STEM CELLS®
Rapid Communication

This material is protected by U.S. Copyright law.
Unauthorized reproduction is prohibited.
For reprints contact: Reprints@AlphaMedPress.com

Human Embryonic Stem Cell Lines Derived from Discarded Embryos

MAISAM MITALIPOVA,[a] JOHN CALHOUN,[b] SOOJUNG SHIN,[c] DAVID WININGER,[d] THOMAS SCHULZ,[c] SCOTT NOGGLE,[e,f] ALISON VENABLE,[b] IAN LYONS,[a] ALLAN ROBINS,[a] STEVEN STICE[c]

[a]BresaGen, Inc., Athens, Georgia, USA; [b]Department of Biochemistry and Molecular Biology, University of Georgia, Athens, Georgia, USA; [c]Department of Animal and Dairy Sciences, University of Georgia, Athens, Georgia, USA; [d]Reproductive Biology Associates, Atlanta, Georgia, USA; [e]Institute of Molecular Medicine and Genetics, Medical College of Georgia, Augusta, Georgia, USA; [f]Department of Genetics, University of Georgia, Athens, Georgia, USA

Key Words. *Embryonic stem cell · Differentiation · Discarded embryo*

ABSTRACT

Human pluripotent embryonic stem (ES) cells have important potential in regenerative medicine and as models for human preimplantation development; however, debate continues over whether embryos should be destroyed to produce human ES cells. We have derived four ES cell lines on mouse embryonic fibroblast cells in medium supplemented with basic fibroblast growth factor, human recombinant leukemia inhibitory factor, and fetal bovine serum. The source of these cell lines was poor-quality embryos that in the course of routine clinical practice would have been discarded. After continuous proliferation in vitro for more than 12 months, these ES cell lines maintained their developmental potential to form trophoblast and somatic cells, including cardiac muscle and neuronal tissue. *Stem Cells* *2003;21:521-526*

INTRODUCTION

Although the National Institutes of Health (NIH) registry of human embryonic stem (hES) cell lines lists 78 lines isolated in five countries, it seems likely that more will need to be isolated. Of the 78 NIH registered lines, only 11 have been significantly characterized and are currently available to researchers (http://stemcells.nih.gov/registry/). In addition to the fact that relatively few hES cell lines are currently available, these lines were derived on mouse feeder layers, and would be treated as a xenotransplant product by regulatory authorities. Established hES cells can be maintained on human feeders [1-3] or without feeders on Matrigel [4], and new cell lines have been derived using human feeders without exposure to animal cell types [2].

Embryos previously used to generate hES cell lines were in excess of clinical requirements, but their developmental stages and morphological characteristics were not documented [2, 5, 6]. Typically, in human fertility clinics, eight-cell or blastocyst-stage embryos with cell number and morphology appropriate to their age are transferred to the patient, donated, or cryopreserved. Embryos lagging behind normal development, with poor morphology, or, in the case of blastocysts, lacking a distinct inner cell mass (ICM), or with low cell numbers are discarded, since they are unlikely to establish pregnancy.

The scoring systems used to assess such embryos vary among individual in vitro fertilization facilities, though

Correspondence: Maisam Mitalipova, Ph.D., BresaGen, Inc., 111 Riverbend Road, Georgia Biobusiness Center, Athens, Georgia 30605, USA. Telephone: 706-613-9878, ext. 279; Fax: 706-613-9879; e-mail: mitalipova@hotmail.com *Received March 6, 2003; accepted for publication July 22, 2003. ©AlphaMed Press 1066-5099/2003/$12.00/0*

© AlphaMed Press

standardized methods have been proposed. This has allowed the correlation between embryo quality and pregnancy outcome to be demonstrated: good-quality blastocysts establish pregnancy with twice the frequency of slightly less advanced embryos [7]. However, experiments that assess the developmental potential of embryos that are days behind normal development and have very few ICM cells, are not feasible, and so these embryos are discarded. Here, we report the establishment of four hES cell lines from 19 such embryos.

Materials and Methods

Derivation and Culture of hES Cells

Embryos were donated to this study with the informed consent of couples that had already undergone an embryo transfer at day 3 or 5 postfertilization of the same cycle. Any good-quality embryos that were not transferred were cryopreserved for the couples' future use. Remaining embryos were graded at day 6 or 7 by the scale of *Gardner et al*. [7], which assesses the extent of blastocyst expansion on a scale of 1 to 6, with 3 being a full blastocyst, and, for embryos of grade 3 or better, the size and quality of ICM and trophectoderm (TE) are each assessed on a three-point scale. The embryos used in this study normally would have been discarded since they were of such poor quality that there was little likelihood of them surviving freezing and thawing.

Zona pelucidae were removed by Pronase (Sigma; St. Louis, MO; http://www.sigmaaldrich.com) digestion (1 mg/ml) for 2 minutes. TEs were removed by immunosurgery [8] using antiplacental alkaline phosphatase antibody (DAKO; Carpenteria, CA; http://www.dakocytomation.com; 1:10 dilution) and guinea pig complement (GIBCO/BRL; Grand Island, NY; http://www.invitrogen.com; 1:4 dilution) in 50-μl droplets under oil. Cell lines were established and maintained on mitotically inactivated mouse embryonic fibroblast (MEF) feeder layers derived from E13.5 random bred mouse fetuses, as previously described [9], in hES cell culture medium: knockout Dulbecco's-modified Eagle's medium (DMEM; GIBCO/ BRL) supplemented with 20% fetal bovine serum (FBS; HyClone; Logan, UT; http://www.hyclone.com), 2 mM L-glutamine (GIBCO/BRL), 1× minimal essential medium nonessential amino acids (GIBCO/BRL), 50 U/ml penicillin, 50 μg/ml streptomycin, 1,000 U/ml recombinant human leukemia inhibitory factor (hLIF; Chemicon; Temecula, CA; http://www.chemicon.com), 0.1 mM β-mercaptoethanol (GIBCO/BRL), and 4 ng/ml basic fibroblast growth factor (bFGF; GIBCO/BRL).

In Vitro Characterization

Before immunostaining, colonies of ES cells were plated on MEF feeder layers in Permanox Chamber Slides (Nalge Nunc; Rochester, NY; htpp://www.nalgenunc.com) fixed in 4% paraformaldehyde in phosphate-buffered saline for 30 minutes. Antibodies used were against: Oct-4 (rabbit polyclonal antigen; Santa Cruz Biotechnology, Inc.; Santa Cruz, CA; http://www.scbt.com; 1:500 dilutions), stage-specific embryonic antigen SSEA-1 (MC480, Developmental Studies Hybridoma Bank [DSHB]; Iowa City, IA; http://www.uiowa.edu/~dshbwww; 1:10), SSEA-3 (MC631, DSHB; 1:10), SSEA-4 (MC 813-70, DSHB; 1:10), TRA-1-60 and TRA-1-81 (a gift of *Dr. Peter Andrews*, University of Sheffield; at 1:10 and 1:12 dilutions, respectively), microtubule-associated protein (MAP)2 (Sigma; 1:500), and nestin (Chemicon; 1:200). Each antibody was detected using appropriate secondary antibodies conjugated to Alexafluor 488 or 594 (Molecular Probes; Eugene, OR; http://www.probes.com; 1:1000). Alkaline phosphatase was detected with the Vector Red substrate kit (Vector Laboratories; Burlingame, CA; http://www.vectorlabs.com). Karyotypes were determined by a standard G-banding method by the Wisconsin State Laboratory of Hygiene.

In Vitro Differentiation

Trophectoderm

Colonies of BG01 passage 65 ES cells on MEFs were grown to confluence over 3 weeks, with the medium replaced every 2 days. Medium, conditioned by the differentiated cells for 48 hours, was concentrated 10-fold with a 10-kD centrifuge filtration device (Millipore; Billerica, MA; http://www.millipore.com), and the amount of human chorionic gonadotropin (hCG) was measured with a radioimmunoassay kit (ICN Pharmaceuticals; Costa Mesa, CA; http://www.icnpharm.com) recognizing the β subunit of hCG. Samples were tested in duplicate both undiluted (after concentration) and diluted twofold with the control serum provided in the kit, to ensure the concentration was within the linear range of the assay.

Neural Cells

Embryoid bodies (EBs) were generated by cutting colonies into pieces with a solid glass needle made from a Pasteur pipette and cultured in suspension in ES cell medium. At day 12, EBs were seeded onto polyornithine/laminin-coated slides and cultured in knockout DMEM, 10% FBS, 1× nonessential amino acids, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. EBs were fixed at day 18 for immunostaining.

Cardiac Muscle

Clumps of 150-200 cells were cultured in nonadherent 35-mm culture dishes in hES cell medium to form EBs.

After 6 days, EBs were plated onto 0.1% gelatin-coated culture dishes. EB explants were observed for 3 weeks. Five days after plating, some colonies contained beating cells.

Results

Donated embryos were scored after *Gardner et al*. [7], who developed a grading system that assesses the extent of blastocyst expansion on a scale of 1 to 6, with 3 being a fully expanded blastocyst. Blastocysts that are full, expanded, or hatched are classified further to assess ICM and TE. All embryos used in this study had very poor blastocyst scores despite having been cultured an extra 24-48 hours; 7 of 19 did not have a full blastocoel (grade 1 or 2), five were full blastocysts (grade 3), six were expanded (grade 4), and one was hatching (grade 5). Two embryos had an ICM of grade B ("loosely grouped, several cells"), while the others were of grade C ("very few cells"). TE of all embryos was graded B ("few cells forming a loose epithelium") or C ("very few large cells").

After removal of the zona pelucidae by Pronase digestion and trophoblast cells by immunosurgery [8], the remaining cells were cultured on mitotically inactivated MEFs isolated from 13.5-day postcoitum fetuses of outbred mice. The human embryos were poorly developed compared with normal embryos 5 or 6 days after in vitro fertilization, and in most cases, there were few ICM cells visible.

Since the ICMs from these embryos were lacking the cell numbers and quality found in good-quality embryos, we added recombinant hLIF and bFGF into the culture medium as a mitogen, as previously described, to isolate mouse and human primordial germ cell-derived pluripotent cell lines [10-12].

When colonies of small compact cells were observed 7 to 10 days after seeding (Fig. 1A), they were manually dissociated into clumps of 10-100 cells and replated on fresh feeder layers. The cells had a high ratio of nucleus to cytoplasm and prominent nucleoli (Fig. 1A). Colonies of cells were maintained and expanded in this way for four

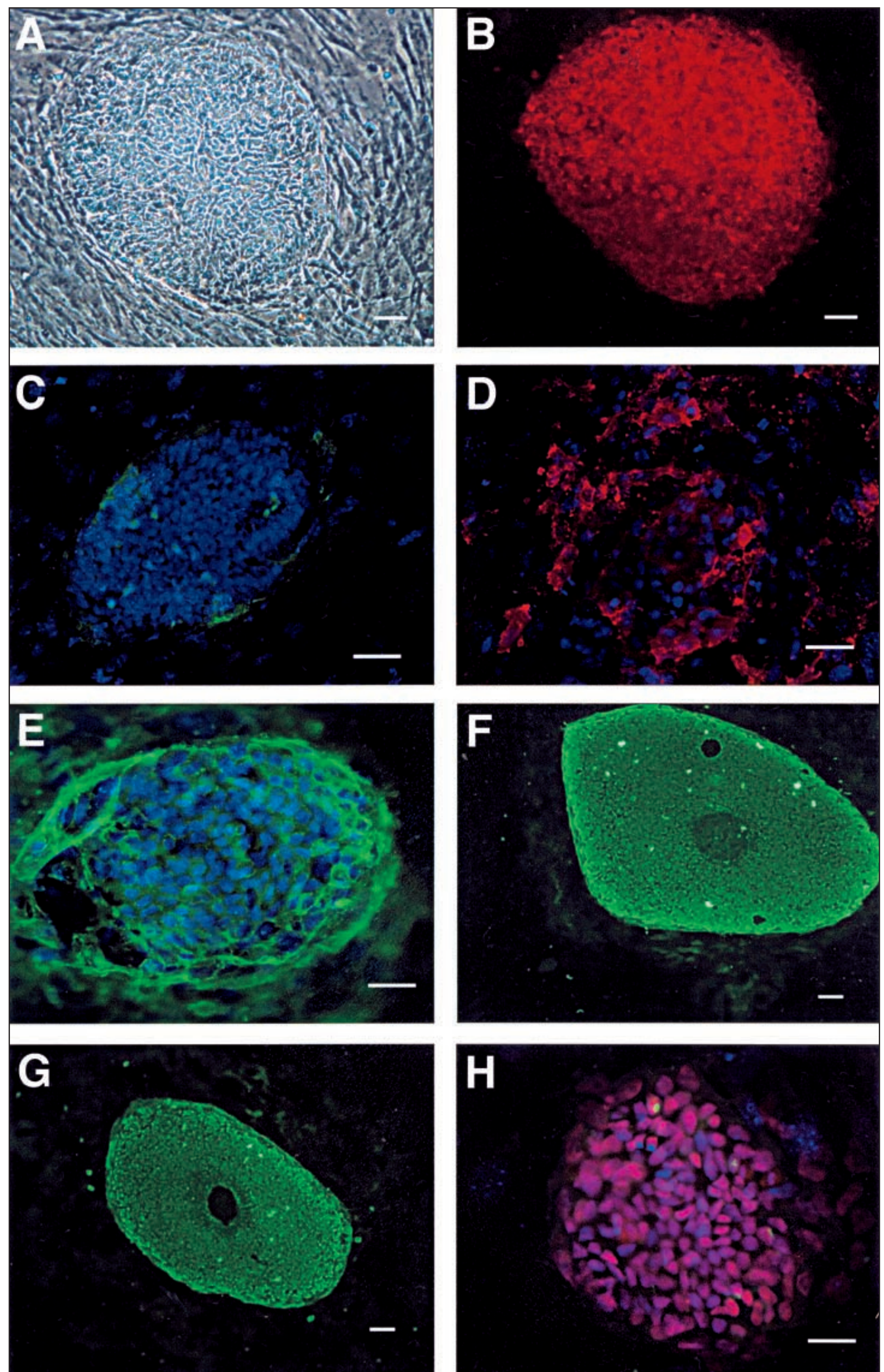

Figure 1. Expression of cell markers by hES cells. *A) Phase contrast micrograph of hES cell colony. B) Alkaline phosphatase. C) SSEA-1. D) SSEA-3. E) SSEA-4. F) TRA-1-60. G) TRA-1-81. H) Oct-4. Scale bars are 100 µm.*

Table 1. Establishment of human ES cell lines

Embryo	Day	Embryo Grade Blastocyst (1-6) ICM (A, B, C) TE (A, B, C)	Attach	Outgrowth	Cell line
1	6	2	Y	Y	
2	7	1			
3	7	3CC	Y	Y	BG01
4	6	4CB	Y	Y	
5	7	4CC	Y		
6	7	3BC	Y		
7	7	4CC	Y		
8	7	3CC			
9	7	2			
10	6	4CB			
11	6	4CC	Y	Y	
12	6	1			
13	6	2			
14	6	3CC	Y	Y	BG02
15	7	2			
16	6	3CC	Y	Y	BG03
17	6	5CB	Y	Y	BG04
18	7	4BC	Y	Y	
19	7	2			

Y = yes

passages (about 25-30 days), at which stage the cell lines were cryopreserved.

No cell lines were isolated from blastocysts of grade 1 or 2, though a cluster of cells from one of the seven grade 1 and 2 embryos attached and proliferated after immunosurgery. The cell lines BG01, BG02, and BG03 were isolated from embryos graded as 3CC, and BG04 was isolated from a 5CB embryo (Table 1).

Of these four cell lines, two have been thawed and extensively characterized. The cell line BG01 has been grown for over 80 passages, and BG02 for over 30 passages in vitro, without loss of their initial ES cell morphology, expression of appropriate markers, or the ability to differentiate to multiple lineages. Marker expression analysis was performed with BG01 ES cells at passages 4-85, with BG02 at passages 4-35, and with BG03 and BG04 at passages 3-5. These hES cell lines expressed cell surface markers that characterize undifferentiated nonhuman primate and human ES, embryonic carcinoma (EC), and embryonic germ (EG) cells [13], including alkaline phosphatase, transcription factor Oct-4, SSEA-3 and SSEA-4, and TRA-1-60 and TRA-1-81 (Fig. 1B-H). As shown in Fig.1 B-H, antibody binding to colonies of human ES cells was strong for five of the six antibodies. The antibody recognizing SSEA-3 antigen exhibited weak binding to ES cells (Fig. 1D), as previously described, for human ES, EC, and EG cells [5, 6, 12].

Two of the four cell lines, BG01 and BG02, have been cytogenetically analyzed and have a normal 46XY karyotype after 35 passages (6 months in culture). The other two ES cell lines have not yet been propagated beyond 5 passages.

In Vitro Differentiation

Spontaneous differentiation of colonies of ES cells was induced by allowing the cells to grow beyond confluence over 2-3 weeks with the medium changed every 2 days. After 15 days at confluence, the level of hCG was measured in conditioned medium from ES cell line BG01 at passage 65. The cells produced 3.3 ± 0.8 mIU hCG/ml of conditioned medium. No hCG was detected in control medium conditioned only by undifferentiated ES cells and a feeder layer.

Like other hES cell lines, BG01 and BG02 are capable of forming EBs in vitro (Fig. 2A) and can differentiate to neural cell types expressing the neural progenitor marker, nestin, and the neuronal marker, MAP2 (Fig. 2B, 2C) [14-17]. This demonstrates that BG01 and BG02 can form tissue that is a derivative of ectoderm.

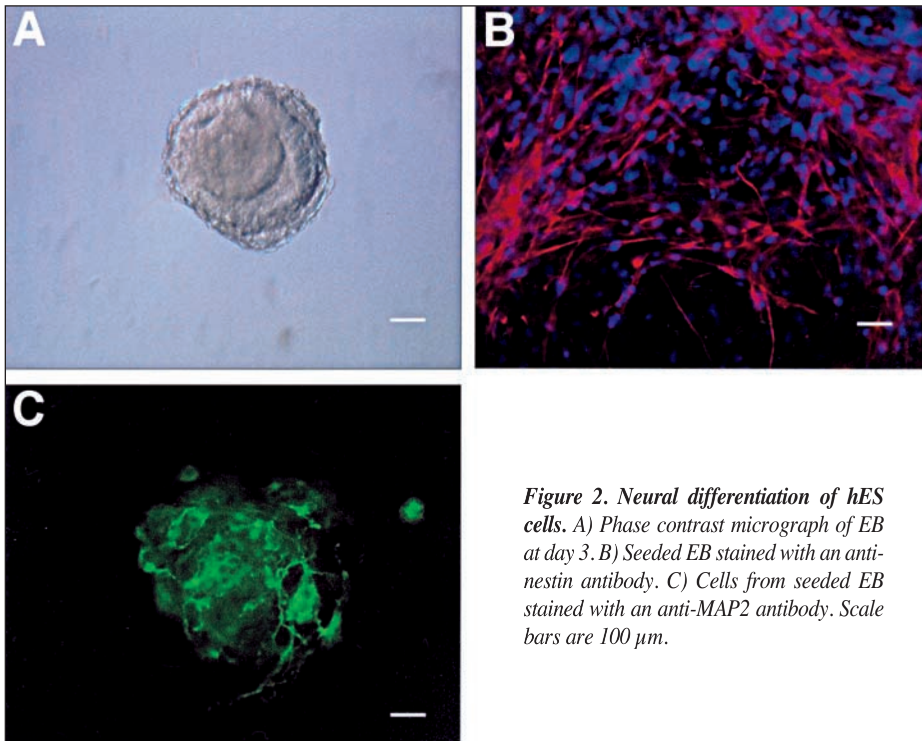

Figure 2. ***Neural differentiation of hES cells.*** *A) Phase contrast micrograph of EB at day 3. B) Seeded EB stained with an anti-nestin antibody. C) Cells from seeded EB stained with an anti-MAP2 antibody. Scale bars are 100 µm.*

BG01 cells can also differentiate to cardiac muscle cells, a mesodermal derivative. When EBs were transferred to adherent culture in hES medium, rhythmically contracting regions of cells typical of cardiomyocytes were observed (data not shown) [18].

Discussion

The embryos used in this study were poorly developed at day 5 of development and were not transferred or cryopreserved. By day 6 or 7, some had reached a stage of cavitation that might be expected at day 5, but none had the tight and even ICM seen in good-quality blastocysts. The pregnancy establishment rate is nearly double when high-quality blastocysts (3AA or better) are transferred at day 5, compared with the outcome when embryos poorer than 3AA are transferred. In this case, the patients had received higher quality embryos on day 3 or 5 of the same cycle, and other embryos had been frozen for future use. What remained was a group of poorly developed embryos that, from previous experience, were unlikely to survive freezing and thawing. No published data exist describing establishment of pregnancy with embryos that achieve 3CC only after 6 or 7 days, and though this is conceivable, the additional impact of a freeze/thaw cycle makes this even less likely, and so in clinical practice, such embryos are discarded.

We added bFGF and recombinant hLIF to the medium to enhance the proliferation of ICM cells from these blastocyst-stage embryos with limited cell numbers. Two cell lines were derived from day-6 embryos while the other two lines were derived from day-7 embryos. Limited embryo numbers prohibited a direct comparison of various culture conditions, including media that did not contain bFGF or hLIF. No cell lines were isolated from embryos without a full blastocoel, although, from one such embryo, cells attached and began to proliferate. The four cell lines were derived from 19 embryos in total, but from only 11 full blastocysts or better. This frequency is comparable with that reported by *Thomson* and coworkers [5] , and suggests that the culture conditions and methods used may have facilitated hES cell line isolation. The human embryo-derived cell lines described here meet criteria used to define human ES cells. These include expression of markers commonly used to identify hES cells, normal and stable karyotype, and demonstrated ability to differentiate in vitro into a variety of cell types. Specifically, BG01 has now been cultured past 85 passages and still maintains the same ES cell marker characteristics as it exhibited in the initial cell passages. This same cell line can differentiate into cardiac and neural-like cell types in vitro. The cell lines described here are karyotypically stable, express markers associated with human pluripotent stem cells, and can differentiate in vitro to a variety of cell types.

We have demonstrated that hES cell lines can be derived from embryos that may be discarded in the usual course of events due to low cell numbers and slow development. Most fertility clinics discard embryos that are unlikely to produce satisfactory pregnancy outcomes after assessment by a published scoring system. We report here that a portion of the embryos that are discarded because of a predicted unsatisfactory pregnancy outcome can alternatively produce viable embryonic stem cell lines.

Acknowledgments

We gratefully acknowledge the assistance of *Dr. Lorraine Meisner, Dr. Julie Johnson*, and the staff of the cytogenetic laboratory at Wisconsin State Laboratory of Hygiene in the analysis of the karyotypes of the cell lines; *Dr. Lisa S. Kelly* and *Ms. Marina Kozak* from the Department of Biochemistry and Molecular Biology at the University of Georgia, Athens, for immunoassay of hCG; and *Dr. Brian Condie* for critically reading the manuscript.

References

1 Cheng L, Hammond H, Ye Z et al. Human adult marrow cells support prolonged expansion of human embryonic stem cells in culture. Stem Cells 2003;21:131-142.

2 Richards M, Fong CY, Chan WK et al. Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. Nat Biotechnol 2002;20:933-936.

3 Amit M, Margulets V, Segev H et al. Human feeder layers for human embryonic stem cells. Biol Reprod 2003;68:2150-2156.

4 Xu C, Inokuma MS, Denham J et al. Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol 2001;19:971-974.

5 Thomson JA, Itskovitz-Eldor J, Shapiro SS et al. Embryonic stem cell lines derived from human blastocysts. Science 1998;282:1145-1147.

6 Reubinoff BE, Pera MF, Fong CY et al. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 2000;18:399-404.

7 Gardner DK, Lane M, Stevens J et al. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. Fertil Steril 2000;73:1155-1158.

8 Solter D, Knowles BB. Immunosurgery of mouse blastocyst. Proc Natl Acad Sci USA 1975;72:5099-5102.

9 Abbondanzo SJ, Gadi I, Stewart CL. Derivation of embryonic stem cell lines. Methods Enzymol 1993;225:803-823.

10 Resnick JL, Bixler LS, Cheng L et al. Long-term proliferation of mouse primordial germ cells in culture. Nature 1992;359:550-551.

11 Matsui Y, Zsebo K, Hogan BL. Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell 1992;70:841-847.

12 Shamblott MJ, Axelman J, Wang S et al. Derivation of pluripotent stem cells from cultured human primordial germ cells. Proc Natl Acad Sci USA 1998;95:13726-13731.

13 Pera MF, Reubinoff B, Trounson A. Human embryonic stem cells. J Cell Sci 2000;113:5-10.

14 Zhang SC, Wernig M, Duncan ID et al. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol 2001;19:1129-1133.

15 Carpenter MK, Inokuma MS, Denham J et al. Enrichment of neurons and neural precursors from human embryonic stem cells. Exp Neurol 2001;172:383-397.

16 Schuldiner M, Eiges R, Eden A et al. Induced neuronal differentiation of human embryonic stem cells. Brain Res 2001;913:201-205.

17 Reubinoff BE, Itsykson P, Turetsky T et al. Neural progenitors from human embryonic stem cells. Nat Biotechnol 2001;19:1134-1140.

18 Xu C, Police S, Rao N et al. Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. Circ Res 2002;91:501-508.