# Embryonic Stem Cell Lines Derived from Human Blastocysts

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**Human blastocyst-derived, pluripotent cell lines are described that have normal karyotypes, express high levels of telomerase activity, and express cell surface markers that characterize primate embryonic stem cells but do not characterize other early lineages. After undifferentiated proliferation in vitro for 4 to 5 months, these cells still maintained the developmental potential to form trophoblast and derivatives of all three embryonic germ layers, including gut epithelium (endoderm); cartilage, bone, smooth muscle, and striated muscle (mesoderm); and neural epithelium, embryonic ganglia, and stratified squamous epithelium (ectoderm). These cell lines should be useful in human developmental biology, drug discovery, and transplantation medicine.**

Embryonic stem (ES) cells are derived from totipotent cells of the early mammalian embryo and are capable of unlimited, undifferentiated proliferation in vitro (*1*, *2*). In chimeras with intact embryos, mouse ES cells contribute to a wide range of adult tissues, including germ cells, providing a powerful approach for introducing specific genetic changes into the mouse germ line (*3*). The term "ES cell" was introduced to distinguish these embryo-derived pluripotent cells from teratocarcinoma-derived pluripotent embryonal carcinoma (EC) cells (*2*). Given the historical introduction of the term "ES cell" and the properties of mouse ES cells, we proposed that the essential characteristics of primate ES cells should include (i) derivation from the preimplantation or periimplantation embryo, (ii) prolonged undifferentiated proliferation, and (iii) stable developmental potential to form derivatives of all three embryonic germ layers even after prolonged culture (*4*). For ethical and practical reasons, in many primate species, including humans, the ability of ES cells to contribute to the germ line in chimeras is not a testable property. Nonhuman primate ES cell lines provide an accurate in vitro model for understanding the differentiation of human tissues (*4*, *5*). We now describe human cell lines that fulfill our proposed criteria to define primate ES cells.

Fresh or frozen cleavage stage human embryos, produced by in vitro fertilization (IVF) for clinical purposes, were donated by individuals after informed consent and after institutional review board approval. Embryos were cultured to the blastocyst stage, 14 inner cell masses were isolated, and five ES cell lines originating from five separate embryos were derived, essentially as described for nonhuman primate ES cells (*5*, *6*). The resulting cells had a high ratio of nucleus to cytoplasm, prominent nucleoli, and a colony morphology similar to that of rhesus monkey ES cells (Fig. 1). Three cell lines (H1, H13, and H14) had a normal XY karyotype, and two cell lines (H7 and H9) had a normal XX karyotype. Each of the cell lines was successfully cryopreserved and thawed. Four of the cell lines were cryopreserved after 5 to 6 months of continuous undifferentiated proliferation. The other cell line, H9, retained a normal XX karyotype after 6 months of culture and has now been passaged continuously for more than 8 months (32 passages). A period of replicative crisis was not observed for any of the cell lines.

The human ES cell lines expressed high levels of telomerase activity (Fig. 2). Telomerase is a ribonucleoprotein that adds telomere repeats to chromosome ends and is involved in maintaining telomere length, which plays an important role in replicative life-span (*7*, *8*). Telomerase expression is highly correlated with immortality in human cell lines, and reintroduction of telomerase activity into some diploid human somatic cell lines extends replicative life-span (*9*). Diploid human somatic cells do not express telomerase, have shortened telomeres with age, and enter replicative senescence after a finite proliferative life-span in tissue culture (*10*–*13*). In contrast, telomerase is present at high levels in germ line and embryonic tissues (*14*). The high level of telomerase activity expressed by the human ES cell lines therefore suggests that their replicative life-span will exceed that of somatic cells.

The human ES cell lines expressed cell surface markers that characterize undifferentiated nonhuman primate ES and human EC cells, including stage-specific embryonic antigen (SSEA)–3, SSEA-4, TRA-l-60, TRA-1-81, and alkaline phosphatase (Fig. 3) (*4*, *5*, *15*, *16*). The globo-series glycolipid GL7, which carries the SSEA-4 epitope, is formed by the addition of sialic acid to the globo-series glycolipid Gb5, which carries the SSEA-3 epitope (*17*, *18*). Thus, GL7 reacts with antibodies to both SSEA-3 and SSEA-4 (*17*, *18*). Staining intensity for SSEA-4 on the human ES cell lines was consistently strong, but staining intensity for SSEA-3 was weak and varied both within and among colonies (Fig. 3, D and C). Because GL7 carries both the SSEA-4 and SSEA-3 epitopes and because staining for SSEA-4 was consistently strong, the relatively weak staining for

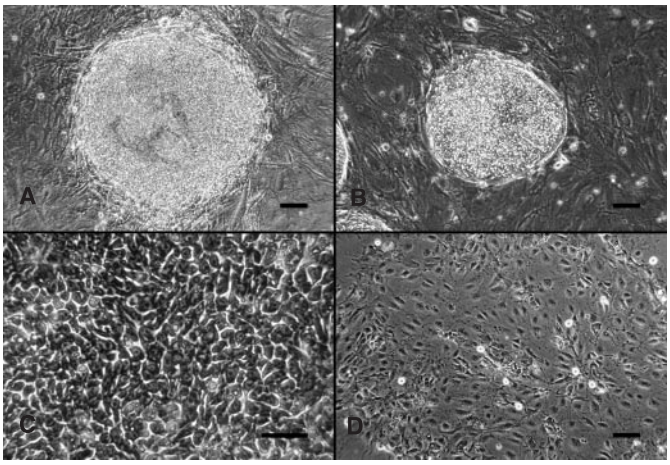


**Fig. 1.** Derivation of the H9 cell line. (**A**) Inner cell mass–derived cells attached to mouse embryonic fibroblast feeder layer after 8 days of culture, 24 hours before first dissociation. Scale bar, 100 μm. (**B**) H9 colony. Scale bar, 100 μm. (**C**) H9 cells. Scale bar, 50 μm. (**D**) Differentiated H9 cells, cultured for 5 days in the absence of mouse embryonic fibroblasts, but in the presence of human LIF (20 ng/ml; Sigma). Scale bar, 100 μm.

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SSEA-3 suggests a restricted access of the antibody to the SSEA-3 epitope. In common with human EC cells, the undifferentiated human ES cell lines did not stain for SSEA-1, but differentiated cells stained strongly for SSEA-l (*15*) (Fig. 3). Mouse inner cell mass cells, ES cells, and EC cells express SSEA-1 but do not express SSEA-3 or SSEA-4 (*17*, *19*), suggesting basic species differences between early mouse and human development.

The human ES cell lines were derived by the selection and expansion of individual colonies of a uniform, undifferentiated morphology, but none of the ES cell lines was derived by the clonal expansion of a single cell. The uniform undifferentiated morphology that is shared by human ES and nonhuman primate ES cells and the consistent expression by the human ES cell lines of cell surface markers that uniquely characterize primate ES and human EC cells make it extremely unlikely that a mixed population of precursor cells was expanded. However, because the cell lines were not cloned from a single cell, we cannot rule out the possibility that there is some variation in developmental potential among the undifferentiated cells, in spite of their homogeneous appearance.

The human ES cell lines maintained the potential to form derivatives of all three embryonic germ layers. All five cell lines produced teratomas after injection into severe combined immunodeficient (SCID)–beige mice. Each injected mouse formed a teratoma, and all teratomas included gut epithelium (endoderm); cartilage, bone, smooth muscle, and striated muscle (mesoderm); and neural epithelium, embryonic ganglia, and stratified squamous epithelium (ectoderm) (Fig. 4). In vitro, the ES cells differentiated when cultured in the absence of mouse embryonic fibroblast feeder layers, both in the presence and absence of human leukemia inhibitory factor (LIF) (Fig. 1). When grown to confluence and allowed to pile up in the culture dish, the ES cell lines differentiated spontaneously even in the presence of fibroblasts. After H9 cells were allowed to differentiate for 2 weeks, both α-fetoprotein (350.9 ± 14.2 IU/ml) and human chorionic gonadotropin (hCG, 46.7 ± 5.6 mIU/ml) were detected in conditioned culture medium, indicating endoderm and trophoblast differentiation (*20*).

Human ES cells should offer insights into developmental events that cannot be studied directly in the intact human embryo but that have important consequences in clinical areas, including birth defects, infertility, and pregnancy loss. Particularly in the early postimplantation period, knowledge of normal human development is largely restricted to the description of a limited number of sectioned embryos and to analogies drawn from the experimental embryology of other species (*21*). Although the mouse is the mainstay of experimental mammalian embryology, early structures including the placenta, extraembryonic membranes, and the egg cylinder all differ substantially from the corresponding structure of the human embryo. Human ES cells will be particularly valuable for the study of the development and function of tissues that differ between mice and humans. Screens based on the in vitro differentiation of human ES cells to specific lineages could identify gene targets for new drugs, genes that could be used for tissue regeneration therapies, and teratogenic or toxic compounds.

Elucidating the mechanisms that control differentiation will facilitate the efficient, directed differentiation of ES cells to specific cell types. The standardized production of large, purified populations of euploid human cells such as cardiomyocytes and neurons will provide a potentially limitless source of cells for drug discovery and

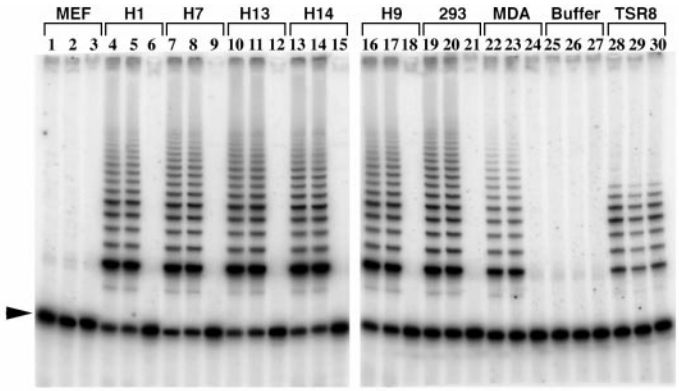


**Fig. 2.** Telomerase expression by human ES cell lines. MEF, irradiated mouse embryonic fibroblasts used as a feeder layer for the cells in lanes 4 to 18; 293, adenovirus-transformed kidney epithelial cell line 293; MDA, breast cancer cell line MDA; TSR8, quantitation control template. Telomerase activity was measured with the TRAPEZE Telomerase Detection Kit (Oncor, Gaithersburg, Maryland). The ES cell lines were analyzed at passages 10 to 13. About 2000 cells were assayed for each telomeric repeat amplification protocol assay, and 800 cell equivalents were loaded in each well of a 12.5% nondenaturing polyacrylamide gel. Reactions were done in triplicate with the third sample of each triplet heat inactivated for 10 to 15 min at 85°C before reaction to test for telomerase heat sensitivity (lanes 6, 9, 12, 15, 18, 21, 24, and 27). A 36–base pair internal control for amplification efficiency and quantitative analysis was run for each reaction as indicated by the arrowhead. Data were analyzed with the Storm 840 Scanner and ImageQuant package (Molecular Dynamics). Telomerase activity in the human ES cell lines ranged from 3.8 to 5.9 times that observed in the immortal human cell line MDA on a per cell basis.

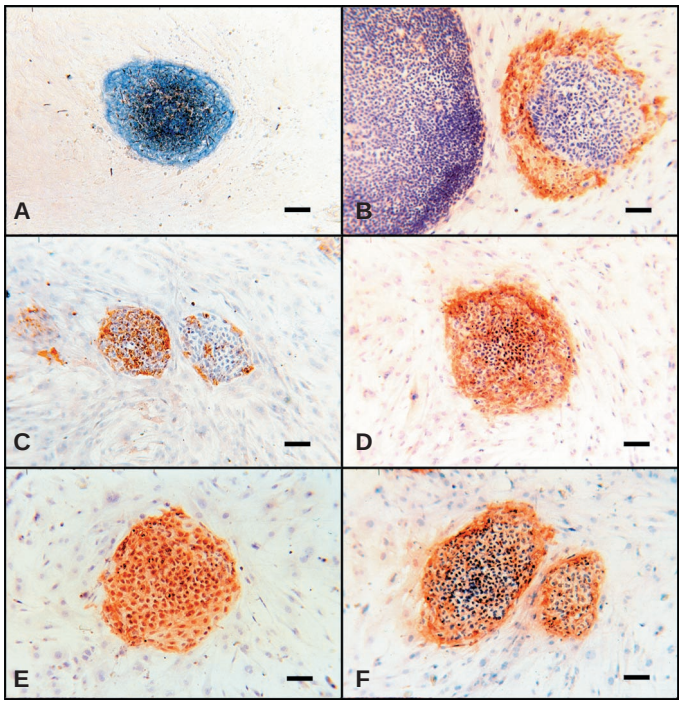


**Fig. 3.** Expression of cell surface markers by H9 cells. Scale bar, 100 μm. (**A**) Alkaline phosphatase. (**B**) SSEA-1. Undifferentiated cells failed to stain for SSEA-1 (large colony, left). Occasional colonies consisted of nonstained, central, undifferentiated cells surrounded by a margin of stained, differentiated, epithelial cells (small colony, right). (**C**) SSEA-3. Some small colonies stained uniformly for SSEA-3 (colony left of center), but most colonies contained a mixture of weakly stained cells and a majority of nonstained cells (colony right of center). (**D**) SSEA-4. (**E**) TRA-1-60. (**F**) TRA-1-81. Similar results were obtained for cell lines H1, H7, H13, and H14.

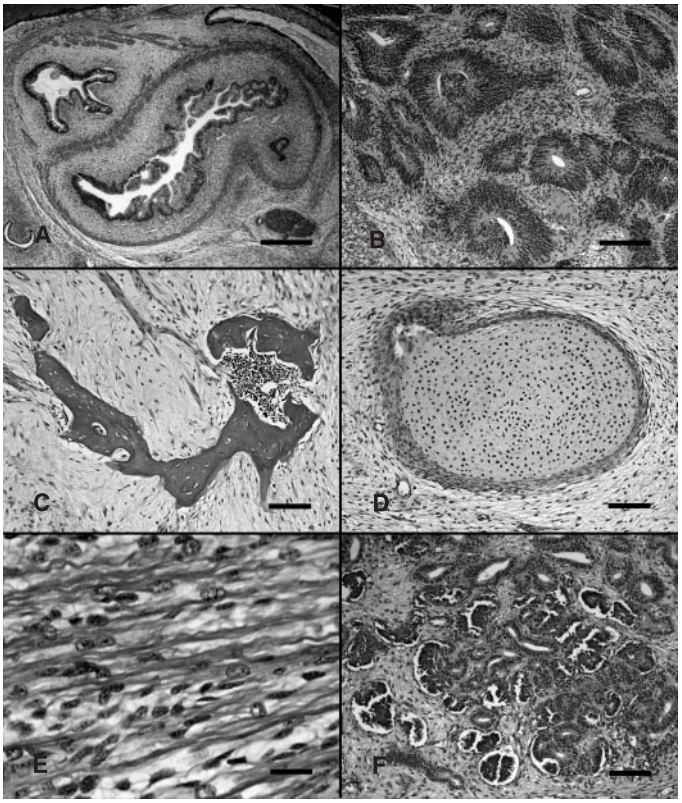


**Fig. 4.** Teratomas formed by the human ES cell lines in SCID-beige mice. Human ES cells after 4 to 5 months of culture (passages 14 to 16) from about 50% confluent six-well plates were injected into the rear leg muscles of 4-week-old male SCID-beige mice (two or more mice per cell line). Seven to eight weeks after injection, the resulting teratomas were examined histologically. (**A**) Gutlike structures. Cell line H9. Scale bar, 400 μm. (**B**) Rosettes of neural epithelium. Cell line H14. Scale bar, 200 μm. (**C**) Bone. Cell line H14. Scale bar, 100 μm. (**D**) Cartilage. Cell line H9. Scale bar, 100 μm. (**E**) Striated muscle. Cell line H13. Scale bar, 25 μm. (**F**) Tubules interspersed with structures resembling fetal glomeruli. Cell line H9. Scale bar, 100 μm.

transplantation therapies. Many diseases, such as Parkinson's disease and juvenile-onset diabetes mellitus, result from the death or dysfunction of just one or a few cell types. The replacement of those cells could offer lifelong treatment. Strategies to prevent immune rejection of the transplanted cells need to be developed but could include banking ES cells with defined major histocompatibility complex backgrounds or genetically manipulating ES cells to reduce or actively combat immune rejection. Because of the similarities to humans and human ES cells, rhesus monkeys and rhesus ES cells provide an accurate model for developing strategies to prevent immune rejection of transplanted cells and for demonstrating the safety and efficacy of ES cell–based therapies. Substantial advances in basic developmental biology are required to direct ES cells efficiently to lineages of human clinical importance. However, progress has already been made in the in vitro differentiation of mouse ES cells to neurons, hematopoietic cells, and cardiac muscle (*22–24*). Progress in basic developmental biology is now extremely rapid; human ES cells will link this progress even more closely to the prevention and treatment of human disease.

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6. Thirty-six fresh or frozen-thawed donated human embryos produced by IVF were cultured to the blastocyst stage in G1.2 and G2.2 medium (*25*). Fourteen of the 20 blastocysts that developed were selected for ES cell isolation, as described for rhesus monkey ES cells (*5*). The inner cell masses were isolated by immunosurgery (*26*), with a rabbit antiserum to BeWO cells, and plated on irradiated (35 grays gamma irradiation) mouse embryonic fibroblasts. Culture medium consisted of 80% Dulbecco's modified Eagle's medium (no pyruvate, high glucose formulation; Gibco-BRL) supplemented with 20% fetal bovine serum (Hyclone), 1 mM glutamine, 0.1 mM β-mercaptoethanol (Sigma), and 1% nonessential amino acid stock (Gibco-BRL). After 9 to 15 days, inner cell mass–derived outgrowths were dissociated into clumps either by exposure to $Ca^{2+}$/$Mg^{2+}$-free phosphate-buffered saline with 1 mM EDTA (cell line H1), by exposure to dispase (10 mg/ml; Sigma; cell line H7), or by mechanical dissociation with a micropipette (cell lines H9, H13, and H14) and replated on irradiated mouse embryonic fibroblasts in fresh medium. Individual colonies with a uniform undifferentiated morphology were individually selected by micropipette, mechanically dissociated into clumps, and replated. Once established and expanded, cultures were passaged by exposure to type IV collagenase (1 mg/ml; Gibco-BRL) or by selection of individual colonies by micropipette. Clump sizes of about 50 to 100 cells were optimal. Cell lines were initially karyotyped at passages 2 to 7.
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16. Alkaline phosphatase was detected with Vector Blue substrate (Vector Labs). SSEA-1, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 were detected by immunocytochemistry with specific primary monoclonal antibodies and localized with a biotinylated secondary antibody and then an avidin or biotinylated horseradish peroxidase complex (Vectastain ABC system; Vector Laboratories) as previously described (*5*). The ES cell lines were at passages 8 to 12 at the time markers were analyzed.
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20. hCG and α-fetoprotein were measured by specific radioimmunoassay (double AB hCG and AFP-TC kits; Diagnostic Products, Los Angeles, CA). hCG assays used the World Health Organization Third International Standard 75/537. H9 cells were allowed to grow to confluence (day 0) on plates of irradiated mouse embryonic fibroblasts. Medium was replaced daily. After 2 weeks of differentiation, medium in triplicate wells conditioned for 24 hours was assayed for hCG and α-fetoprotein. No hCG or α-fetoprotein was detected in unconditioned medium.
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27. We thank the personnel of the IVF clinics at the University of Wisconsin School of Medicine and at the Rambam Medical Center for the initial culture and cryopreservation of the embryos used in this study; D. Gardner and M. Lane for the G1.2 and G2.2 media; P. Andrews for the NTERA2 cl.D1 cells and the antibodies used to examine cell surface markers; C. Harris for karyotype analysis; and Geron Corporation for the 293 and MDA cell pellets and for assistance with the telomerase TRAP assay. Supported by the University of Wisconsin (UIR grant 2060) and Geron Corporation (grant 133-BU18).

5 August 1998; accepted 7 October 1998