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Human embryonic stem cell lines derived from single blastomeres

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The derivation of human embryonic stem (hES) cells currently requires the destruction of *ex utero* embryos[1–4]. A previous study in mice indicates that it might be possible to generate embryonic stem (ES) cells using a single-cell biopsy similar to that used in preimplantation genetic diagnosis (PGD), which does not interfere with the embryo's developmental potential[5]. By growing the single blastomere overnight, the resulting cells could be used for both genetic testing and stem cell derivation without affecting the clinical outcome of the procedure. Here we report a series of ten separate experiments demonstrating that hES cells can be derived from single blastomeres. Nineteen ES-cell-like outgrowths and two stable hES cell lines were obtained. The latter hES cell lines maintained undifferentiated proliferation for more than eight months, and showed normal karyotype and expression of markers of pluripotency, including Oct-4, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, nanog and alkaline phosphatase. These cells retained the potential to form derivatives of all three embryonic germ layers both *in vitro* and in teratomas. The ability to create new stem cell lines and therapies without destroying embryos would address the ethical concerns of many, and allow the generation of matched tissue for children and siblings born from transferred PGD embryos.

A series of experiments was carried out to determine whether hES cells can be derived from single blastomeres (Supplementary Table 1). Unused embryos produced by *in vitro* fertilization (IVF) for clinical purposes were obtained with full informed consent and used in compliance with Advanced Cell Technology's ethics advisory board and institutional review board. Sixteen pronuclear- and multicell-stage embryos were thawed and cultured to the 8–10-cell stage in 20-μl microdrops of Quinn's cleavage medium under paraffin oil (see Methods). Six of these embryos were grade I or II (symmetrical and even cell division with little or no cytoplasmic fragmentation), whereas the remaining ten embryos were grade III (variable fragmentation), using a standard scoring system[6]; embryos with blastomeres of unequal size and moderate-to-severe fragmentation (grades IV and V) were excluded from this study. The zona pellucida was disrupted and individual blastomeres mechanically separated from the denuded embryos using a micropipette and gentle tapping of the pipette holder. The separated blastomeres were cultured together in the same medium, arranged so as to avoid contact with each other using depressions created in the bottom of the plastic tissue culture plate, as described previously[7].

The majority (58%) of the isolated blastomeres divided at least once, and approximately half of these (28 out of 53) formed vesicles/clumps that produced outgrowths within two days (Fig. 1). Previous experiments in mice[5] indicated that cell co-culture is important for ES cell derivation from single blastomeres. However, the aggregation system used in these previous studies could not be employed, because—unlike in the mouse—human blastomeres do not form tight aggregates with ES cells. Therefore, microdrops containing the blastomere-derived vesicles/clumps were merged with microdrops seeded with mitomycin-C-treated mouse embryonic fibroblasts (MEFs) and green fluorescent protein (GFP)-positive hES cells. When the outgrowths reached approximately 50–100 cells, they were mechanically passaged onto fresh feeders in microdrops containing hES cell medium. Although the initial outgrowths generally contained cells of different morphologies, over a period of several days we observed a number of fates: (1) cells resembling trophectoderm took over the culture, (2) cells that initially resembled ES cells differentiated within the culture, or (3) ES-cell-like cells continued undifferentiated proliferation. All of these outcomes are typical of derivation of ES cells from human embryos, especially when intact blastocysts are plated out without the removal of the trophectoderm using immunosurgery.

Routine passaging of putative hES cells was performed by mechanical dispersion of the colonies and transfer to fresh feeders every 2–3 days, until there were enough cells for trypsinization (after passage 10; see ref. 8). The colony morphology, growth rate, and procedures and culture media used were very similar to those of blastocyst-derived ES cells[3,8,9]. Two of the six grade I/II embryos generated stable hES cell lines that showed normal karyotype (line MA01, 46,XX; line MA09, 46,XX; Fig. 2h) and molecular markers of pluripotency (Fig. 2a–g). Both lines stain for alkaline phosphatase, and express octamer binding protein 4 (Oct-4), stage-specific embryonic

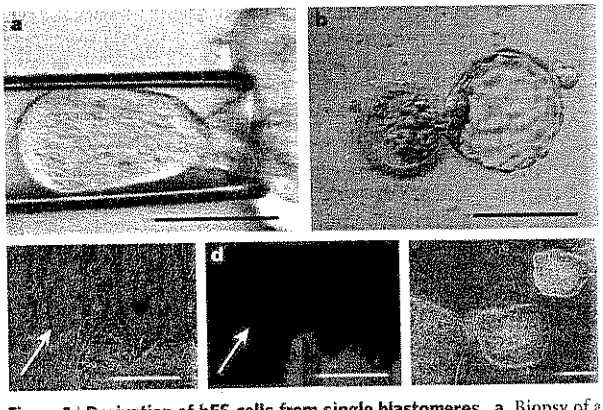

Figure 1 | Derivation of hES cells from single blastomeres. a, Biopsy of a single blastomere. **b**, Development of a blastomere-biopsied embryo into a hatching blastocyst. **c**, **d**, Blastomere-derived outgrowth (arrows) close to a colony of GFP-positive hES cells. The same field of cells is shown under phase contrast (**c**) and fluorescence (**d**). **e**, Morphology of blastomere-derived hES cell colonies.

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antigen (SSEA)-3, SSEA-4, tumour-rejection antigen (TRA)-1-60, and TRA-1-81. Karyotype analysis ruled out fusion (both new lines were female and the WA01 hES cells used for co-culture were male; Fig. 2h), and microsatellite analysis confirmed that MA01 and MA09 were genetically distinct from other hES cell lines grown in our laboratory (Fig. 3d). Polymerase chain reaction (PCR) analysis further confirmed this, indicating the absence of GFP and Y chromosome gene sequences in both of the blastomere-derived hES cell lines (Fig. 3a–c).

When the hES cell cultures were allowed to overgrow or form embryoid bodies, they readily differentiated into cells of all three germ layers, as evidenced by immunostaining with antibodies to α-fetoprotein (primitive endoderm; Fig. 4b), smooth-muscle actin (mesoderm; Fig. 4c) and β III tubulin (ectoderm; Fig. 4d). The single-blastomere-derived hES cells could also be differentiated *in vitro* into cells of specific therapeutic interest, including endothelial cells that, after replating on Matrigel, formed typical capillary/vascular-like structures (Fig. 4e) that expressed high levels of von Willebrand factor and took up acetylated low-density lipoprotein (Fig. 4f). Retinal pigment epithelium (RPE) clusters also appeared in adherent hES cell cultures and in embryoid bodies, and were used to establish, as described previously[10], passageable RPE cell lines that displayed a pigmented phenotype and typical 'cobblestone' morphology (Fig. 4g), bestrophin immunostaining (Fig. 4h), and expressed bestrophin, *RPE65*, *CRALBP* and *PEDF* as shown by PCR with reverse transcription (RT–PCR; Fig. 4i). To demonstrate the pluripotency of the putative ES cells derived in this study, cells were injected into NOD-SCID mice forming teratomas containing tissues from all three germ layers including neural rosettes (ectoderm), haematopoietic cells (mesoderm), and liver, respiratory and intestinal epithelia (endoderm), among others (Fig. 4a). The presence of all three germ layers was also confirmed by immunostaining (Fig. 4a, insets). Although only two of the six (33%) grade I/II embryos (or 2 out of 35 blastomeres; 2 out of 91 blastomeres including grade III–V embryos) used in the current study generated hES cell lines, this success rate is similar to that reported by other investigators using conventional derivation methods[2,3,11,12]. The grade of the leftover embryos proved crucial, and we believe the success rate can be further increased by optimizing conditions at the earliest stages of blastomere outgrowth.

According to the Food and Drug Administration (FDA) regulations, the presence of animal cells in culture systems is permissible under current xenotransplantation guidelines[13]. We are in the process of adapting both of the blastomere-derived hES cell lines (which were derived on mouse feeders) to animal-free conditions under current good manufacturing practice (cGMP) to comply with these requirements. However, it may be desirable to derive future lines using human feeder cells, or feeder-free systems such as those that have been described previously by our group[9] and others[14–16].

Additional studies will also be necessary to determine whether blastomere-derived hES cell lines differ from conventional hES cell lines in their ability to form functional differentiated cell types. Blastomere-derived lines MA01 and MA09 were observed to differentiate, at the very least, in the same manner and at the same rate as all other hES cell lines studied in our laboratory. Furthermore, it seems that they may more readily differentiate into certain vital cell types, although it is unclear whether this is related to the derivation technique or number of passages by trypsinization. For instance, neural progenitors were readily generated without the need for embryoid-body intermediates, stromal feeder layers, or low-density passaging. When transferred to a laminin-coated substrate and maintained in defined medium containing laminin and basic fibroblast growth factor (FGF2), they began to express neural and neural progenitor markers, including nestin, β III tubulin and Pax6, at the second passage. MA01 hES cells also formed haematopoietic colony forming units (CFUs) 3–5 times more efficiently than WA01 GFP cells and 5–10 times more efficiently than WA09 cells. (NIH-registered WA01 and WA09 hES cell lines were formerly known as H1 and H9, respectively[2].) MA09 hES cells showed similar potential as WA09 cells for haematopoietic differentiation, but demonstrated a higher capability to differentiate towards the endothelial lineage as compared with both WA01-GFP and WA09 cells (data not shown).

Concerns have been raised as to whether individual eight-cell-stage blastomeres, such as those used in the current study, are totipotent and could potentially generate a human being. A recent report shows the localization of cell fate determinants (*Cdx2*) in the late-dividing blastomeres of the two-cell-stage mouse embryo[17]. Other studies show that at the four-cell stage, blastomeres have different developmental properties[18], and that individual human

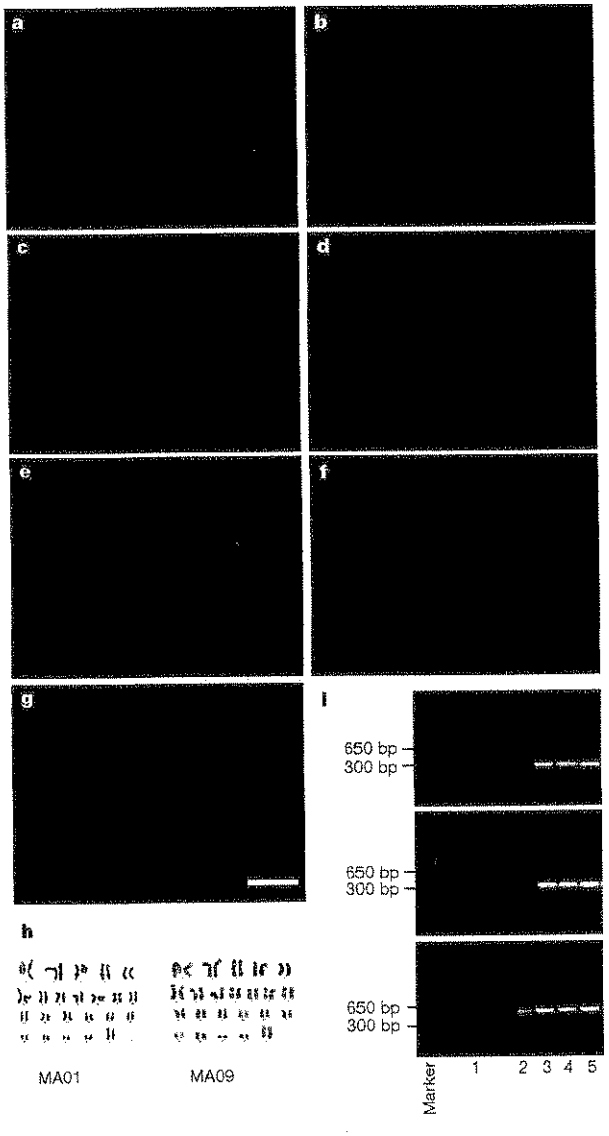

Figure 2 | Characterization of hES cells from single blastomeres. **a–g**, Staining for markers of pluripotency, showing Oct-4 (**a**) and corresponding DAPI staining (**b**), TRA-1-60 (**c**), TRA-1-81 (**d**), SSEA-3 (**e**), SSEA-4 (**f**) and alkaline phosphatase (**g**). Scale bar, 200 μm. **h**, Representative chromosome spreads of the two single-blastomere-derived hES cell lines. **i**, RT–PCR analysis of the expression of markers of pluripotency in single-blastomere-derived hES cell lines. Top panel, *Oct-4*; centre panel, nanog; bottom panel, *GAPDH*. Lane 1, no template; lane 2, negative control (MEFs); lane 3, MA01; lane 4, MA09; lane 5, WA01.

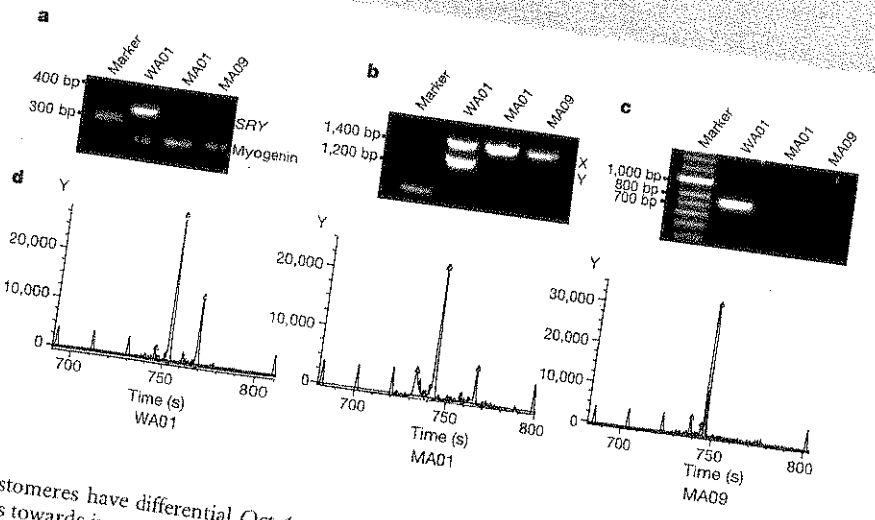

Figure 3 | Microsatellite and PCR analysis of single-blastomere-derived hES cells versus the cell line (WA01) used for co-culture. **a**, **b**, *SRY* (**a**) and amelogenin (**b**) genes (cropped image). **c**, GFP sequences (cropped image). **d**, Microsatellite analysis. Full-length gel images are presented in Supplementary Fig. 1.

blastomeres have differential *Oct-4* expression that seems to direct cells towards inner cell mass or trophoectoderm lineages[19]. Notably, individual morula (8–16-cell)-stage blastomeres have never been shown to have the intrinsic capacity to generate a complete organism in any mammalian species.

Previous attempts to induce isolated human blastomeres to proliferate into pluripotent stem cell lines have failed[20,21]. One study[20] observed proliferation of blastomeres (in the range of 1–8 cells per blastomere) from cleavage-stage human embryos *in vitro*, although in all cases differential labelling indicated that they had generated trophoectodermal cells. Here we demonstrate that single blastomeres can be used to establish hES cell lines using an approach that does not

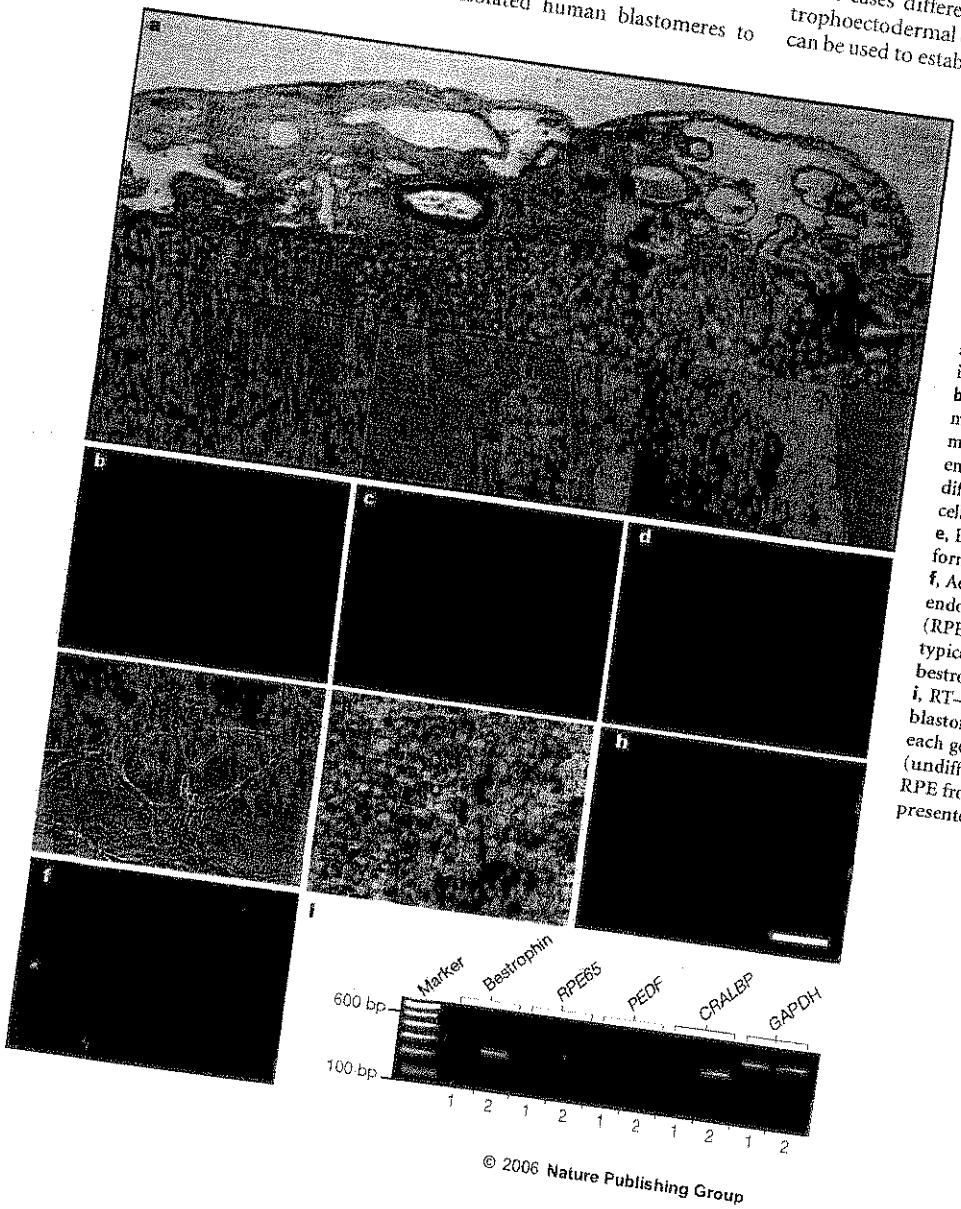

Figure 4 | *In vitro* differentiation of single-blastomere-derived hES cells into all three germ layers. **a**, Teratoma formation after transplantation of hES cells under the kidney capsule of NOD-SCID mice for seven weeks. Insets show enlargements of adjacent sections stained for molecular markers to confirm the presence of all three germ layers: left inset, neural tissue stained for nestin (ectoderm); centre inset, smooth-muscle actin (mesoderm); right inset, intestine stained for cdx2 (endoderm). **b–d**, Immunofluorescence analysis of the molecular markers of ectoderm (**b**, β III tubulin), mesoderm (**c**, smooth-muscle actin) and primitive endoderm (**d**, α-fetoprotein). **e–i**, *In vitro* differentiation of single-blastomere-derived hES cells into cells of specific therapeutic interest. **e**, Endothelial cells plated on Matrigel showing formation of typical capillary-tube-like structures. **f**, Acetylated low-density lipoprotein uptake by endothelial cells. **g**, **h**, Retinal pigment epithelium (RPE) showing a pigmented phenotype and typical 'cobblestone' morphology (**g**) and bestrophin staining (**h**). Scale bar, 100 μm. **i**, RT–PCR analysis for markers of RPE in single-blastomere-derived RPE (cropped image). For each gene: lane 1, negative control (undifferentiated hES cells, line WA09); lane 2, RPE from hES cells. A full-length gel image is presented in Supplementary Fig. 1.

interfere with the developmental capacity of the parent embryo. The biopsy procedure is similar to that used in IVF clinics worldwide, and could be used without affecting the clinical outcome. Blastomeres grown overnight could be used for both genetic testing and stem cell generation, thereby allowing selection for a day-5 blastocyst transfer. Numerous reports suggest that neither the survival rate nor the subsequent development and chances of implantation differ between intact human embryos at the blastocyst stage and those following blastomere biopsy for PGD[22–25]. However, until remaining doubts about safety are resolved, we do not recommend this procedure be applied outside the context of PGD. Blastomere-derived hES cells could be of great potential benefit for medical research, as well as for children and siblings born from transferred PGD embryos.

METHODS

Derivation of hES cells. Human embryos were thawed and cultured until the 8–10-cell stage at 37 °C in 20-μl drops of Quinn's cleavage medium under paraffin oil in a highly humidified incubator with 5.5% CO_2 in air. The zona pellucida was disrupted using either acidic Tyroide's solution or multiple piezo-pulses, and individual blastomeres were mechanically separated from the denuded embryos by holding the cell with a micropipette and gently tapping the pipette holder. The separated blastomeres were cultured together in the same medium, arranged so as to avoid contact with each other using depressions created in the bottom of the plastic tissue culture plate as described previously[5]. During this process, sets of microdrops were prepared, consisting of a 50-μl drop of Quinn's blastocyst medium (Cooper Surgical) supplemented with 5 μg ml^{-1} human placental laminin and human plasma fibronectin (Sigma), surrounded by several microdrops of hES cell culture medium[6] containing GFP-labelled hES cells growing on a MEF feeder layer. The microdrops containing the blastomere-derived aggregates were merged with one or two of the surrounding microdrops by scraping the bottom of the plate between the drops with a glass capillary, and fresh MEFs added to the drops in 24 h. After the formation of initial outgrowths, approximately half of the medium was changed every other day until the outgrowths reached approximately 50–100 cells. They were then mechanically passaged onto fresh MEF feeder layers in hES cell culture medium, which was changed every 1–2 days. The colonies were passaged by mechanical dispersion until enough cells were produced to initiate adaptation to trypsin. Thereafter, they were cultured as described previously[6]. We will provide a detailed protocol elsewhere for the derivation and maintenance of these cells (*Nature Protocols*, manuscript in preparation).

Immunostaining. Cells were fixed with 2% (w/v) paraformaldehyde, permeabilized with 0.1% (v/v) NP-40, and blocked with 10% (v/v) goat serum, 10% (v/v) donkey serum. Incubation with primary antibodies was carried out overnight at 4 °C. The antibodies used in this study were anti-Oct-4 (Santa Cruz Biotechnology), anti-SSEA-3 and anti-SSEA-4 (developed by Solter and Knowles and obtained from the Developmental Studies Hybridoma Bank, University of Iowa), anti-TRA-1-60 and anti-TRA-1-81 (Chemicon), anti-β-III-tubulin (Covance), anti-α-fetoprotein (DAKO) and anti-smooth-muscle-actin (DAKO). After washing in PBS containing 0.1% (v/v) Tween-20 (PBST), fluorescently labelled or biotinylated secondary antibodies were added for 1 h; some samples were subsequently incubated for 15 min with fluorescently labelled streptavidin. After additional washing in PBST, specimens were mounted using Vectashield with DAPI (4,6-diamidino-2-phenylindole; Vector Laboratories) and observed using a fluorescent microscope.

Karyotyping. The cells were passaged onto gelatin in ES cell culture medium, which was replaced the day before harvest when the cells were approximately 50% confluent. Colcemid was added to the culture at a concentration of 0.12 μg ml^{-1} for 40 min, then the cells were rinsed twice with PBS, trypsinized and centrifuged in DMEM with 10% (v/v) fetal bovine serum. KCl (0.075 M) was added to the pellet, and the cells were incubated for 10 min at 37 °C, centrifuged and fixed with 3:1 methanol:acetic acid for 10 min, then centrifuged again and suspended in this fixative. Cytogenetic analysis was performed on metaphase cells using G-banding on ten cells.

Microsatellite and PCR analyses. For microsatellite analysis, genomic DNA (MA01, passage 18; MA09, passage 23) was extracted with a DNeasy Tissue kit (Qiagen). Conventional PCR reactions were performed with 100 ng gDNA, Amplitaq Gold polymerase (ABI), and primer pairs specific for FES/FPS, vWA31, D22S417, D10S526 and D5S592 genomic microsatellite sequences (Coriell). Single primers in each pair were end-labelled with a 6-Fam fluorescent label. After incubation for 10 min at 94 °C to activate the polymerase, amplification was performed with 30 cycles of 94 °C for 45 s, 56 °C for 60 s, and 72 °C for 60 s. Labelled amplicons were separated and sized using an ABI 3730 sequencer (ABI). For amplification of enhanced GFP, amelogenin and *SRY* genes, gDNA (MA01, passage 40; MA09, passage 44) was isolated using a QIAamp DNA Mini kit (Qiagen), and 200 ng gDNA per reaction in 50 μl was used for GFP, amelogenin and *SRY* amplification. The primers used for GFP were 5′-TTGAATTCGCCACCATGGTGAGC-3′ (forward) and 5′-TTGAATTCTT ACTTGTACAGCTCGTCC-3′ (reverse), and PCR reactions were performed as described previously[5]. For sex determination, both the amelogenin and *SRY* genes were amplified by PCR as described previously[26,27]. The primers used for the amelogenin gene were 5′-CTCATCCTGGGCACCCTGGTTATATC-3′ (forward) and 5′-GGTACCACTTCAAAGGGGTAAGCAC-3′ (reverse), which generated a fragment of 1,310 base pairs (bp) for the Y chromosome and a fragment of 1,490 bp for the X chromosome. For the Y-chromosome-specific *SRY* gene, the primers used were 5′-GATCAGCAAGCAGCTGGGATACCAGTG-3′ (forward) and 5′-CTGTAGCGGTCCCGTTGCTGCGGTG-3′ (reverse), which amplified a DNA fragment of 330 bp. As a control for the PCR reactions, myogenin primers (5′-TCACGGTGGAGGATATGTCT-3′ (forward) and 5′-GAGTCAGCT AAATTCCCTCG-3′ (reverse)) were included in *SRY* PCR reactions, which generated a fragment of 245 bp. PCR products were separated on an agarose gel and visualized by ethidium bromide staining.

Total RNA was isolated using the RNase Mini kit (Qiagen); for RPE cells, a Trizol purification step was included. RT–PCR was performed using the Qiagen One Step RT–PCR kit for hES cell markers using the following conditions: 50 °C for 30 min for reverse transcription; 95 °C for 15 min for inactivation of the reverse transcriptase and activation of the polymerase; 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min; and 72 °C for 10 min for final extension. The primers used were for *Oct-4* (5′-GAAGGTATTCAGCCAAACGAC-3′ (forward) and 5′-GTTACAGAACCACACTCGGA-3′ (reverse); 315 bp), nanog (5′-TGCAAATGTCTTCTGCTGAGAT-3′ (forward) and 5′-GTTCAGGATGTTGG AGAGTTC-3′ (reverse); 285 bp) and *GAPDH* (5′-GTCCATGCCATCACTG CCA-3′ (forward) and 5′-TTACTCCTTGGAGGCCATG-3′ (reverse); 513 bp). For RPE markers, complementary DNA was made using the SuperScript III CellDirect Synthesis system (Invitrogen). RT–PCR was performed using Platinum Blue PCR SuperMix (Invitrogen). After incubation at 94 °C for 3 min, 30 cycles of 94 °C for 35 s, 55 °C for 35 s, and 72 °C for 50 s were performed, followed by a final extension at 72 °C for 10 min. An undifferentiated hES cell line WA09 was used as a negative control. The primers used were *RPE65* (5′-ATGGAC TTGGCTTGAATCACTT-3′ (forward) and 5′-GAACAGTCCATGAAAGGTG ACA-3′ (reverse); 285 bp), bestrophin (5′-ATCAGAGGCCAGGCTACTACAG-3′ (forward) and 5′-TCCACAGTTTTCCTCCTCACTT-3′ (reverse); 235 bp), *RALBP* (*CRALBP*) (5′-AAATCAATGGCTTCTGCATCATT-3′ (forward) and 5′-CCAAAGAGCTGCTCAGCAAC-3′ (reverse); 340 bp), *PEDF* (5′-AAGCTG AGTTATGAAGGCGAAG-3′ (forward) and 5′-TCTGTGTCCCTCAGTACG AAGA-3′ (reverse); 255 bp) and *GAPDH* (5′-CGATGCTGGCGCTGAGTAC-3′ (forward) and 5′-GTTTCTTACTCCTTGGAGGC-3′ (reverse); 465 bp).

Teratomas. Small clumps of 50–100 cells were mechanically removed from the culture and transplanted under the kidney capsules of 6–8-week-old NOD-SCID mice under anaesthesia. Between 18–57 days after transplantation, the kidneys were removed, fixed with 4% (w/v) paraformaldehyde overnight, washed for 24 h in PBS, embedded in paraffin, sectioned and analysed for the presence of the derivatives of the three germ layers.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information The hES cell lines generated in this study will be made available to investigators under a material transfer agreement (an application has been submitted for deposition of the hES cell lines into the UK stem Cell Bank). Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details accompany the paper at www.nature.com/nature. Correspondence and requests for materials should be addressed to R.L. (rlanza@advancedcell.com).