Serum-free derivation of human embryonic stem cell lines on human placental fibroblast feeders

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Objective: To derive new human embryonic stem cell (hESC) lines on pathogen-free human placental fibroblast feeders under serum-free conditions. Because the embryo develops in close contact with extraembryonic membranes, we hypothesized that placental mesenchyme might replicate the stem cell niche in situ.

Design: We isolated and characterized human placental fibroblast lines from individual donors and tested their ability to support growth of federally registered hESC lines. Moreover, we performed extensive pathogen testing to ensure their suitability as feeders for the derivation of therapy-grade hESCs.

Result(s): Human placental fibroblasts were comparable or superior to mouse embryo fibroblasts as hESC feeders. We used these qualified placental fibroblasts to derive two new hESC lines in knockout Dulbecco's modified Eagle's medium with serum-free 20% knockout serum replacement. The cells, which had a normal karyotype, were grown for more than 25 passages, expressed markers of stemness including Oct-3/4, Tra 1-60, Tra 1-80, and SSEA-4, exhibited high telomerase activity, and differentiated in vitro and in vivo into cells derived from all three germ layers, confirming their pluripotency. Additionally, newly derived hESCs were adapted to growth on a human placental laminin substrate in a defined medium.

Conclusion(s): To our knowledge, this is the first report of hESC derivation in the absence of serum on qualified pathogen-free human feeders. (Fertil Steril® 2005;83:1517–29. ©2005 by American Society for Reproductive Medicine.)

Key Words: Human embryonic stem cells, human placental fibroblasts, laminin, nonxenogeneic culture, serum-free medium

Thus far, much of the intellectual driving force behind human embryonic stem cell (hESC) work has come from the murine embryonic stem (ES) cell field (reviewed by 1–3). The wealth of existing knowledge about murine cells has allowed investigators to capitalize on the similarity between ES cells from both species to jump-start the human ES cell field. For example, mouse embryo fibroblast (MEF) feeders are commonly used in the derivation of ES cell lines from both species (4–6). However, this approach has a significant downside in the derivation of hESCs. To realize the enormous potential benefits of hESC therapy, banks of cell lines that express different combinations of the major histocompatibility genes should be constructed, preferably without exposing embryos and lines derived from them to animal cells and proteins.

At the same time, it has become apparent that there are important fundamental differences between the culture conditions required for propagation of human and murine ES cells. For example, leukemia inhibitory factor, which allows the growth of murine ES cells in an undifferentiated state, does not have the same effect on hESCs (5). The concept of species specialization in stem cell niches is likely one very important reason for the observed differences.

Researchers have long appreciated the importance of the microenvironment in regulating stem cell fate, that is, the decision to proliferate and self-renew or to enter a multistep differentiation pathway. The concept of specialized niches originated in the hematopoiesis field, where a great deal is known about the in vitro conditions that mimic the in vivo environment. Secreted molecules, such as colony-stimulat-

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ing factor and stem cell factor (Kit ligand), play especially important roles, particularly in survival (reviewed by 7, 8). Very recent work highlights this critical principle: matrix metalloproteinase-9 expression induced in bone marrow cells releases soluble Kit ligand, permitting the transfer of endothelial and hematopoietic stem cells from the quiescent to the proliferative niche. Bone marrow ablation induces stromal cell–derived factor-1, which upregulates matrix metalloproteinase-9 expression and causes shedding of soluble Kit ligand and recruitment of c-Kit$^+$ stem/progenitors (9).

Contact with the extracellular matrix and with other cells is another important mechanism for sensing the microenvironment and making decisions that govern cell fate. Heterodimeric integrin cell adhesion receptors are one class of molecules that mediate stem cell-matrix interactions. For example, high expression of β1 integrins is required for maintenance of the epidermal stem cell pool (10).

Interactions with other cells also play an important role. The Drosophila Ca^{2+}-dependent cell-cell adhesion molecule is required for recruiting and anchoring germ line stem cells to their niche (11). Cell-cell interactions involving other classes of molecules are also important. Interestingly, interactions between Eph tyrosine kinase receptors and their ephrin transmembrane ligands regulate neural stem cell proliferation and migration (12).

Compared with somatic stem cells, we know a great deal less about the specialized microenvironment that gives rise to the transient stem cell population of the early-stage human embryo, which, in turn, forms the three primordial germ layers. Here we used early-gestation placental fibroblasts in an attempt to recreate this specialized niche. The results showed that feeders formed from these cells can be used to propagate established hESC lines and derive new ones. Since the placental fibroblasts were screened for human and animal pathogens and no serum was used during the derivation process, this method greatly reduces the risk of zoonoses.

MATERIALS AND METHODS

Materials

All the reagents and materials used were from Invitrogen Chemical Company (Carlsbad, CA) or Sigma Chemical Company (St. Louis, MO), unless otherwise noted.

Establishing Lines of Human Placental Fibroblasts. Human placentas (6–9 weeks of gestation) were obtained individually. The protocol was approved by the University of California's Committee on Human Research. The placentas were immediately transferred to Dulbecco's modified Eagle's H-21 medium containing 2.5% fetal bovine serum (FBS), glutamine, penicillin, streptomycin, and gentamicin. After transport back to the laboratory, the tissue was transferred, in a biosafety cabinet, to a sterile petri dish containing phosphate-buffered saline (PBS).

The chorionic villi, consisting of fibroblast cores with a trophoblast covering, were dissected from the fetal membranes and minced into 3- to 4-mm pieces. The villus fragments were isolated by centrifugation (1,000 $\times$ *g* for 5 minutes) and weighed before they were transferred to a 100-mL flask. Enzyme mixture I (0.0620 g collagenase, 0.04 g DNase, 0.069 g hyaluronidase, and 0.1 g bovine serum albumin in 100 mL of PBS) was added at a ratio of 4 mL/g of tissue. Digestion was carried out by swirling the flask for 6 minutes in a 37°C water bath. Then the suspension was filtered through gauze to collect the tissue fragments, which were transferred to another 100-mL flask. Trypsin solution I (0.0069 g trypsin, 0.04 g Dnase, and 0.02 g EDTA in 100 mL of PBS) was added at a ratio of 3 mL/g of tissue. Digestion was carried out for 5 minutes in a 37°C water bath. The tissue fragments were collected as described above and transferred to another 100-mL flask. Trypsin solution II (8 g/L NaCl, 1.0 g/L glucose, 0.4 g/L KCl, 2.5 g/L trypsin, 4.5 mg/L phenol red, 0.2 g/L EDTA, and 0.58 g/L $NaHCO_3$) was added at a ratio of 2 mL/g of tissue. The digestion was carried out for 45 minutes in a 37°C water bath; 10% FBS was then added to stop enzyme activity. The suspension was passed through gauze, which was washed with PBS, and the filtrate was collected in toto.

Cells were isolated by centrifugation (1,000 $\times$ *g* at 4°C for 10 minutes). The supernatant was discarded, and the pellet was resuspended in 10 mL of Dulbecco's modified Eagle's medium (DMEM), which was passed through a 105-μm mesh Nitex filter (Small Parts Inc., Miami Lakes, FL). Additional medium was added to a final volume of 50 mL. The cell pellet was washed three times by centrifugation before being resuspended in 10 mL of DMEM/M199 (4:1, vol/vol) containing 10% FBS and plated in a T25 flask.

The next day, the medium was changed and the culture was not disturbed for 4–5 days. If growth was observed, the medium was changed at 4-day intervals. Once the cells reached confluence, they were removed from the tissue culture plate with trypsin solution II and split in a 1:2 ratio into T25 flasks. After three passages, the purity of the cells was assessed using immunolocalization methods (13). The absence of staining for cytokeratin (indicative of trophoblast contamination [14]), factor VIII (indicative of endothelial cells; Dako, Carpinteria, CA), and CD45 (indicative of leukocytes; Dako) was verified together with the presence of vimentin staining (indicative of placental fibroblasts; Sigma). In all, three lines from different placentas were established.

Pathogen Testing. A two-phase process was used for pathogen testing. The first phase was initial screening by using the polymerase chain reaction (PCR) for the detection of bacteria and viruses that commonly infect the placenta. Specifically, we assayed for genes encoded by cytomegalovirus, herpes simplex virus types 1 and 2, bacteria, *Chlamydia trachomatis*, *Chlamydia spp*, *Mycoplasma spp*, *Mycoplasma genitalium*, *Mycoplasma hominis*, and *Ureaplasma urealyticum*. With the exception of *Mycoplasma spp* primers (for-