

SPECIAL REPORT

Derivation of Embryonic Stem-Cell Lines from Human Blastocysts

Chad A. Cowan, Ph.D., Irina Klimanskaya, Ph.D., Jill McMahon, M.S., Jocelyn Atienza, B.S., Jeannine Witmyer, Ph.D., Jacob P. Zucker, B.S., Shunping Wang, Ph.D., Cynthia C. Morton, Ph.D., Andrew P. McMahon, Ph.D., Doug Powers, Ph.D., and Douglas A. Melton, Ph.D.

Embryonic stem cells have the unique ability to form all adult cell types. Harnessing this potential may provide a source of cells to replace those that are lost or impaired as a result of disease. Moreover, the derivation of human embryonic stem cells opens a unique window into the study of early human development. At present, approximately 15 human embryonic stem-cell lines are publicly available, and they vary considerably in their usefulness for research and the extent of their characterization (see <http://stemcells.nih.gov/registry/index.asp>). To promote further research with human embryonic stem cells, we sought to derive and characterize more fully cell lines that meet strict criteria for ease of manipulation, including enzymatic passage with trypsin, streamlined freezing and thawing procedures, well-defined culture mediums, and straightforward methods for *in vitro* differentiation. We report the derivation and characterization of 17 additional human embryonic stem cell lines.

We obtained frozen cleavage- and blastocyst-stage human embryos, produced by *in vitro* fertilization for clinical purposes, after obtaining written informed consent and approval by a Harvard institutional review board. A total of 286 frozen and thawed cleaved embryos (6 to 12 cells each) were cultured to the blastocyst stage, and 58 frozen and thawed blastocysts were allowed to re-expand in culture, whereupon they were treated with Tyrode's solution to remove the zona pellucida, followed by immunosurgery to isolate inner cell masses.¹ Many of these embryos were of such poor quality that they did not develop or divide after thawing. Nevertheless, 97 inner cell masses were isolated, and 17 individual human embryonic stem-cell lines (HUES1 through HUES17) were derived according to published protocols that we modified in terms of medium composition, enzymatic dissociation, and pro-

cedures for freezing and thawing (Fig. 1A, 1B, and 1C, next page).²⁻⁴ Too few embryos were available for us to determine systematically whether the procedural changes we used contributed substantially to the success rates we achieved. A detailed manual of our methods for culturing blastocysts and isolating embryonic stem cells is available elsewhere.⁵

Blastocysts representing a wide range of morphologic grades were used for derivation (see Table 1 in Supplementary Appendix 1, available with the full text of this article at www.nejm.org).⁶ Although cleavage-stage embryos that were of extremely poor quality often did not develop to the blastocyst stage, cell lines were produced from three blastocysts that would otherwise have been discarded because of their poor morphologic characteristics. Moreover, four additional cell lines were derived from embryos of intermediate quality. Twelve of the human embryonic stem-cell lines were derived from blastocysts that had been frozen and thawed, and five from blastocysts cultured from cleaved embryos that had been frozen and thawed. These data suggest that human embryonic stem-cell lines may be derived more efficiently from frozen blastocysts than from frozen cleaved embryos.

All 17 human embryonic stem-cell lines were derived on and have since been cultured on mitotically inactivated mouse embryonic fibroblasts in medium supplemented with basic fibroblast growth factor, recombinant human leukemia inhibitory factor, serum replacement, and a human plasma protein fraction (plasmanate). Each cell line was first passaged by mechanical dissociation (usually fewer than five passages) immediately after the initial outgrowth of the inner cell mass. By design, all cell lines were then adapted to enzymatic passage with trypsin. Although this approach may have reduced our efficiency in isolating putative embryonic

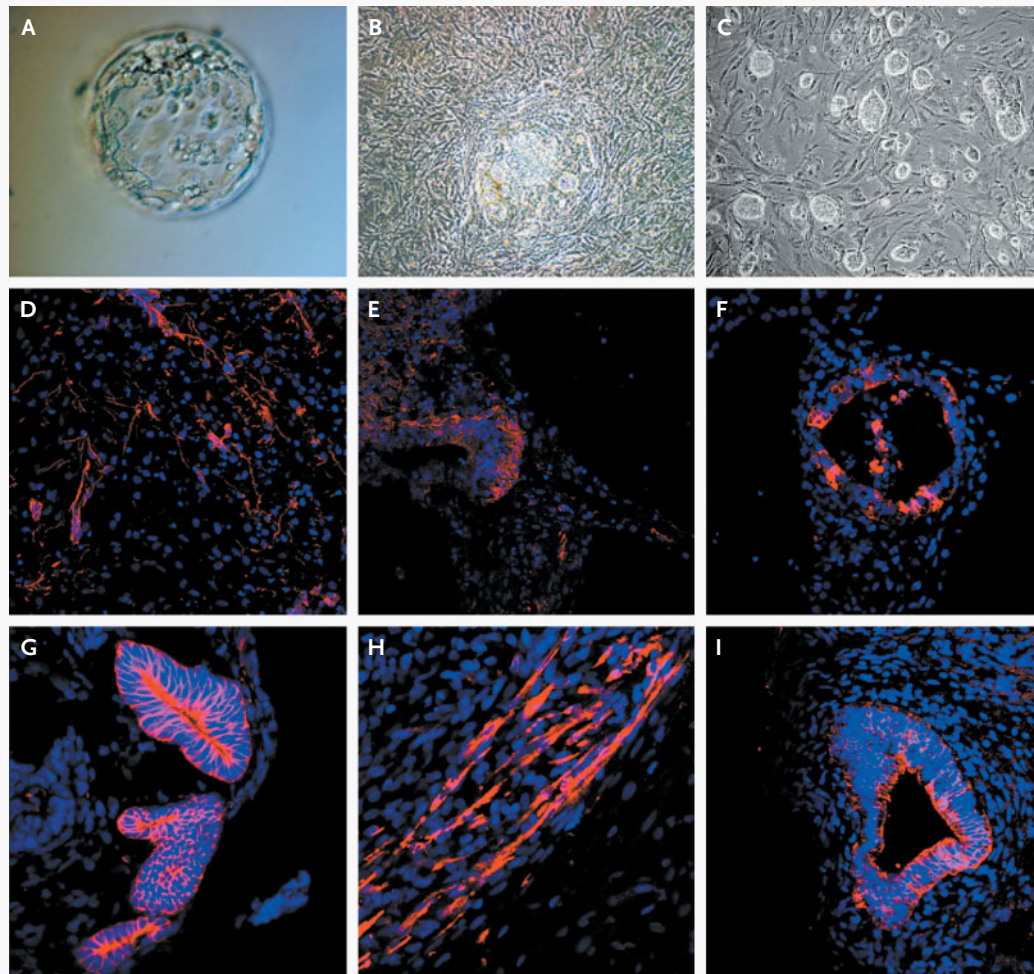


Figure 1. Derivation and Differentiation of Human Embryonic Stem Cells.

Panel A shows a human embryo at the blastocyst stage, Panel B shows initial outgrowth of the inner cell mass after immunosurgery, and Panel C shows colonies of human embryonic stem cells after enzymatic dissociation. Fluorescent immunostaining of cell line HUES6 is shown (red) with anti-Tuj1 antibodies (Panel D, $\times 25$), anti-MF20 antibodies (Panel E, $\times 25$), and anti- α -fetoprotein antibodies (Panel F, $\times 25$). Immunostaining of a teratoma resulting from cell line HUES2 is shown (red) with anti-Tuj1 antibodies (Panel G, $\times 25$), anti-MF20 antibodies (Panel H, $\times 40$), and anti- α -fetoprotein antibodies (Panel I, $\times 40$). In Panels D, E, F, G, H, and I, nuclei are stained blue with 4',6-diamidino-2-phenylindole dihydrochloride.

stem-cell lines, it produced cell lines that are easily and routinely cultured *in vitro*. These human embryonic stem cells have a high ratio of nucleus to cytoplasm, prominent nucleoli, and compact colony structure, as reported for other embryonic stem-cell lines.^{3,4} These human embryonic stem-cell lines were strongly positive for a number of molecular markers of undifferentiated pluripotent human stem cells, including octamer binding protein 3/4, stage-specific embryonic antigen (SSEA)-3,

SSEA-4, TRA-1-60, TRA-1-81, and alkaline phosphatase (Table 1 and Fig. 3 in Supplementary Appendix 1).⁷⁻¹¹

The population doubling time for HUES1, HUES2, HUES4, HUES6 through HUES9, and HUES13 through HUES16 is approximately 24 to 48 hours (Fig. 2 and Table 1 in Supplementary Appendix 1). Three cell lines, HUES3, HUES5, and HUES10, have slightly longer population doubling times — between 60 and 72 hours. We found that

low-passage human embryonic stem cells had longer population doubling times (approximately 150 hours) and that with additional passages, the population doubling times shortened and stabilized (Table 1 and Fig. 3 in Supplementary Appendix 1, and additional data not shown). With continued culturing (usually after more than 40 passages), the population doubling times shorten, which may be attributable to karyotypic changes. Five human embryonic stem-cell lines (HUES1 through HUES4 and HUES6) have undergone more than 50 passages in culture (with population doubling more than 130 times) without replicative crisis.¹²

Karyotype analysis revealed that all 17 human embryonic stem-cell lines had a normal complement of 46 chromosomes (HUES1, 2, 5, 6, 9, 12, 14, and 15 were 46,XX, and HUES3, 4, 7, 8, 10, 11, 13, 16, and 17 were 46,XY) (Fig. 2 in Supplementary Appendix 1). After prolonged culture, we observed karyotypic changes involving trisomy of chromosome 12 (HUES3 and HUES4), as well as other changes (additions to chromosome 2 in HUES1) (Table 1 in Supplementary Appendix 1). These karyotypic abnormalities are accompanied by a proliferative advantage and a noticeable shortening in the population doubling time. Chromosomal abnormalities are commonplace in human embryonal carcinoma cell lines and in mouse embryonic stem-cell lines and have recently been reported in human embryonic stem-cell lines.¹³⁻¹⁷ Trisomy 12 mosaicism has been repeatedly observed in different laboratories, indicating its potential importance in the growth and passage of human embryonic stem cells in culture.

To assess the capacity of our human embryonic stem-cell lines to form differentiated cell types *in vitro*, we induced differentiation of HUES1 through HUES16 by culturing the cells in suspension and allowing them to form cystic embryoid bodies.¹⁸ Cryosections of embryoid bodies cultured for 30 days reacted positively with mouse monoclonal antibodies that detect neuron-specific β -tubulin (Tuj1, ectoderm), myosin heavy chain (MF20, mesoderm), and a rabbit polyclonal antibody that reacts with alpha-fetoprotein (endoderm) (Fig. 1D, 1E, and 1F and Fig. 4 in Supplementary Appendix 1).¹⁹⁻²¹ In addition, we investigated the potential of these human embryonic stem-cell lines to differentiate into ectodermal, mesodermal, and endodermal cell types *in vivo* through teratoma formation after placement of subcutaneous xenografts in immunocompromised mice. Cryosections of each terato-

ma were positive on staining for Tuj1, MF20, and alpha-fetoprotein (Fig. 1G, 1H, and 1I and Fig. 5 in Supplementary Appendix 1). These data indicate that the human embryonic stem-cell lines reproducibly differentiate *in vitro* and *in vivo* into cell types from all three embryonic germ layers.

The 17 new cell lines described here should facilitate our understanding of the mechanisms by which differentiation of embryonic stem cells may be controlled to produce cell types for drug development and for transplantation in the treatment of disease. Under current regulations, the HUES cell lines cannot be used in research that is funded, even in part, by federal funds. The cells are being made available to researchers by Dr. Melton's laboratory under a Material Transfer Agreement. Complete information on how to obtain the cells and detailed protocols regarding their growth and maintenance are available at <http://www.mcb.harvard.edu/melton/hues>. These detailed protocols are also provided in Supplementary Appendix 1.

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Drs. Cowan, Klimanskaya, and McMahon contributed equally to this article.

From the Howard Hughes Medical Institute (C.A.C., I.K., J.M., J.A., J.P.Z., D.A.M.) and the Department of Molecular and Cellular Biology (C.A.C., I.K., J.M., J.A., J.P.Z., A.P.M., D.A.M.), Harvard University, Cambridge, Mass.; Boston IVF, Waltham, Mass. (J.W., S.W., D.P.); and the Department of Obstetrics, Gynecology, and Reproductive Biology and Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston (C.C.M.).

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1. Solter D, Knowles BB. Immunotherapy of mouse blastocyst. *Proc Natl Acad Sci U S A* 1975;72:5099-102.
2. Bongso A, Fong CY, Ng SC, Ratnam S. Isolation and culture of inner cell mass cells from human blastocysts. *Hum Reprod* 1994;9:2110-7.
3. Thomson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocysts. *Science* 1998;282:1145-7. [Erratum, *Science* 1998;282:1827.]
4. Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation *in vitro*. *Nat Biotechnol* 2000;18:399-404. [Erratum, *Nat Biotechnol* 2000;18:559.]
5. Klimanskaya I, McMahon J. Approaches for derivation and maintenance of human ES cells: detailed procedures and alternatives. In: Lanza R, ed. *Handbook of embryonic stem cells*. San Diego, Calif.: Academic Press (in press).
6. Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. *Fertil Steril* 2000;73:1155-8.

7. Brivanlou AH, Gage FH, Jaenisch R, Jessell T, Melton D, Rossant J. Stem cells: setting standards for human embryonic stem cells. *Science* 2003;300:913-6.
8. Andrews PW, Banting GS, Damjanov I, Arnaud D, Avner P. Three monoclonal antibodies defining distinct differentiation antigens associated with different high molecular weight polypeptides on the surface of human embryonal carcinoma cells. *Hybridoma* 1984;3:347-61.
9. Kannagi R, Cochran NA, Ishigami F, et al. Stage-specific embryonic antigens (SSEA-3 and -4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells. *EMBO J* 1983;2:2355-61.
10. Shevinsky L, Knowles BB, Damjanov I, Solter D. Monoclonal antibody to murine embryos defines a stage-specific embryonic antigen expressed on mouse embryos and human teratocarcinoma cells. *Cell* 1982;30:697-705.
11. Nichols J, Zevnik B, Anastasiadis K, et al. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* 1998;95:379-91.
12. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. *Exp Cell Res* 1961;25:585-621.
13. Rodriguez E, Houldsworth J, Reuter VE, et al. Molecular cytogenetic analysis of i(12p)-negative human male germ cell tumors. *Genes Chromosomes Cancer* 1993;8:230-6.
14. Skotheim RJ, Monni O, Mousses S, et al. New insights into testicular germ cell tumorigenesis from gene expression profiling. *Cancer Res* 2002;62:2359-64.
15. Longo L, Bygrave A, Grosveld FG, Pandolfi PP. The chromosome make-up of mouse embryonic stem cells is predictive of somatic and germ cell chimaerism. *Transgenic Res* 1997;6:321-8.
16. Eggan K, Rode A, Jentsch I, et al. Male and female mice derived from the same embryonic stem cell clone by tetraploid embryo complementation. *Nat Biotechnol* 2002;20:455-9.
17. Draper JS, Smith K, Gokhale P, et al. Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. *Nat Biotechnol* 2003;22:53-4.
18. Itskovitz-Eldor J, Schuldiner M, Karsenti D, et al. Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Mol Med* 2000;6:88-95.
19. Easter SS Jr, Ross LS, Frankfurter A. Initial tract formation in the mouse brain. *J Neurosci* 1993;13:285-99.
20. Bader D, Masaki T, Fischman DA. Immunochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. *J Cell Biol* 1982;95:763-70.
21. MacDonald DJ, Kelly AM. The rapid quantification of serum alpha-fetoprotein by two-site micro enzyme immunoassay. *Clin Chim Acta* 1978;87:367-72.

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