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Human Embryonic Stem Cell Lines Generated without Embryo Destruction

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To date, the derivation of all human embryonic stem cell (hESC) lines has involved destruction of embryos. We previously demonstrated that hESCs can be generated from single blastomeres (Klimanskaya et al., 2006). In that "proof-of-principle" study, multiple cells were removed from each embryo and none of the embryos were allowed to continue development. Here we report the derivation of five hESC lines without embryo destruction, including one without hESC coculture. Single blastomeres were removed from the embryos by using a technique similar to preimplantation genetic diagnosis (PGD). The biopsied embryos were grown to the blastocyst stage and frozen. The blastomeres were cultured by using a modified approach aimed at recreating the ICM niche, which substantially improved the efficiency of the hESC derivation to rates comparable to whole embryo derivations. All five lines maintained normal karyotype and markers of pluripotency for up to more than 50 passages and differentiated into all three germ layers.

Although Thomson et al. (1998) first reported the derivation of hESCs in 1998, the need to destroy or disaggregate embryos (Cowan et al., 2004; Hovatta et al., 2003; Klimanskaya et al., 2005; Lanzendorf et al., 2001; Reubinoff et al., 2000) continues to cause ethical concerns and is prohibited by law in many countries. In the United States, federal funding is not permitted for research that involves the destruction of human embryos or the use of hESC lines derived from those embryos (Dickey-Wicker, 1996).

The strategy we employed previously for derivation of hESCs from single blastomeres (Klimanskaya et al., 2006) frequently led to the formation of trophectodermal-like vesicles that formed small sheets of cells with trophoblastic morphology similar to that described in previous studies (Geber et al., 1995; Rossant, 1976; Takeuchi et al., 2007; Wilton and Trounson, 1989) This procedure was, therefore, highly inefficient (only ~2% of blastomeres generated hESC lines), and multiple biopsies were taken from each embryo to minimize the number of embryos destroyed in the experiment. To increase the efficiency of hESC derivation, in this study we employed a modified approach aimed at recreating the ICM niche by preventing trophectoderm differentiation.

A series of nine initial experiments separated into two groups were carried out with pronuclear stage embryos that were thawed and cultured to the eight-cell stage (Table S1 available online). As in PGD, only one (or in a few [7/41] cases, two) blastomere was removed from each embryo by using a biopsy procedure. For both groups, the derivation process included coculture with GFP-labeled hESCs. In the first set of experiments, the parental embryos and blastomeres were cultured together in the original microdrop for 12 to 24 hr, and then the parental embryos were transferred to blastocyst medium for an additional 48 hr. 22 of the 26 biopsied embryos (85%) continued development to the blastocyst stage, and most (21/31) of the single blastomeres divided, forming either cell clumps or vesicles comprising four to eight cells. They were transferred to microdrops of blastocyst medium supplemented with laminin and fibronectin and seeded with mitotically inactivated mouse embryonic fibroblasts (MEFs). The following day, the microdrops were merged with GFP-hESC-seeded microdrops as described in the Supplemental Experimental Procedures. Under these conditions, the vast majority of single-blastomere-derived cell aggregates formed cavitated trophectodermal-like vesicles. Vesicles that did not attach spontaneously within 28 hr after plating were forced to attach by poking them with a 26 G needle. Only 1 out of 26 biopsied embryos generated an hESC line (no embryo destruction [NED1]) with this method.

In the second set of experiments, the parental embryos and biopsied blastomeres were only cocultured together for 12 hr before transfer of the blastomeres to blastocyst medium microdrops supplemented with laminin and fibronectin. Importantly, trophectodermal-like vesicles did not form under these conditions, but almost all (9/11) blastomere-derived cell aggregates produced outgrowths that had either an ESC-like or nontrophoblast cell morphology. In both sets of experiments, the parental embryos were allowed to develop to the blastocyst stage and frozen. 80%–85% of the biopsied embryos formed healthy blastocysts (Table S1 and Figure 1B, row a), a rate consistent with or higher than previously reported for both biopsied and nonbiopsied embryos (Geber and Sampaio, 1999; Palmer et al., 2002).

29 of the 32 (91%) blastomere-derived aggregates generated cellular outgrowths, of which 4/20 (20%) and 4/9 (44%) morphologically resembled hESCs in the first and second sets of experiments, respectively (Table S1 and Figure 1A). In the first set of experiments, only 1 of the 26 embryos (3.8%) generated a stable hESC

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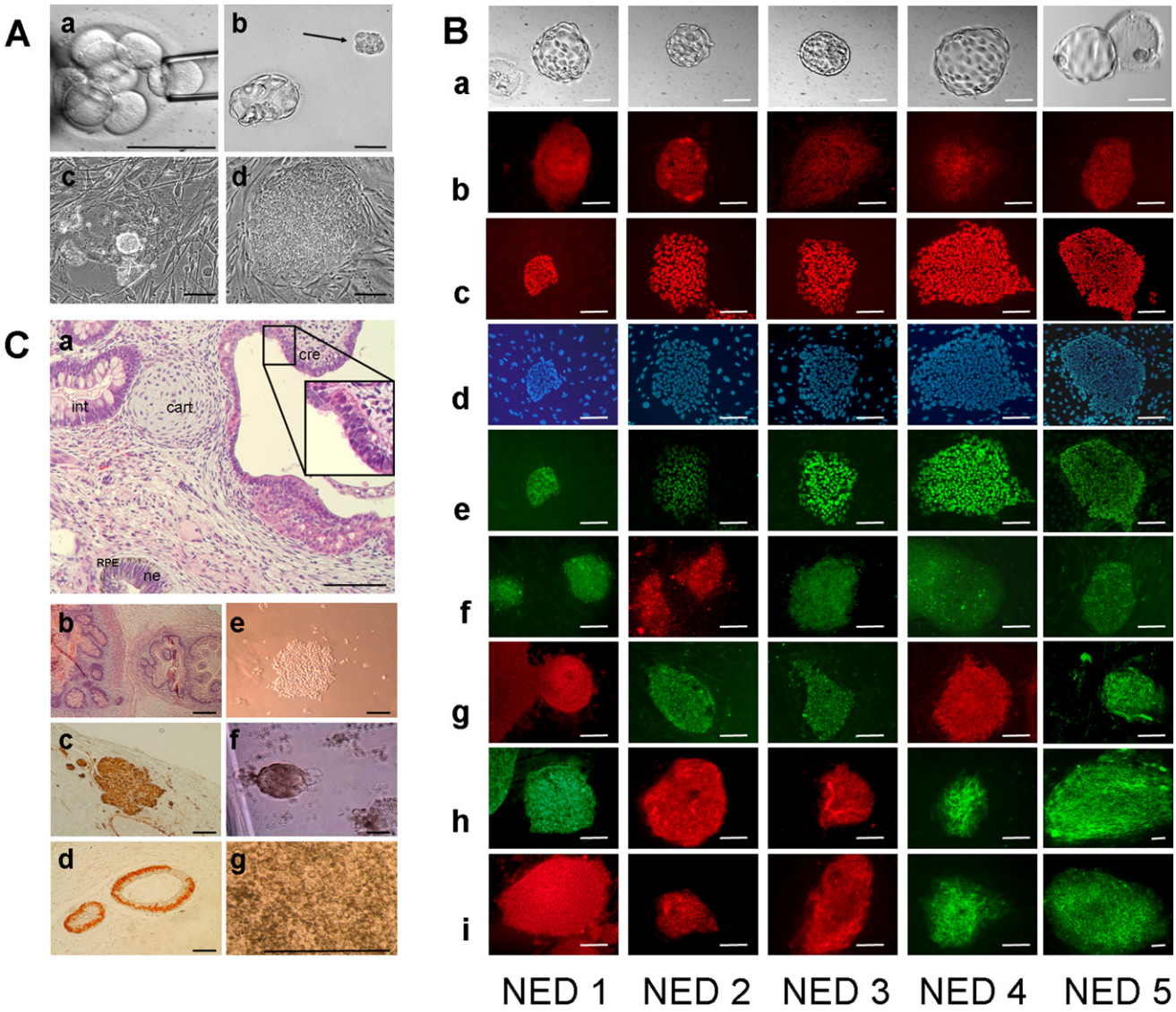

Figure 1. Derivation and Characterization of hESC Lines from Single Blastomeres without Embryo Destruction

(A) Stages of derivation of hES cells from single blastomere. (a) Blastomere biopsy, (b) biopsied blastomere (arrow) and parent embryo are developing next to each other, (c) initial outgrowth of single blastomere on MEFs, 6 days, and (d) colony of single blastomere-derived hES cells.

(B) Blastocysts formed by the biopsied parental embryos (a) and markers of pluripotency in single blastomere-derived hES cell lines (b–i); (b) alkaline phosphatase, (c) Oct-4, (d) DAPI corresponding to Oct-4 and Nanog (e), (f) SSEA-3, (g) SSEA-4, (h) TRA-1-60, and (i) TRA-1-81.

(C) Differentiation of single-blastomere-derived hESCs into three germ layers in vivo (a–d) and in vitro (e–g). (a) Teratoma showing derivatives of all three germ layers. cre, ciliated respiratory epithelium, including inset at higher magnification showing cilia; int, intestinal epithelium; cart, cartilage; and ne, columnar neuroepithelium with associated retinal pigmented epithelium (rpe). (b–d) Examples from other teratomas: (b) bronchiolar nests, (c) muscle stained for smooth muscle actin, and (d) intestinal epithelium stained for cdx2. (e–g) Examples of in vitro differentiated derivatives of clinical value: (e) hemangioblast colony with both hematopoetic and endothelial potential, (f) an embryoid body with beating heart cells, and (g) retinal pigment epithelium. Scale bars, 100 μm.

line, which is consistent with the low efficiency previously reported (Klimanskaya et al., 2006). However, in the second set of experiments, 3 out of 15 embryos (20%) generated stable hESC lines, a derivation rate comparable with standard ESC efficiency from blastocysts (Cowan et al., 2004; Hovatta et al., 2003; Lanzendorf et al., 2001; Simon et al., 2005; Thomson et al., 1998).

When the blastomere-derived (hESC-like) colonies reached ~50 cells or more, they were mechanically dispersed and the clumps plated next to the initial outgrowths. Secondary colonies were also allowed to grow to a similar size and mechanically passaged onto fresh MEFs every 3–5 days until they adapted to routine passaging with trypsin and could be frozen (usually after 7–10 passages) as previously described (Klimanskaya et al., 2007). At each passage, the colonies were screened under a fluorescent microscope for the absence of GFP-positive cells. All four hESC lines were positive for Oct-4, Nanog, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, and alkaline phosphatase (Figure 1B). In vitro differentiation confirmed the presence of derivatives from all three germ layers, including

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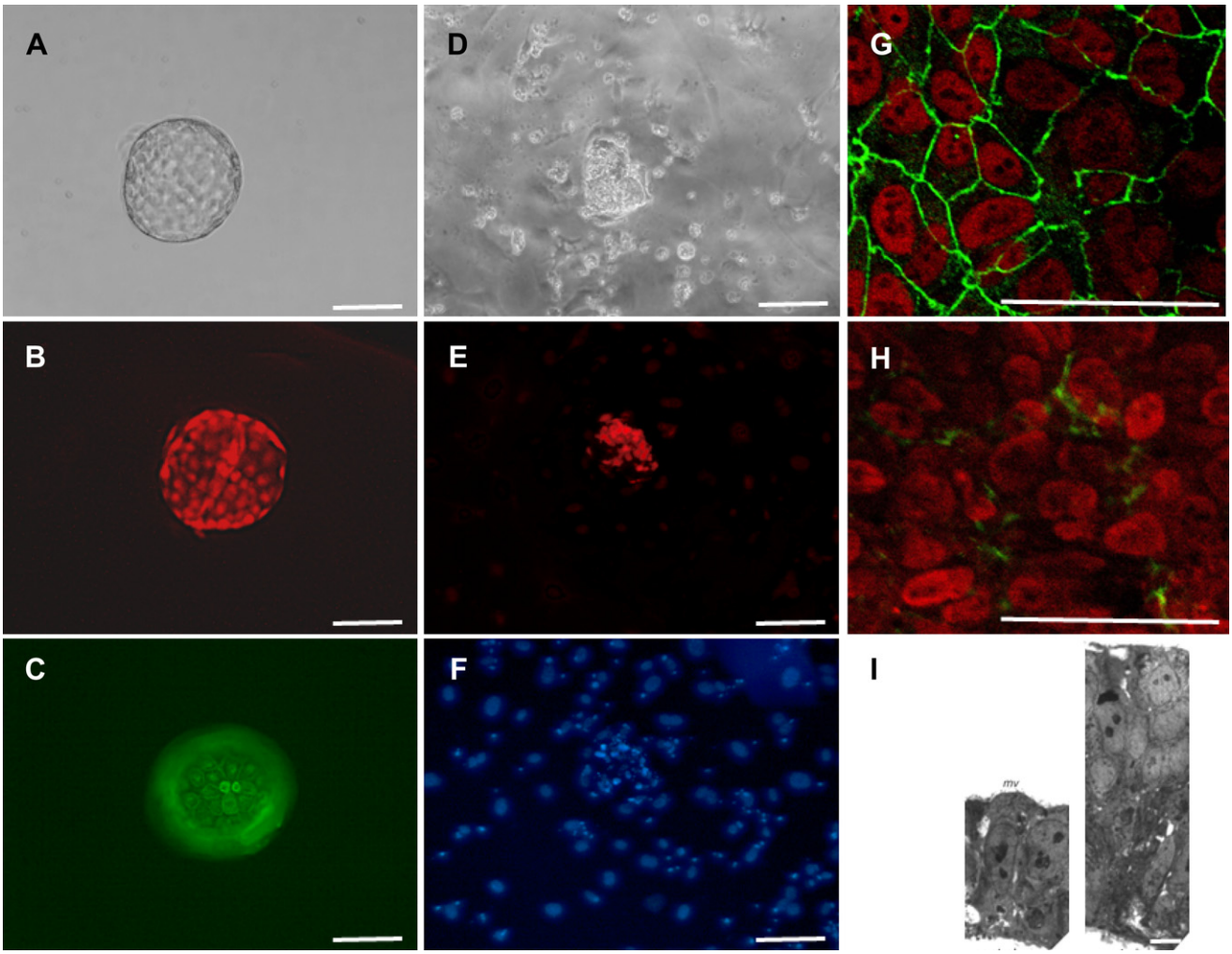

Figure 2. Effects of Laminin on Single Blastomere Development and hESCs

(A–C) Formation of trophectoderm-like vesicles in the absence of laminin. (A) Hoffman modulation contrast, (B) immunostaining for cdx2, and (C) immunostaining for cytokeratin 8.

(D–F) Formation of ICM-like outgrowth in the presence of laminin. (D) Phase contrast, (E) immunostaining for Oct-4, and (F) corresponding DAPI image.

(G–I) Depolarization effects of laminin on hESCs. (G and H) Confocal microscopy of the control (G) and laminin (H) overlaid hESC (WA07) costained with tight junction marker ZO-1(green) and pluripotency marker Oct-4 (red). Ultrastructural analysis (semithin sections) of the cross section of the control (left) and laminin overlaid (right) hESC colony (WA09). The control colony is organized into a semistratified epithelium. Presence of apical microvilli (mv) and tight junctions (data not shown) indicate structural specialization typical for epithelial-like polarization. Laminin overlay induced cell depolarization as shown by lack of microvilli on the cell surface and piling of cells to form multilayered structures. Scale bars, (A–H) 50 μm and (I) 7 μm.

hematopoietic and endothelial cells, neurons, retinal pigment epithelium (RPE), beating heart cells, and other cell types of therapeutic importance (Figure 1C). To assess the in vivo differentiation potential, the cells were injected under the kidney capsules of NOD-SCID mice, where they formed teratomas in ~7–12 weeks, differentiating into structures of all three germ layers (Figure 1B).

All four of the newly established hESC lines had normal karyotypes (NED1, 46 XY; NED2, 46 XY; NED3, 46 XX; and NED4, 46 XY (Figure S1). PCR analysis confirmed the absence of GFP DNA, which eliminated the possibility of cross-contamination or fusion with the GFP-hESCs used for coculture (Figure S2). Further genotyping showed the unique identity of the new hESC lines, ruling out any potential crosscontamination with other hESC lines currently maintained in our laboratory (Table S2).

Finally, in a third set of experiments, we tested whether GFP-hESC coculture is necessary for successful derivation. An experiment was carried out with blastomeres removed from two frozen cleavage-stage embryos that were thawed and cultured in blastocyst medium for 2 hr prior to biopsy. A single blastomere was removed from one embryo, and two blastomeres were removed from the second embryo. The remaining biopsied embryos were allowed to continue development and were frozen at the blastocyst stage. Extracted blastomeres were cultured under the same conditions as described for the second set of experiments except that no GFP-hESCs were present. Both blastomere-derived aggregates generated cellular outgrowths, whereas one (50%) generated a stable hESC line. Immunostaining of the stable hESC line established from this colony

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(NED5) confirmed the expression of markers of pluripotency, including Oct-4, Nanog, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, and alkaline phosphatase (Figure 1C). The newly established hESC line had a normal male (46 XY) karyotype (Figure S1) and differentiated into derivatives from all three germ layers, including immunostaining with antibodies to tubulin β III (ectoderm), smooth muscle actin (mesoderm), and α fetoprotein (primitive endoderm).

Separate studies were carried out to investigate the basis of enhanced blastomere differentiation into ICM. In the absence of laminin and fibronectin, dissociated single blastomeres uniformly differentiated into trophectoderm (16/16 [100%] of blastomere outgrowths contained trophectodermal cells versus 0/16 [0%] ICM-like cells). By contrast, when laminin was added to the medium, only 1/14 (7%) of blastomere-derived aggregates gave rise to trophectoderm-like vesicles and 13/14 (93%) yielded ICM-like cells. The addition of fibronectin alone had little or no effect on lineage specification (5/6 [83%] of outgrowths containing trophectodermal cells versus 1/6 [17%] ICM-like cells). This suggests that laminin may play a key role in directing blastomere differentiation toward ICM. To test this hypothesis, we immunostained blastomere-derived vesicles formed in the absence of laminin (Figure 2A) and ICM-like cells (Figure 2D) derived in the presence of laminin for the markers of trophectoderm and ICM/ESC, respectively. As expected, blastomere-derived vesicles that formed without laminin expressed key trophectoderm markers, including cdx2 and cytokeratin 8 (Figures 2B and 2C), whereas ICM-like outgrowths formed in the presence of laminin, expressed Oct-4 (Figure 2E). Interestingly, immunostaining for the tight junction marker ZO-1 and ultrastructural analysis by transmission electron microscopy and semithin sections (Krtolica et al., 2007; Figures 2G–2I) revealed that the addition of laminin to the culture medium of established hESC lines disrupts tight junctions and depolarizes ESCs, inducing them to assume an ICM-like phenotype. Furthermore, staining for ZO-1 confirmed that the addition of laminin to the culture medium of the blastomeres disrupted tight junctions.

Since the publication of our previous report showing the derivation of hESCs from single blastomeres (Klimanskaya et al., 2006), concerns have been raised that none of the embryos used in the experiments were allowed to develop and that the survival of the biopsied embryos was only demonstrated in control experiments. Another concern was that several (four to seven) blastomeres from each embryo were cocultured together and that this was essential to the successful derivation of hESCs. And finally, there were ethical objections that the derivation system required coculture with hESCs from other embryos that were destroyed. Here we clearly show that hESC lines can be derived without embryo destruction and that the biopsy procedure did not appear to interfere with subsequent good blastocyst development of the parent embryo. These results were achieved without culturing multiple blastomeres together, and at an efficiency substantially higher (20% or 50% versus 2%) than in our previous report. The success rate of this new procedure is similar to that of conventional hESC derivation techniques using blastocysts. In addition, we show that hESC coculture is not an essential part of the derivation procedure.

Our current results demonstrate that hESC lines can be generated far more readily when the isolated blastomeres are cultured in medium supplemented with laminin. Laminin is a common component of basement membranes (Aumailley and Smyth, 1998), including those of preimplantation embryos, and is associated with the induction of apical/basal polarity (Bissell and Bilder, 2003; Klein et al., 1988; Miner and Yurchenco, 2004; Schuger et al., 1996). By exposing extracted blastomeres to laminin during the first day of their postbiopsy development, we enhanced their ability to give rise to hESCs. We hypothesize that laminin partially recapitulates the ICM niche and suppresses trophectoderm differentiation by preventing polarization of the blastomeres. Indeed, our results demonstrate that, unlike vesicles formed in the absence of laminin, cell clumps formed in the presence of laminin are not polarized and express markers of ICM/ES cells.

The stem cell lines generated in this study appear to have the same characteristics as other hESC lines, including expression of the same markers of pluripotency, self-renewing capacity, karyotypic stability, and ability to differentiate into derivatives of all three germ layers both in vitro and in teratomas. The improved efficiency of the derivation procedure may also allow translation of the technique to the IVF clinic.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, two figures, and two tables and can be found with this article online at http://www.cellstemcell.com/cgi/content/full/2/2/■■■/DC1/.

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