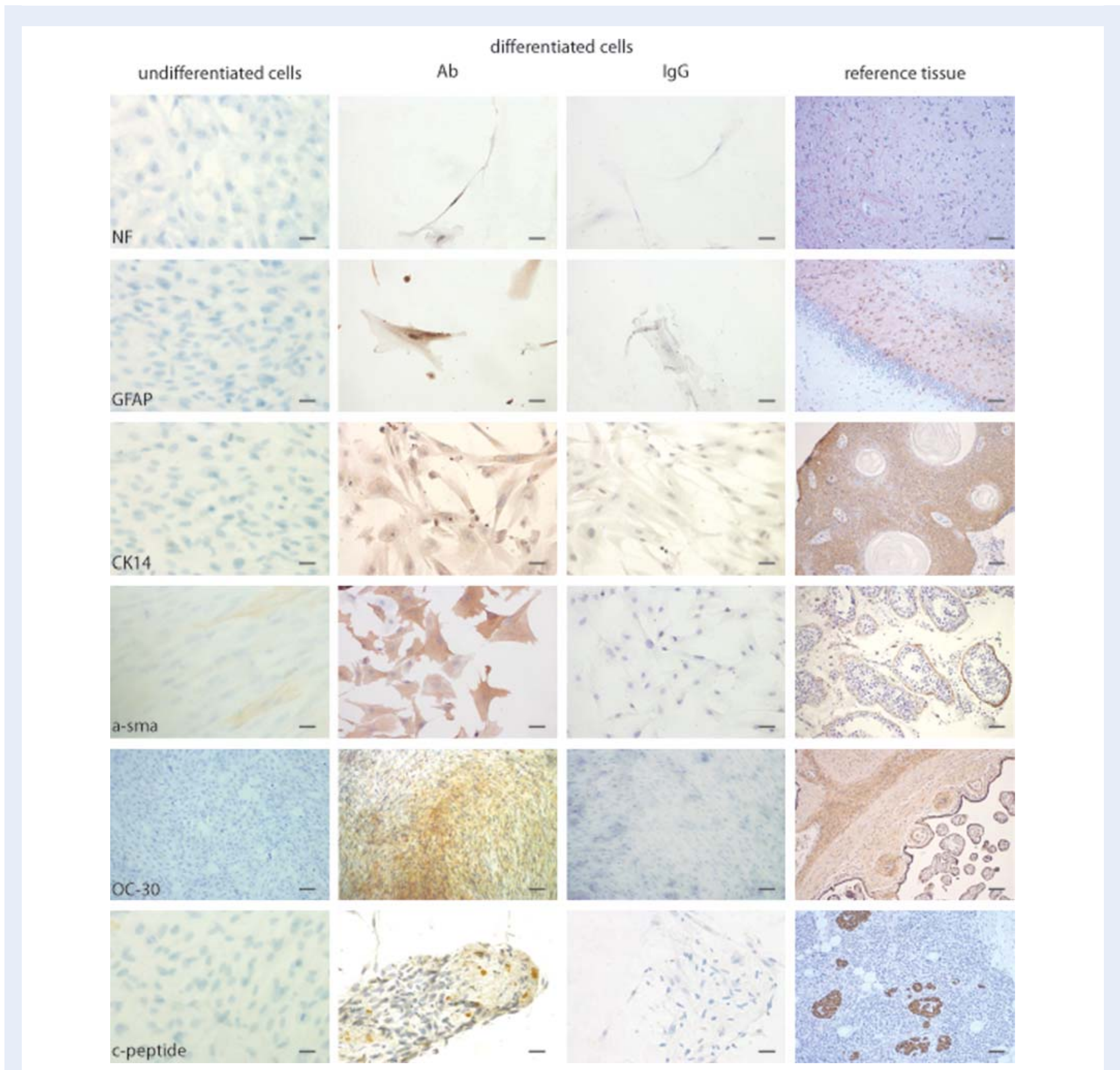

Figure 3 Differentiation potential of ES-like cells *in vitro*. Bright field images showing differentiation into the neural (NF and GFAP) epidermal (CK14), myogenic (α-SMA), osteogenic (OC-30) and pancreatic (c-peptide) lineages. The left panel shows undifferentiated ES-like cells, the second panel shows differentiated cells, the third panel shows the IgG isotype negative control for each staining and the right panel shows the reference tissues as a positive control (from top to bottom: brain tissue for NF and GFAP stainings, skin tissue for CK14 staining, testis tissue for α-SMA staining, placenta tissue for OC-30 staining, pancreas tissue for c-peptide staining). Bars represent 40 μm in NF, GFAP, CK14 and α-SMA images and c-peptide staining on undifferentiated cell images and 60 μm in c-peptide stainings on differentiated cell images and 80 μm in OC-30 staining panels and reference tissue panels.

Cells were treated with Colcemid (Gibco 15 210-057), trypsinized, resuspended in 75-mM KCl hypotonic solution, fixed in MeOH/acetic Acid (3:1) and stained for metaphase spreads using a standard technique (Barch *et al.*, 1997). For each cell line, at least 30 metaphase spreads were examined.

Bisulphite sequencing

Methylation status of *H19*, *Pou5f1* and *Nanog* was investigated in ES-like cells using enriched SSCs as a reference. To obtain enriched SSCs, we performed CD49f (Biolegend 313604) positive cell selection on testicular cells using miniMACS^{TM} separation system (Miltenyi Biotec 130-090-312) in accordance with manufacture's protocol.

Genomic DNA was extracted and conversion of unmethylated cytosines to uracil was carried out using the EpiTect Bisulphite kit (#59 104, QIAGEN) according to the manufacturer's protocol. For each reaction 2 μg of DNA was treated with bisulfite and one-fifth of that converted DNA was used as input for each PCR reaction. Primers for *Pou5f1* and *Nanog* were as described by Deb-Rinker *et al.* (2005) and the primers for H19 were as described by Conrad *et al.* (2008). PCR was performed as follows: initial denaturation for 10 min at 95°C and 38 cycles of 95°C for 1 min, 58°C for 1 min and 72°C for 1 min for *Pou5f1* and *Nanog* and 38 cycles of 95°C for 1 min, 53°C for 1 min and 72°C for 1 min for H19 and followed by 72°C for 10 min. The resultant products were cleaned up with QIAquick PCR purification kit (#28 104, QIAGEN) and cloned (TOPO-TA cloning, Invitrogen) and after that were sequenced. Resultant sequences were aligned to the DNA sequences of the selected loci of the gene of interest on the website of quantification tool for methylation analysis (http://quma.cdb.riken.jp/top/index.html).

Results

Testicular cells were isolated and cultured from testis samples with normal or focal spermatogenesis of four men (Table I). In the cultures of all four samples, we observed the appearance of sharp-edged and compact colonies (Fig. 1A) that resembled true hES colonies. We designated these colonies as ES-like colonies. The number of human testis-derived ES-like colonies that developed in each culture experiment varied between one and more than 90 colonies (Table I). All colonies appeared between 3 and 8 weeks after the initiation of the primary cultures and these colonies could be further subcultured using hES cell-specific conditions on matrigel-coated dishes without a feeder layer for at least 15 weeks for 13 passages (Fig. 1B). Cells were passaged 1:3 every week with an estimated doubling time of 96 h (http://www.chestx-ray.com/SPN/DoublingTime.html) and could be successfully frozen and thawed multiple times.

When subcultured under hES cell-specific conditions, the ES-like cells displayed strong alkaline phosphatase activity (Fig. 1C) and expressed hES cell-specific transcription factors (POU5F1, NANOG, SOX2) and surface markers (SSEA4, TRA-1-60, TRA-1-81) (Fig. 1D). Similarly to hES cells, human testicular-derived ES-like cells were negative for SSEA1 (Fig. 1D). At the transcript level, ES-like cells showed expression of hES cell-specific genes that remained expressed throughout the length of the cultures (Fig. 2).

Upon exposure to *in vitro* directed differentiation conditions, ES-like cells showed the potential to differentiate into derivatives of all three germ layers. More precisely, we confirmed differentiation into the

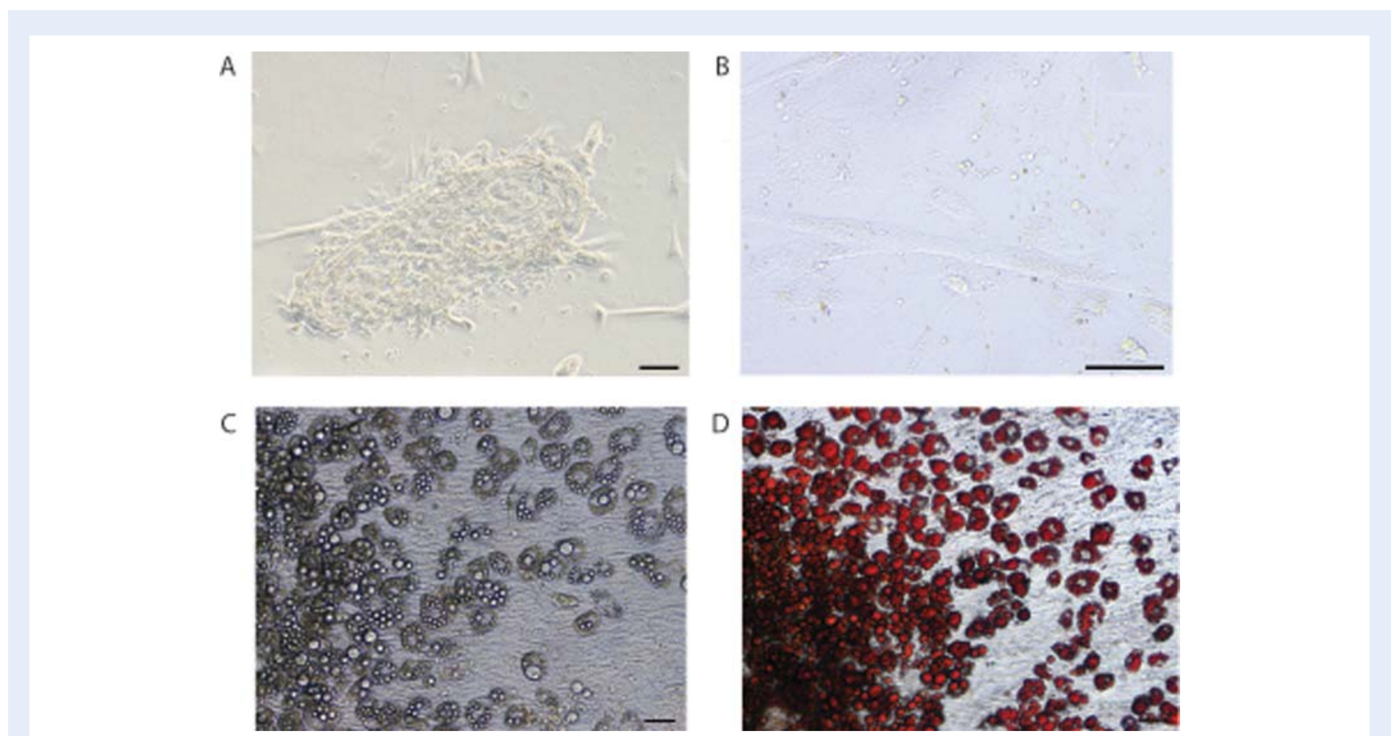

Figure 4 Morphological view and chemical stainings for differentiation. (**A**) Digital interference contrast image showing morphology of an *in vitro*-formed pancreatic islet, bar represents 100 μm. (**B**) Binucleated, elongated muscle cell, bar represents 100 μm. (**C**) Spontaneous differentiation of ES-like cells into adipocytes showing vacuolization in the cytoplasm of the cells, bar represents 50 μm. (**D**) Spontaneous differentiation of ES-like cells into adipocytes showing lipid drops with Oil Red O staining, bar represents 50 μm.

neural lineage by staining with neurofilament (NF) and glial fibrillary acidic protein (GFAP) (Fig. 3). ES-like cells that differentiated into the epithelial lineage stained positive for cytokeratin14 (CK14) while ES-like cells that differentiated into the myogenic lineage stained positive for alpha smooth muscle actin (α-SMA) (Fig. 3). Osteogenic cells derived from ES-like cells clearly showed a layered structure typical of cartilage and stained positive for OC-30, and ES-like cells also had the potential of differentiation into the pancreatic lineage as demonstrated by the presence of c-peptide, i.e. the precursor of insulin (Fig. 3).

Besides the changes in expression upon differentiation as described above, the ES-like cells also showed morphological changes like the formation of islets upon pancreatic differentiation (Fig. 4A) and the presence of multinucleated elongated cells after myogenic differentiation (Fig. 4B). Furthermore, we occasionally observed spontaneous differentiation of ES-like cells into cells with typical adipocyte morphology and accumulation of lipid (Fig. 4C and D).

We then studied the functionality of the differentiated ES-like cells. ES-like cells directed towards pancreatic differentiation were able to increase insulin secretion upon exposure to glucose (Fig. 5A). In addition, ES-like cells directed towards osteogenic differentiation deposited both calcium and phosphate (Fig. 5B).

ES-like cells from three patients (URO0008 ES-like clone 2 passage 8, URO0012 ES-like clone 2 passage 12 and URO0021 ES-like clone 2 passage 9) were karyotyped and all three showed a normal 46 XY karyotype (Fig. 6).

We also studied the methylation status of *H19*, *Pou5f1* and *Nanog* in enriched SSCs and ES-like clones from all patients. As compared with enriched SSCs, the ES-like cells showed increased demethylation for all three genes (Fig. 7).

To investigate the ability of ES-like cells to give rise to teratomas, we injected a total of 44 mice of two different immunocompromised strains commonly used to assay teratoma (NMRI nu/nu and SCID) with varying amounts of cells (from 10^6 to 10^7 cells per injection) from early and later passages (passage number ranging from 4 to 7) at several injection sites (subcutaneous, i.m. and intratesticular). Mice were sacrificed and analyzed for teratoma formation after 9–20 weeks (Supplementary Table S2). Although we observed small areas with differentiated cell types of human origin (Fig. 8), we did not observe extensive teratomas in any of the 44 injected mice, whereas hES or iPS cells injected in parallel readily gave rise to complex teratomas (data not shown).

Discussion

Our results demonstrate the spontaneous *in vitro* generation of ES-like cells from adult human testis under conditions known to support long-term proliferation of SSCs. These ES-like cells expressed hES cell-specific markers, and could be subcultured for 15 weeks and stay karyotypically normal. In addition these ES-like cells could differentiate into cells of all germ layers *in vitro* and produce functional beta cells and adipocytes, but did not contribute to teratoma formation *in vivo*. As such, these ES-like cells could potentially be highly useful for future stem cell-based clinical therapies.

The difference between our study and the very recently published studies (Conrad *et al.*, 2008; Golestaneh *et al.*, 2009; Kossack *et al.*, 2009) on human ES-like cells from adult testis lies in the culture method used. To generate ES-like colonies, we used culture conditions known to support the long-term proliferation of SSCs (Kanatsu-Shinohara *et al.*, 2004), whereas the others used culture conditions that favor the growth of ES cells. Our results together with the previous papers on ES-like cells derived from adult human testis indicate that the culture conditions used to generate ES-like cells are not that critical. Future studies should determine the most efficient method to generate ES-like cells from human testis.

Although the ES-like colonies in our experiments arose from human testicular cells that support SSCs, the precise origin of these cells remains to be established since we did not use a rigorous preselection procedure but rather used a mixed culture system of various testicular

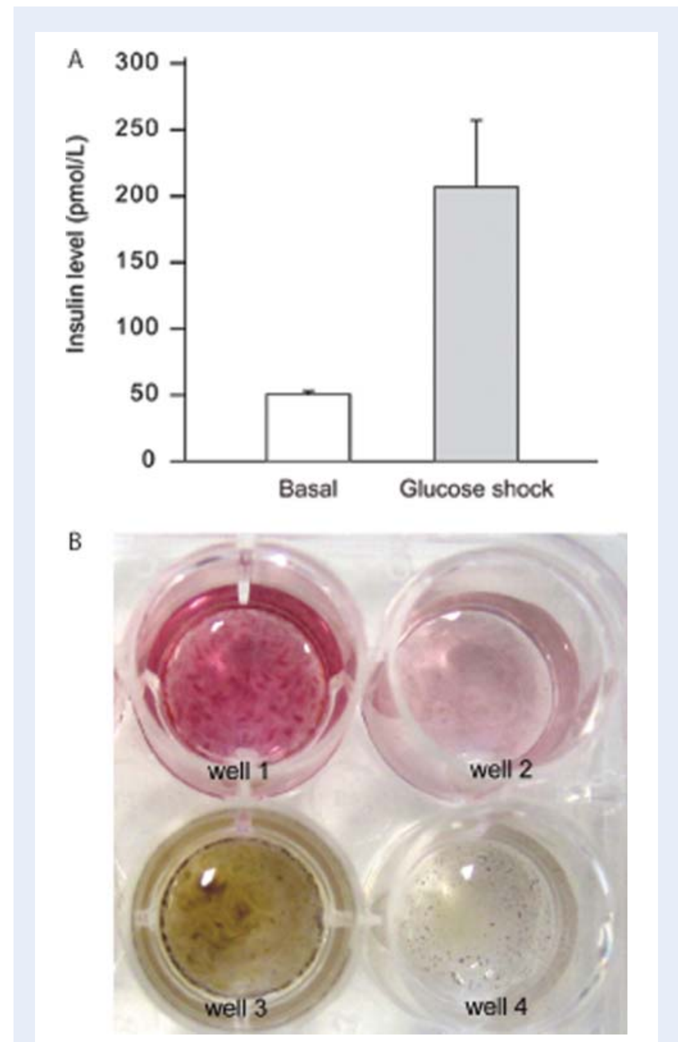

Figure 5 Functionality of *in vitro* differentiated ES-like cells. (**A**) Histogram showing the active secretion of insulin in response to glucose by ES-like cells differentiated into functional beta cells. (**B**) Histochemical analysis of ES-like cells differentiated into the osteogenic lineage. Wells 1 and 2 show calcium deposition (Alizarin Red staining). Well 1 shows the *in vitro* differentiated ES-like cells while well 2 shows undifferentiated parallel cultured cells from the same patient as a negative control. Phosphate deposition was detected with von Kossa staining in osteogenic differentiated ES-like cells (Well 3), and undifferentiated ES-like cells were used as controls (Well 4).

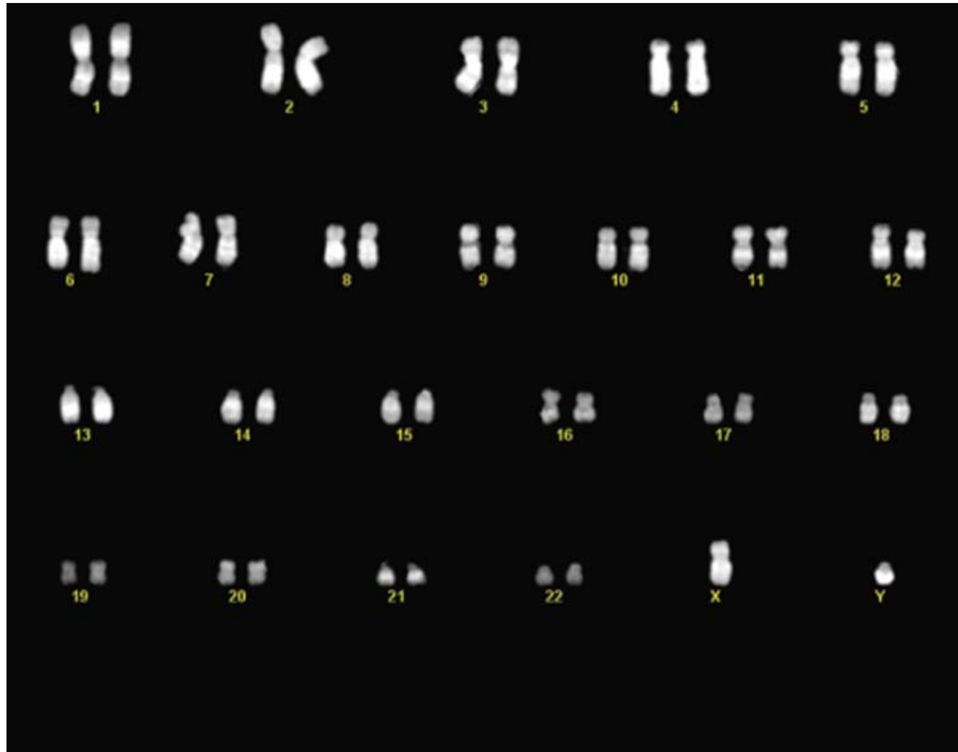

Figure 6 Karyotype analysis of one of the ES-like cell lines. The ES-like clones showed a normal 46 XY karyotype.

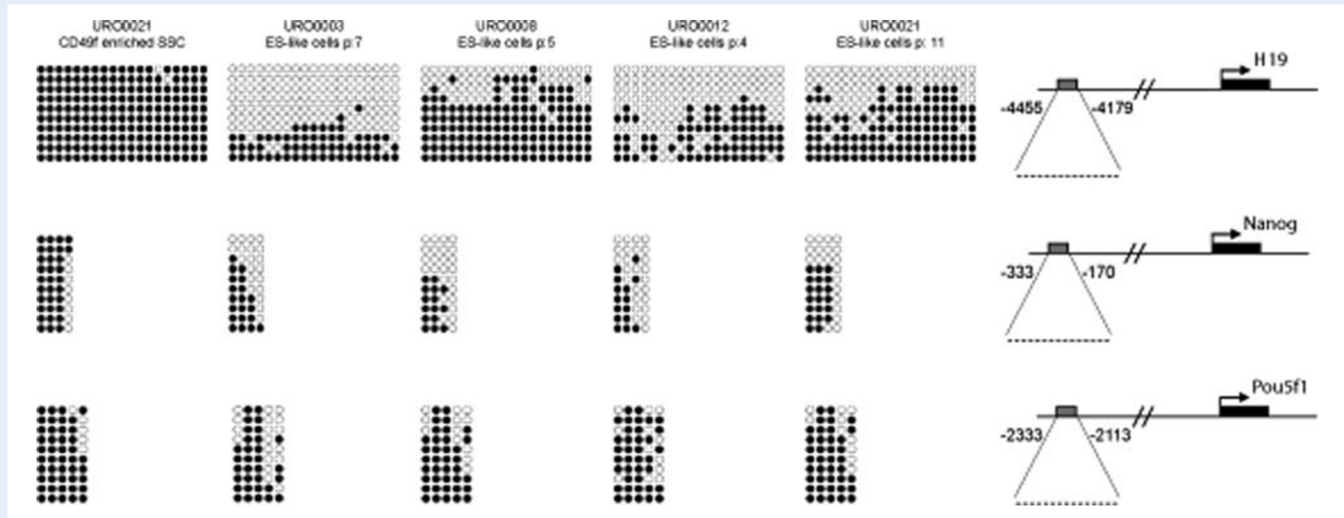

Figure 7 Epigenetic status of H19, Nanog and Pou5f1 genes in the ES-like cell lines. Methylation status of *H19*, *Pou5f1* and *Nanog* in ES-like cells, as compared with the enriched SSCs, showed increased demethylation.

cells, including Sertoli and peritubular cells. Studies in mice using GPR125 as a germ cell marker indicate that the ES-like cells are of germ cell origin, making SSCs the most likely candidates (Guan *et al.*, 2006; Seandel *et al.*, 2007). Unfortunately, no unique human or mouse SSC markers are currently available to address this question directly.

Although we succeeded in the generation of ES-like colonies from all four men included in our study, the occurrence of these colonies seemed a stochastic, and as yet uncontrollable, process and as a result the number of colonies derived from each sample varied. This variation might depend on the age of the patient and/or the number of stem cells present within the testis biopsy. Future studies

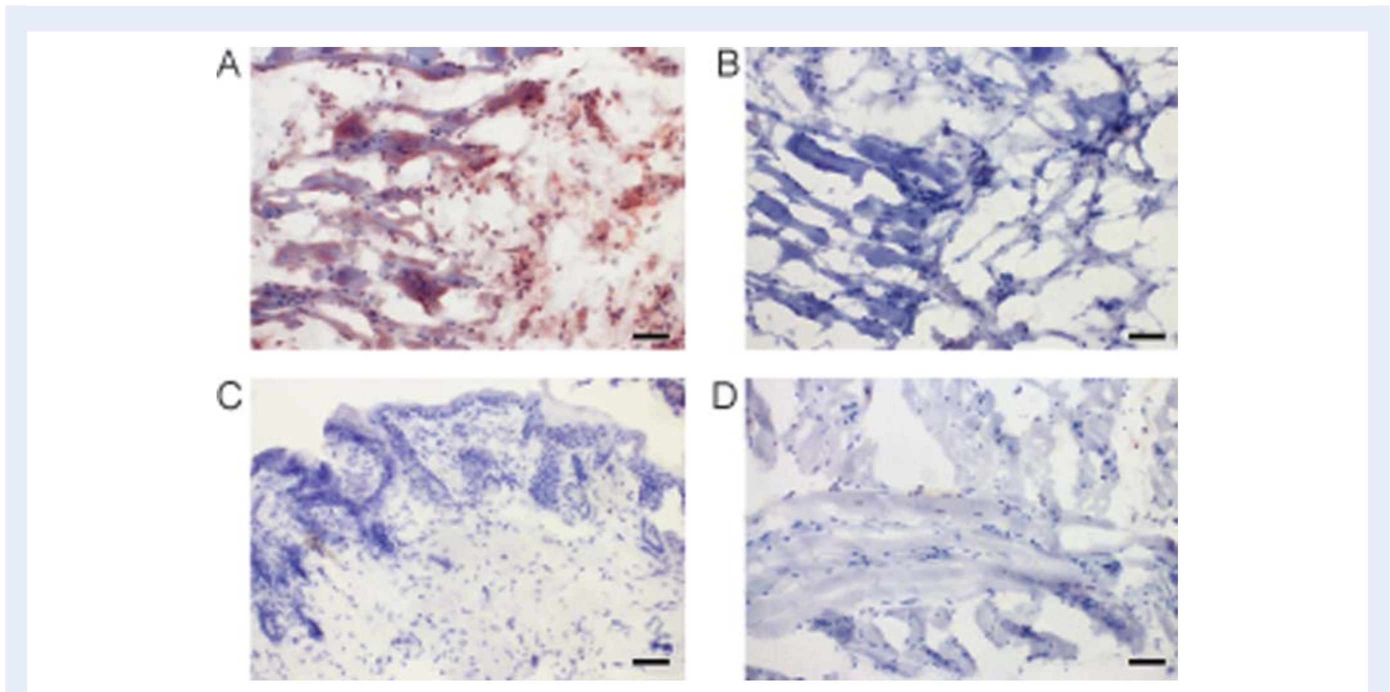

Figure 8 HLA staining of *in vivo* differentiated ES-like cells to detect cells of human origin. (**A**) HLA staining on *in vivo* differentiated ES-like cells. (**B**) IgG application on *in vivo* differentiated ES-like cells as a negative control. Mouse skin (**C**) and muscle (**D**) are shown to indicate HLA specificity to human cells. No cross-reaction on mouse tissue was seen indicating the specificity of HLA staining for human tissue.

will need to determine the precise pathway by which ES-like cells arise from human testis to both control the timing of their occurrence and to increase the efficiency of the procedure.

Teratoma formation is a hallmark of pluripotent cells but, at the same time, constitutes a major drawback for their potential therapeutic application. Although we were able to show the presence of human cells at the injection site 9 weeks post-injection, we did not observe teratomas in any of the 44 injected mice. The human testis-derived ES-like cells recently described showed in general organized but rather small-sized non-extensive teratoma formation upon injection into immunocompromised mice and not in all cases (Conrad *et al.*, 2008; Golestaneh *et al.*, 2009; Kossack *et al.*, 2009). This suggests that the ES-like cells derived from human testis might have lost some of their pluripotency or may have not been fully reprogrammed to pluripotency during their culture *in vitro*.

In conclusion, our results show that adult human testis-derived ES-like cells can differentiate into cell types of all germ layers with functional capabilities *in vitro*. Thus, testis-derived human ES-like cells are potentially useful for future stem cell-based clinical therapies as part of regenerative medicine. Their easy accessibility and ethical acceptance make these cells an attractive alternative to human ES cells, at least for male patients.

Authors' Roles

S.C.M.: conception and design, collection and/or assembly of the data, data analysis and interpretation, manuscript writing, final approval of the manuscript. J.V.C., H.S-A., S.D., C.M.K., S.E.H., H.L.R.-G., K.F. and A.C.K.: collection and/or assembly of the data. A.R.: collection and/or assembly of the data, final approval of the manuscript. Th.M.R. and J.J.M.C.H.R.: provision of study material or patients. J.C.B.: final approval of the manuscript. F.V.: manuscript writing, final approval of the manuscript. D.G.R., S.R. and A.M.M.P.: conception and design, data analysis and interpretation, manuscript writing, final approval of the manuscript.

Supplementary Data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Acknowledgements

We thank H. Skaletsky for providing us with the primer sequences for RT–PCR, R. Vassena for help with the teratoma experiments, T. de Haas for help with histological analysis of testis sections, F.M.F. van Dissel-Emiliani, J. Middeldorp and K.W.A. van Grinsven for providing antibodies and reagents, M. Visser for providing sections of reference tissues, E.G. de Boer for karyotyping experiments and Yueh-Chiang Hu and Gregoriy Dokshin for contribution to the epigenetics studies. Human brain tissue was obtained from The Netherlands Brain Bank, Netherlands Institute for Neuroscience.

Funding

This study was supported by the European Science Foundation EUROCORES Programme EuroSTELLS, is granted by ZonMW 910-20-032.

References

Bagutti C, Wobus AM, Fassler R, Watt FM. Differentiation of embryonal stem cells into keratinocytes: comparison of wild-type and beta 1 integrin-deficient cells. *Dev Biol* 1996;**179**:184–196.

Baharvand H, Jafary H, Massumi M, Ashtiani SK. Generation of insulin-secreting cells from human embryonic stem cells. *Dev Growth Differ* 2006;**48**:323–332.

Barch MJ, Knutsen T, Spurbeck JL. *The AGT Cytogenetics Laboratory Manual*. Philadelphia: Lippincott-Raven Publishers, 1997.

Conrad S, Renninger M, Hennenlotter J, Wiesner T, Just L, Bonin M, Aicher W, Buhring HJ, Mattheus U, Mack A *et al*. Generation of pluripotent stem cells from adult human testis. *Nature* 2008; **456**:344–349.

Cowan CA, Klimanskaya I, McMahon J, Atienza J, Witmyer J, Zucker JP, Wang S, Morton CC, McMahon AP, Powers D *et al*. Derivation of embryonic stem-cell lines from human blastocysts. *N Engl J Med* 2004; **350**:1353–1356.

Deb-Rinker P, Ly D, Jezierski A, Sikorska M, Walker PR. Sequential DNA methylation of the Nanog and Oct-4 upstream regions in human NT2 cells during neuronal differentiation. *J Biol Chem* 2005;**280**: 6257–6260.

de Rooij DG, Mizrak SC. Deriving multipotent stem cells from mouse spermatogonial stem cells: a new tool for developmental and clinical research. *Development* 2008;**135**:2207–2213.

Gearhart J, Pashos EE, Prasad MK. Pluripotency redux–advances in stem-cell research. *N Engl J Med* 2007;**357**:1469–1472.

Golestaneh N, Kokkinaki M, Pant D, Jiang J, Destefano D, Fernandez-Bueno C, Rome JD, Haddad BR, Gallicano GI, Dym M. Pluripotent stem cells derived from adult human testes. *Stem Cells Dev* 2009. Epub ahead of print. PMID: 19281326.

Guan K, Nayernia K, Maier LS, Wagner S, Dressel R, Lee JH, Nolte J, Wolf F, Li M, Engel W *et al*. Pluripotency of spermatogonial stem cells from adult mouse testis. *Nature* 2006;**440**:1199–1203.

Huitema LF, Vaandrager AB, van Weeren PR, Barneveld A, Helms JB, van de Lest CH. The nitric oxide donor sodium nitroprusside inhibits mineralization in ATDC5 cells. *Calcif Tissue Int* 2006;**78**:171–177.

Hyun I, Hochedlinger K, Jaenisch R, Yamanaka S. New advances in iPS cell research do not obviate the need for human embryonic stem cells. *Cell Stem Cell* 2007;**1**:367–368.

Kanatsu-Shinohara M, Ogonuki N, Inoue K, Miki H, Ogura A, Toyokuni S, Shinohara T. Long-term proliferation in culture and germline transmission of mouse male germline stem cells. *Biol Reprod* 2003; **69**:612–616.

Kanatsu-Shinohara M, Inoue K, Lee J, Yoshimoto M, Ogonuki N, Miki H, Baba S, Kato T, Kazuki Y, Toyokuni S *et al*. Generation of pluripotent stem cells from neonatal mouse testis. *Cell* 2004;**119**:1001–1012.

Kossack N, Meneses J, Shefi S, Nguyen HN, Chavez S, Nicholas C, Gromoll J, Turek PJ, Reijo-Pera RA. Isolation and characterization of pluripotent human spermatogonial stem cell-derived cells. *Stem Cells* 2009;**27**:138–149.

Lowry WE, Richter L, Yachechko R, Pyle AD, Tchieu J, Sridharan R, Clark AT, Plath K. Generation of human induced pluripotent stem cells from dermal fibroblasts. *Proc Natl Acad Sci U S A* 2008; **105**:2883–2888.

Mali P, Ye Z, Hommond HH, Yu X, Lin J, Chen G, Zou J, Cheng L. Improved efficiency and pace of generating induced pluripotent stem cells from human adult and fetal fibroblasts. *Stem Cells* 2008;**26**: 1998–2005.

Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, Okita K, Mochiduki Y, Takizawa N, Yamanaka S. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat Biotechnol* 2008;**26**:101–106.

Park IH, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, Lerou PH, Lensch MW, Daley GQ. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* 2008;**451**:141–146.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999; **284**:143–147.

Raya A, Rodriguez-Piza I, Aran B, Consiglio A, Barri PN, Veiga A, Izpisua-Belmonte JC. Generation of cardiomyocytes from new human embryonic stem cell lines derived from poor-quality blastocysts. *Cold Spring Harb Symp Quant Biol* 2008;**73**:127–135.

Sagev H, Fishman B, Ziskind A, Shulman M, Iskovitz-Eldor J. Differentiation of human embryonic stem cells into insulin-producing cells. *Stem Cells* 2004;**22**:265–274.

Seandel M, James D, Shmelkov SV, Falciatori I, Kim J, Chavala S, Scherr DS, Zhang F, Torres R, Gale NW *et al*. Generation of functional multipotent adult stem cells from GPR125+ germline progenitors. *Nature* 2007; **449**:346–350.

Sottile V, Thomson A, McWhir J. In vitro osteogenic differentiation of human ES cells. *Cloning Stem Cells* 2003;**5**:149–155.

Strubing C, Ahnert-Hilger G, Shan J, Wiedenmann B, Hescheler J, Wobus AM. Differentiation of pluripotent embryonic stem cells into the neuronal lineage in vitro gives rise to mature inhibitory and excitatory neurons. *Mech Dev* 1995;**53**:275–287.

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007;**131**:861–872.

Wernig M, Meissner A, Cassady JP, Jaenisch R. c-Myc is dispensable for direct reprogramming of mouse fibroblasts. *Cell Stem Cell* 2008; **2**:10–12.

Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, Carpenter MK. Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol* 2001;**19**:971–974.

Zheng JK, Wang Y, Karandikar A, Wang Q, Gai H, Liu AL, Peng C, Sheng HZ. Skeletal myogenesis by human embryonic stem cells. *Cell Res* 2006;**16**:713–722.

Submitted on October 15, 2008; resubmitted on August 20, 2009; accepted on September 1, 2009