

Embryonic and extraembryonic stem cell lines derived from single mouse blastomeres

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The most basic objection to human embryonic stem (ES) cell research is rooted in the fact that ES cell derivation deprives embryos of any further potential to develop into a complete human being^{1,2}. ES cell lines are conventionally isolated from the inner cell mass of blastocysts^{3–5} and, in a few instances, from cleavage stage embryos^{6–9}. So far, there have been no reports in the literature of stem cell lines derived using an approach that does not require embryo destruction. Here we report an alternative method of establishing ES cell lines—using a technique of single-cell embryo biopsy similar to that used in pre-implantation genetic diagnosis of genetic defects¹⁰—that does not interfere with the developmental potential of embryos. Five putative ES and seven trophoblast stem (TS) cell lines were produced from single blastomeres, which maintained normal karyotype and markers of pluripotency or TS cells for up to more than 50 passages. The ES cells differentiated into derivatives of all three germ layers *in vitro* and in teratomas, and showed germ line transmission. Single-blastomere-biopsied embryos developed to term without a reduction in their developmental capacity. The ability to generate human ES cells without the destruction of *ex utero* embryos would reduce or eliminate the ethical concerns of many.

A series of six separate experiments was carried out to determine whether stem cell lines can be generated from single blastomeres (Supplementary Table 1). Eight-cell stage 129/Sv-ROSA26:*lacZ* mouse embryos were biopsied through a hole made in the zona pellucida with piezo-pulse drilling, and the biopsied (7-cell) embryos transferred to the oviducts of 1.5 days post coitum (d.p.c.) synchronized surrogate mothers. Each isolated blastomere was aggregated with a small clump of green fluorescent protein (GFP)-positive 129Sv/CD-1 mouse ES (mES) cells, and after incubation for 24–48 h, a growing ‘bud’ of GFP-negative cells was observed on the sides of the majority (60%) of GFP-mES clusters (Fig. 1a, b). The cell aggregates were plated onto mitomycin C-treated mouse embryonic fibroblasts (MEFs) and cultured in mES cell growth medium¹¹. Approximately half (36 out of 75) of them formed rapidly growing clumps of cells within 2–4 days, which were separated from GFP-positive mES cells by hand under a fluorescence microscope. The cells were expanded using mechanical and enzymatic methods, while further selecting by eye for the colonies morphologically resembling ES cells and excluding any GFP-positive cells (Fig. 1c–f). In four experiments, putative lines of LacZ⁺ ES cells were produced (Fig. 2a, c) that exhibited normal karyotype (Fig. 3g) and maintained markers of pluripotency after up to >50 passages. Each line expresses octamer binding protein 4 (Oct-4), stage-specific embryonic antigen (SSEA)-1, nanog and alkaline phosphatase (Fig. 2e, g, i).

Polymerase chain reaction (PCR) analysis confirmed the presence of LacZ but not GFP gene sequences in these cells (Supplementary Fig. 1a, b). Together with the karyotype analyses, the absence of GFP also rules out the possibility of contamination and/or fusion of the blastomere-derived lines with the ES cells used for co-culture.

When the putative ES 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 muscle actin (mesoderm, Fig. 3a), α -feto protein (primitive endoderm, Fig. 3c) and β III tubulin (ectoderm, Fig. 3e). Beating heart muscle, extraembryonic endoderm and multiple neuronal cell types were also routinely observed in differentiating cultures. To demonstrate further the pluripotency of the derived putative ES cells, cells were either injected into CD-1 mouse blastocysts or aggregated with 8-cell stage morulae as described¹¹. Forty-eight injected/aggregated embryos (between 8 and 15 per cell line) were transferred to recipient females. 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) staining of the resulting 29 fetuses (followed from mid-gestation through to term) showed that the ES cell lines contributed to all organ systems, including heart, kidney, liver, lung, intestine, brain, blood, skin and genital ridge, among others. Twenty-four of the fetuses (83%) were chimaeric (Fig. 3d, f), and eight out of nine (89%) pups (Fig. 3h) were chimaeric; the latter had the LacZ gene in their gametes (confirmed by PCR analysis; Supplementary Fig. 3), and produced LacZ⁺ offspring when crossed with CD-1 females, confirming the contribution of the blastomere-derived ES cells to the germ line. When the ES cells were injected into NOD-SCID (non-obese diabetic-severe combined immunodeficiency) mice, they formed teratomas containing tissues from all three germ layers, including bone and cartilage (mesoderm), neural rosettes (ectoderm), and ciliated respiratory epithelia (endoderm), among others (Fig. 3b).

Although stable putative ES stem lines were generated in only four of the six experiments, numerous other blastomere-derived outgrowths contained cells with both embryonic and extraembryonic stem-cell-like morphology. When FGF-4 was added to the media, seven putative TS lines were established, which maintained normal karyotype and expressed markers of TS cells (Fig. 2b, d, f, h, j). These cells were negative for Oct-4 (Fig. 2h) and for α -feto protein. RT-PCR analysis confirmed that these cells expressed *Cdx2*, but not Oct-4; *nanog* and *Rex-1* were expressed in both the putative TS and ES cell lines (data not shown). Putative TS cells contributed to the extraembryonic lineage in chimaeric fetuses generated by aggregation with the LacZ⁺ TS cells (Supplementary Fig. 2).

In two control experiments, individual blastomeres ($n = 44$) isolated from 8-cell embryos were plated into 20–100- μ l drops

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containing mES cell culture medium. Most of the blastomeres failed to divide over the 10-day period of culture, whereas 9 (20%) generated small clusters of differentiated trophoblast-like giant cells (Fig. 1g, h) before arresting at the 2–6-cell stage, thus suggesting that cell co-culture was critical to the success of this system.

The blastomere-biopsied embryos in the present study developed to term without a reduction in their developmental capacity (49% (23 out of 47) live young versus 51% (38 out of 75) for control non-biopsied embryos (χ^2 test, $P = 0.85$)). These results are consistent with human data, which indicate that normal and pre-implantation genetic diagnosis (PGD)-biopsied embryos develop into blastocysts with comparable efficiency¹².

Although only 36 out of 125 blastomeres (29%) generated inner cell mass-like outgrowths, and only 12 stable putative ES and TS cell

lines were generated in this study (compared to approximately 25–35% for normal mouse blastocysts), we believe that this success rate can be considerably increased by greater attention to the earliest stages of blastomere outgrowth, as well as the use of various measures that influence the spontaneous differentiation of pluripotent ES cells into trophectoderm and other cell types. Owing to the labour intensiveness of the various steps, only conspicuous outgrowths were selected for further passaging, although many of the GFP-positive ES clusters were observed to contain other blastomere-derived stem cells. Preliminary experiments using all blastomeres at the 8-cell stage indicate that most of the blastomeres give outgrowths

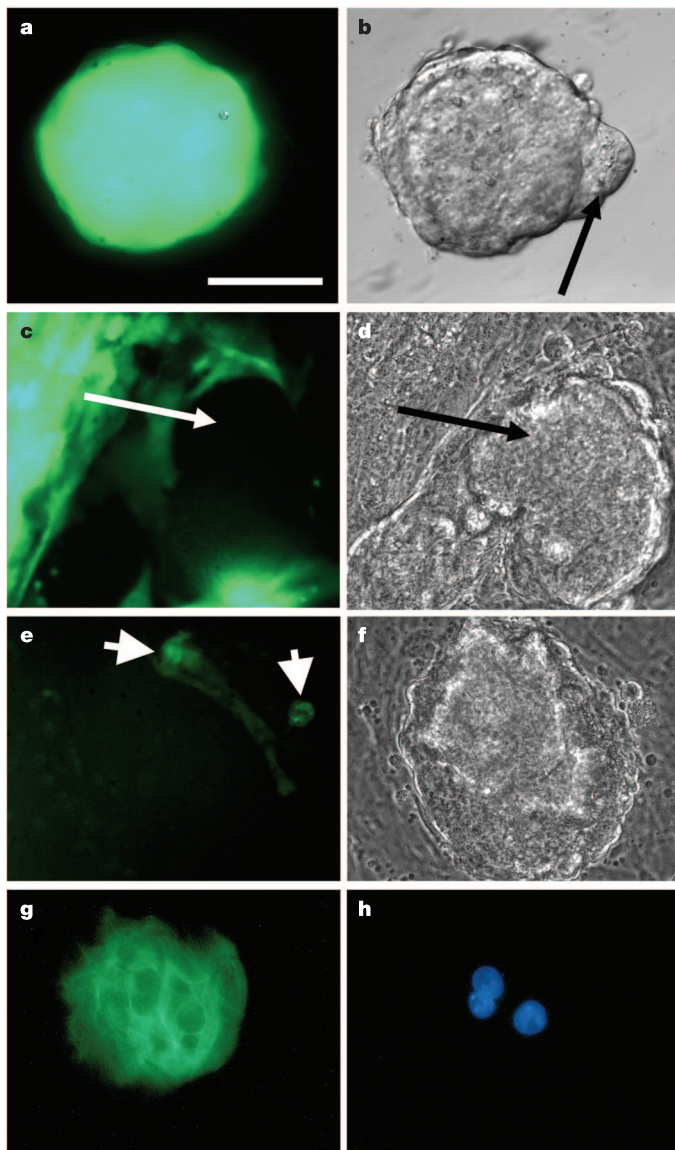


Figure 1 | Stages of single blastomere growth in the presence or absence of mES cells. **a, b**, Clump of GFP-positive mES cells 48 h after aggregation with single blastomeres; arrow shows a protruding cluster of GFP-negative cells. **c, d**, Outgrowth of GFP-negative cells aggregated with GFP-positive mES cells, after being plated on MEFs; arrows point to GFP-negative cells. **e, f**, Passage 1 of the outgrowth; arrows show remaining GFP-positive mES cells. **g, h**, Single blastomere outgrowth on MEFs for 4 days without ES cells, stained with Troma-1 and DAPI. **a, c, e**, Green fluorescence; **b**, Hoffman modulation optics; **d, f**, phase contrast. Scale bar, 100 μm .

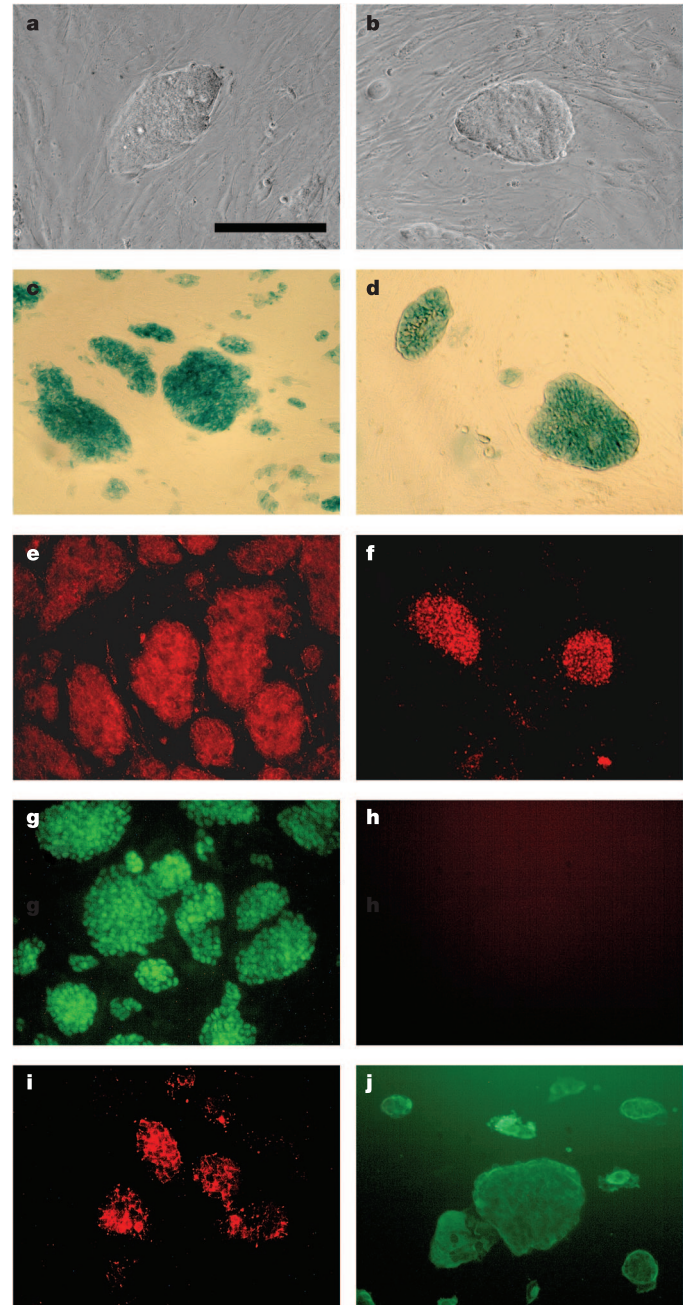


Figure 2 | Comparison of putative ES (left column) and TS (right column) cell lines derived from single blastomeres. **a, b**, Phase contrast photograph of typical colonies. **c, d**, LacZ-stained colonies, showing their single blastomere origin. **e, f**, Alkaline phosphatase staining. **g, h**, Indirect immunofluorescence with antibodies to Oct-4. **i**, SSEA-1 staining of putative ES cells; **j**, Troma-1 staining of putative TS cells (same field as **h**). Scale bar, 200 μm .

that appear to be morphologically identical. However, it is unclear whether the individual blastomeres have different developmental fates based on these observations. The microenvironment is clearly critical, and variables such as ES cell number, the spatial orientation of blastomeres, ES cells and MEFs, as well as media composition, seem to be important.

Previous studies have described different approaches to establish pluripotent stem cell lines from blastocysts^{3–5} and 8–20-cell stage morulae^{6–9,13,14}. In two studies, stem cell lines were established from disaggregated morulae, although the cells from the embryos were cultured together and free to reaggregate^{9,13}. However, none of these strategies uses single isolated cells that could be used in conjunction with assisted reproduction technologies such as PGD, and, importantly, that would not interfere with the embryo's normal development to birth. There have been previous attempts in both humans and animals to induce single isolated blastomeres to proliferate *in vitro*, although none of these investigators derived pluripotent stem cell lines. One study¹⁵ cultured individual biopsied

mouse blastomeres *in vitro* on different extracellular matrix components, including fibronectin and laminin, which have been shown to promote attachment and proliferation of blastomeres *in vitro* and to enhance blastocyst development in various animal species^{16–18}. Most of the isolated blastomeres divided to form small sheets of 6–8 cells with a trophoblastic morphology similar to that described in ref. 19. In the human, another study²⁰ observed proliferation of blastomeres that had been removed and co-cultured with the biopsied embryos (range 1–8 cells per blastomere), although in all cases differential labelling indicated that they had generated trophoblastic cells. Unfortunately, the molecular mechanisms regulating these events are poorly understood, and it is unclear whether the success of the ES co-culture system in the present study is attributable to substances secreted by the ES cells or if cell–cell contact is required.

The developmental capacity of blastomeres isolated from mammalian embryos has been studied extensively, and it is clear that they retain their pluripotency, and, indeed, are capable of regular *in vivo* development upon transfer into mice²¹, rabbits²², sheep²³, swine^{18,24,25} and primates²⁶. We have demonstrated that single pre-implantation blastomeres can also be used to establish embryonic and extra-embryonic stem cell lines using an approach that does not interfere with the developmental potential of the parent embryo. The biopsy procedure described here is carried out in IVF clinics worldwide without a reduction in pregnancy rate. However, further investigations are required to determine whether stem cell lines can be derived from other mammalian species, including humans, using this micromanipulation technique. The ability to generate human ES cells from PGD blastomeres could circumvent the ethical concerns voiced by many, and allow the banking of autologous ES cell lines for children born from transferred embryos.

METHODS

Generation of ES and TS cell lines. Eight-cell stage 129/Sv-ROSA26:*lacZ* mouse embryos were biopsied through a hole in the zona pellucida using piezo-pulse drilling. The biopsied embryos were transferred to the oviducts of 1.5 d.p.c. synchronized surrogates, and each separated blastomere was aggregated with a small clump (≤ 100 cells) of GFP-positive 129Sv/CD-1 mES cells in a 300- μm depression created by pressing a needle into the bottom of a plastic tissue culture plate, as described¹¹. After incubation for 24–48 h in mES cell growth medium¹¹ supplemented with 2,000 U ml⁻¹ mouse leukaemia inhibitory factor (LIF; Chemicon) and 50 μM MEK1 inhibitor (Cell Signaling Technology), a growing bud of GFP-negative cells was observed on the sides of the majority of GFP-mES clusters. The aggregates were plated onto mitomycin C-treated MEFs and cultured in mES cell growth medium until GFP-negative clumps became large enough for dispersion (> 20 cells), which then were separated from GFP-positive mES cells by hand with a microcapillary under a fluorescence microscope. The cells were dissociated and expanded by alternating mechanical dissociation and digestion with 0.05% trypsin (Invitrogen). Blastomere outgrowths that morphologically resembled trophoblast and extraembryonic endoderm but not ES cells were further cultured in the mES cell medium with 50 ng ml⁻¹ FGF-4, and produced TS-like cells that were maintained under these conditions and passaged with trypsin.

Immunofluorescence and alkaline phosphatase staining. Indirect immunofluorescence staining was performed on cells growing on 4-well tissue culture plates as previously described²⁵. The following primary antibodies were used: Oct-4 (Santa Cruz Biotechnology), SSEA-1 (developed by D. Solter and B. Knowles and obtained through the DSHB (Developmental Studies Hybridoma Bank) of the University of Iowa), Troma-1 (raised by P. Brulet and R. Kemler and obtained through DSHB), α -feto protein (DACO), β III tubulin (Covance) and muscle actin (Abcam). Alkaline phosphatase staining was performed using the Vector red kit from Vector Laboratories.

Chimaeras and X-gal staining. Approximately 15 ES cells or TS cells were injected either into the blastocoels of expanded blastocysts or into the perivitelline space of pre-compacting 8-cell stage embryos obtained from CD-1 mice. Blastocyst chimaeras were transferred into the uteri of 2.5 d.p.c. surrogate mice 4 h after the ES cell injections. Most of the 8-cell stage chimaeric embryos developed into expanded blastocysts after overnight culture in mES medium, and were then transferred to surrogate mice. The pregnancies were terminated at days 11.5, 12.5 or 19.5, and the embryos and placentas were fixed overnight in 4%

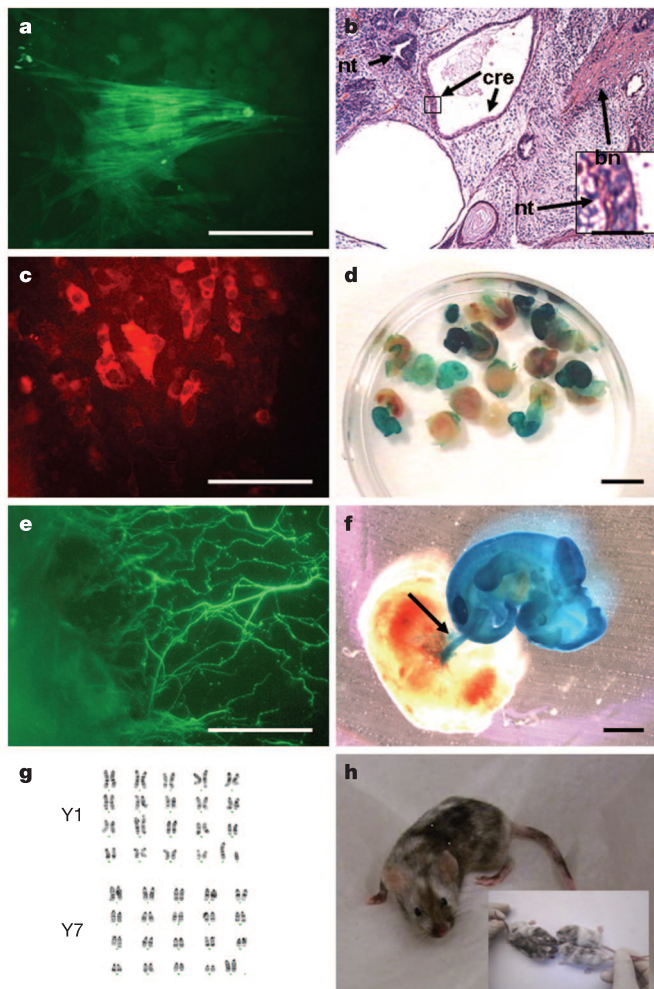


Figure 3 | Differentiation of blastomere-derived mES cells *in vitro* and *in vivo*. **a, c, e,** Immunofluorescence analysis of molecular markers of mesoderm (muscle actin, **a**), primitive endoderm (α -feto protein, **c**) and ectoderm (β III tubulin, **e**). **g,** Representative chromosome spreads of two single-blastomere-derived mES cell lines. **b,** Teratomas section, stained with haematoxylin and eosin. bn, bone (mesoderm); nt, neural tissue (ectoderm); cre and insert, ciliated respiratory epithelium (endoderm). **d, f, h,** 11.5 d.p.c. chimaeric embryos (**d, f**) and chimaeric pups (**h**) produced from three mES cell lines; arrow in **f** shows the extraembryonic mesoderm-derived placental labyrinth, also chimaeric. Scale bars: **a, c,** 100 μm ; **e,** 200 μm ; **d,** 10 mm; **f, h,** 2 mm.

paraformaldehyde and washed with PBS overnight; LacZ staining using a X-gal staining kit (GAL-S, Sigma) was done to test for chimaerism in the embryos.

Teratomas. Approximately 1 million ES cells were injected into the rear thigh of a NOD-SCID mouse. After approximately 2 months the mice were killed and the teratomas excised, fixed in 4% paraformaldehyde, embedded in paraffin and sectioned.

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Competing interests statement:

Y.C., I.K., S.B., J.M., S.-J.L. and R.L. are employees of Advanced Cell Technology, a biotechnology company in the field of regenerative medicine. J.J. and L.M. declare no conflict of interest.