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A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells

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BACKGROUND: Human embryonic stem (hES) cell lines were first cultured using fetal mouse fibroblasts as feeder cells. To avoid feeders and to reduce the amount of xeno-components, Matrigel- and laminin-coated dishes, and conditioned mouse feeder cell medium have been used, and hES cells have also been cultured on human fetal muscle and skin, and adult Fallopian tube epithelial cells. METHODS: We used post-natal, commercially available human foreskin fibroblasts as feeder cells. Inner cell masses (ICM) were isolated from five supernumerary blastocysts, obtained as donations from couples undergoing IVF treatment. RESULTS: Two ICM showed continuous growth. One line, HS181, has been in culture for 41 weeks with a doubling time of 24–36 h. It continues to express stem cell markers alkaline phosphatase, Oct-4, stage-specific embryonic antigen (SSEA)-4 and tumour-related antigen (TRA)-1-60. The karyotype is 46,XX. Pluripotency was demonstrated by teratoma formation in immunodeficient mice. In high-density cultures, spontaneous differentiation to beating cells and neuron-like cells was seen. The second line, HS207, was cultured for 9 weeks and cryopreserved, as were samples of line HS181. Both lines began to grow after thawing. CONCLUSIONS: We used successfully human foreskin fibroblasts as feeder cells for derivation and continued undifferentiated growth of hES cells. These feeder cells are convenient for IVF units, because no fetal human tissues or tissue from operations are needed.

Key words: blastocyst/culture/feeder cells/fibroblasts/human embryonic stem cells

Introduction

In-vitro culture of cells from human blastocysts was first described in the early 1980s, and it was suggested that these cells might have potential in tissue repair (Fishel *et al*., 1984; Edwards, 2001; 2002). Culture of cells from the inner cell mass (ICM) of human blastocysts and subsequent growth of embryonic stem (ES)-like cells for two passages with partial characterization was first described by Bongso *et al*. (1994). The concept of using human embryonic stem (hES) cells in transplantation received greater attention after the first reports of permanent cell lines derived from human blastocysts showing pluripotent developmental capacity (Thomson *et al*., 1998; Reubinoff *et al*., 2000).

On the basis of experience with mouse ES cells (Evans and Kaufman, 1981; Martin, 1981) these first reported hES cell lines were cultured with mitotically inactivated fetal mouse fibroblast feeder cells, which also kept the human ES cells undifferentiated. In contrast to the situation with mouse ES cells (Smith, 1988), leukaemia inhibitory factor (LIF) alone did not keep human ES cells undifferentiated (Thomson *et al*., 1998). The use of mouse fibroblast feeder cells to a large extent inhibits the spontaneous differentiation of human ES cells *in vitro* and removal of the feeder cells leads to markedly enhanced differentiation (Reubinoff *et al*., 2000). This development can be partially guided by adding growth factors to the medium (Schuldiner *et al*., 2000).

The use of non-human materials bears a risk of transmitting pathogens, and they are not optimal in cultures aimed at cell transplantation in humans. Hence, cultures have been developed using extracellular matrix, Matrigel- or laminin-coated dishes and conditioned mouse feeder cell medium (Xu *et al*., 2001). Successful culture of hES cells with human fetal muscle and skin cells and adult Fallopian tube epithelial cells as feeders has recently been described (Richards *et al*., 2002).

With permission from the Ethics Committee of the Karolinska Institutet, we began to derive human embryonic

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stem cell lines from supernumerary blastocysts from our IVF unit. In the initial experiments, we used mouse embryo fibroblasts as feeder cells. Eleven cultures of ICM were kept proliferating for 10 days to 3 months before being cryopreserved. Six such ICM cultures were derived under conditions that fulfilled the criteria for stem cell registry at the National Institutes of Health (http://escr.nih.gov/).

In order to avoid using mouse feeder cells with human fetal cells, and to obtain a culture system which would be simple and feasible for IVF units, we developed the use of post-natal human fibroblasts, which have been used earlier as substrate cells for several cell types (Muggleton-Harris and Aroian, 1982; Muggleton-Harris and Findlay, 1991), in our derivation and culture of human ES cells.

Materials and methods

For this project, we received five donated blastocysts from consenting couples. Only one of the blastocysts had expanded by day 6, and all had only a few cells in the ICM. The donating couples had good quality embryos for transfer and cryopreservation, and the donated blastocysts would have been discarded if not used in research. The embryos had been cultured in IVF medium (Vitrolife, Sweden) individually in 20 µl droplets under oil for the first 2 days, and then transferred to blastocyst medium (CCM, Vitrolife). They were transferred to the research laboratory on day 6 after fertilization. For evaluating the quality of the blastocysts, the scoring system described by Gardner *et al*. (1998) was used.

Isolation of the ICM was carried out by first removing the zona pellucida using 0.5% Pronase (Sigma, USA). The trophectoderm was removed by immunosurgery as described earlier (Solter and Knowles, 1975), using rabbit antihuman whole serum (Sigma) and guinea-pig complement serum (Sigma).

As substrate cells, we decided to use commercially available human foreskin fibroblasts (CRL-2429; ATCC Mananas, USA). The fibroblasts, with an anticipated life span of 61 doublings, were used in our experiments after 9–25 doublings. The cells were grown to form a confluent monolayer, and irradiated (35 Gy) before being used as substrate cells. The medium used in culture of these feeder cells was Iscovs medium (GibcoBRL, Life Technologies, Sweden) supplemented (10%) with fetal calf serum (FCS; SDS, Sweden).

The ICM were transferred to culture dishes containing the feeder cells. The dishes used were plastic dishes tested for use in embryo culture (Falcon; Becton Dickinson, USA). The culture medium used for derivation and culture of the hES cells was Knockout D-MEM 1× (GibcoBRL, Life Technologies), supplemented with 2 nmol/l L-glutamine, 20% FCS (R&D, Sweden), 0.1 mmol/l β-mercaptoethanol (Gibco), 1% non-essential amino acids (Gibco), and recombinant human LIF, 1 µl/ml (Chemicon, UK). The FCS batch used in these experiments had been selected from a large number of tested batches based on the criteria of strong proliferation and induction of a minimum amount of differentiation, in two different mouse ES cell lines (D3 and GS-1). The same selection criteria are routinely used for selection of FCS for mouse ES cell cultures in our core facility for the generation of knock-out mice.

After an initial growth period of 9–19 days, the cell aggregates were removed from the dish by using a mild dispase solution (10 mg/ml; Gibco BRL, Life Technologies) and mechanical slicing using glass capillaries. The cell aggregates were transferred to new plates containing similar feeder cells. After the first splitting, the new growing aggregates were again split and transferred to new dishes every 5 days. Undifferentiated cells, as judged by morphology, were chosen for each further passage.

For freezing of the cells, vitrification in pulled open straws, using ethylene glycol, dimethylsulphoxide (20% each) and 1 mol/l sucrose as cryoprotectants, was carried out as described by Reubinoff *et al*. (2001).

The growing cells were characterized immunohistochemically by using antibodies against markers characteristic of non-differentiated ES cells. The antibody for Oct-4 was a kind gift from Dr G.A.Schulz (University of Calgary, Canada), that for TRA-1-60 was a kind gift from Dr P.Andrews (University of Sheffield, UK), and the antibody for SSEA-4 was from the Developmental Studies Hybridoma Bank, University of Iowa, USA. The expression of alkaline phosphatase was shown by using a Vector Blue/Red substrate kit (Vector Laboratories, USA). The cells were fixed with 4% paraformaldehyde. Fluorescent secondary antibodies, Cy3 (Jackson Immuno Research, USA) or AlexaFluor 488 (Molecular Probes Inc., USA) were used. P19 and TERA-2 cell lines and human foreskin fibroblasts were used as controls. Non-immune serum was also used as a negative control for the human ES cell line in question. The karyotype was determined by standard G-banding.To test pluripotency, ~10^3 cells at passage 20 were injected to the right testis of SCID-beige mice (C.B.-17/GbmsTac-scid-bgDF N7 from M&B, Denmark). The teratoma formation was followed up by palpation and the resulting tumours were fixed, embedded in paraffin and processed for histology.

Results

One of the donated blastocysts, scoring 2BA (not expanded, small number of cells in the ICM but a good number of cells in the trophectoderm) did not grow at all. Two ICM, from blastocysts scoring 2BB (not expanded, small numbers of cells in the ICM and trophectoderm) and 3BB (large blastocoele, but not fully expanded, small numbers of cells in the ICM and trophectoderm) survived on the feeders for 1 and 4 weeks respectively. From two blastocysts we obtained cell lines. The line HS181 was derived from a blastocyst scoring 4BB (expanded, small cell numbers in the ICM and trophectoderm). The line HS207 was obtained from a blastocyst with a score of 2CB (not expanded, hardly any visible ICM, few cells in the trophectoderm). The outgrowth of cells from these two

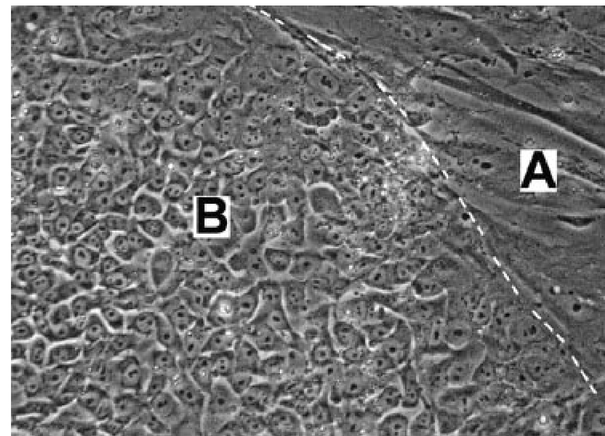

Figure 1. Embryonic stem cells, line HS 181 (**B**), growing on post-natal human fibroblast feeder celler (**A**). Original magnification ×40.

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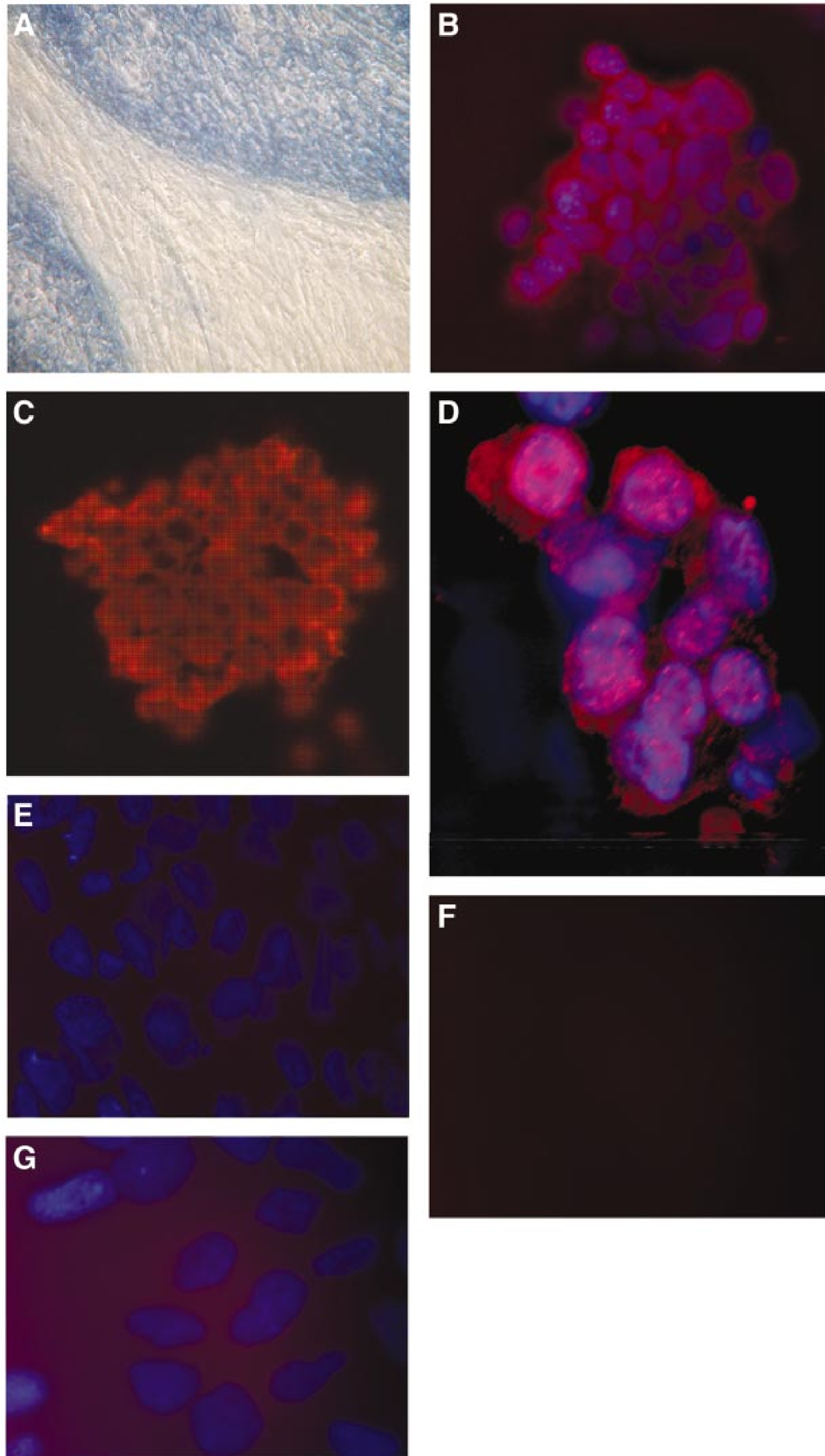

Figure 2. Characterization of HS 181 cells, passage level 30. Alkaline phosphatase activity (**A**); Immunostaining for tumour-related antigen (TRA)-1-60 (**B**); stage-specific embryonic antigen (SSEA)-4 (**C**) and Oct-4 (**D**); negative controls for the corresponding isotype staining for the human embryonic stem cell line in question (HS 181), isotype control for TRA-1-60 mouse IgM (**E**); SSEA-4 mouse IgG (**F**); Oct-4 rabbit IgG (**G**). Original magnification ×40, the diameter of each cell being ~5–10 µm.

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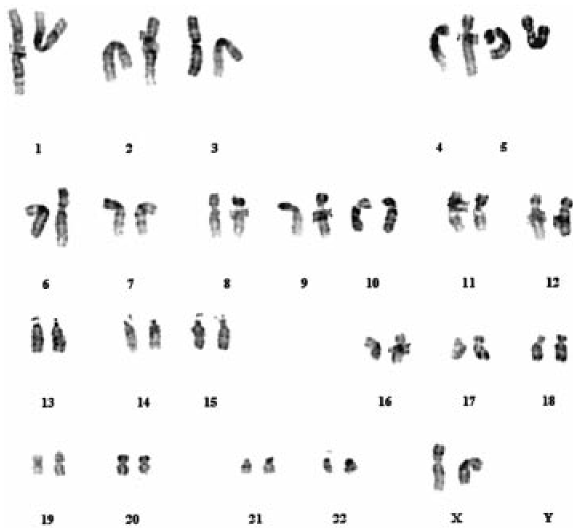

Figure 3. The karyotype 46,XX of the line 181.

blastocysts was seen within 1 week after initial plating on the substrate cells.

The first line, HS181, has been in continuous culture for 41 weeks, with a doubling time of ~24–36 h (Figure 1). This line has been enzymatically and mechanically split and transferred to new dishes by the time the aggregate has been formed by ~$2.5–6.4 \times 10^3$ cells. The size of the transferred aggregates has been ~$1–2 \times 10^3$ cells. Spontaneous differentiation was observed in 10–20% of the cells, in one out of 20 colonies during the 5 day culture period. High-density cultures regularly showed spontaneous development into multinuclear aggregates containing both undifferentiated and differentiated cells, including neural tube-like cells and structures, and beating cells.

After 9 months, the cells (80–100% of the cells in each aggregate) continue to express alkaline phosphatase, Oct-4, SSEA-4 and TRA-1–60 (Figure 2A–G. The karyotype is 46,XX, with no structural aberrations (Figure 3).

Teratoma formation showing pluripotency of the 181 cells was obtained after injection of passage 20 of these cells to the testes of SCID mice. The teratomas were analysed 18 weeks after the injection. They contained macroscopically well circumscribed tumour-like structures, containing cystic cavities and solid areas. Some of the cysts were filled with a gelatinous substance. In Figure 4A, tissue with endodermal differentiation is shown. There is a cavity lined by cylindrical cells with basally located nuclei, and numerous lightly stained goblet cells. Below these structures, there are tissue components showing mesodermal differentiation; connective tissue stroma and cells with spindle form with elongated nuclei, which are typical of smooth muscle layer of the intestine. Differentiation to ectoderm is demonstrated in Figure 4B. Multi-layered nuclei are arranged in rows compatible with formation of neural epithelium or neural rosettes. Differentiation into cartilage is demonstrated in Figure 4C.

The second line (HS207) was not characterized, but it was morphologically similar to the line HS181. Hence, we do not have evidence from the possible pluripotency of this line. It was cultured for 9 weeks and then cryopreserved, as were samples of line HS181. Both of the cell lines have been thawed, and have undergone divisions, showing that these cells survive freezing and thawing.

Discussion

Our results with the human ES cell lines show that post-natal human feeder cells, in this case foreskin fibroblasts, support the derivation of new hES cell lines, and continued undifferentiated growth of these cells. The hES cells were cultured on commercially available human foreskin fibroblasts, which makes the procedure simple and accessible to IVF laboratories starting to derive human ES cells for cell banks. In their recent article, Richards *et al*. (2002) reported successful culture of human ES cells on human fetal muscle and fetal skin cells and human adult tubal epithelial cells. Our approach, involving human skin fibroblast cells, is an even simpler solution, because no fetal material after termination of pregnancy or tissue from gynaecological operations is needed.

For IVF units working without animal facilities, the use of human cells is more convenient than the use of mouse cells as feeders. In addition, the quality control systems of most of the IVF units do not allow culture of mouse cells in the same facility.

Finding optimal techniques to culture human ES cells without a feeder layer, and culturing them without FCS, is important for cell transplantation. Xu *et al*. (2001) have already been successful with non-feeder cultures, but the conditioned medium from mouse cell cultures is not optimal as regards human transplantation. The risk of transmitting pathogens still exists when conditioned medium from mouse fibroblast cultures is used. In the present study we used FCS in the cultures, and the next step is to replace it with human serum, and find serum-free conditions with synthetic serum supplements. The use of human serum has already been successful when using other feeder cells (Richards *et al*., 2002). Optimally, also the feeder cells should also be cultured in serum-free medium, which has been tested (Amit *et al*., 2000). The serum-free system is hence already well established for the culture of human ES cells. On the other hand, FCS has been accepted as an ingredient of culture media for transplantable adult stem cells. To obtain transplantable cells, good manufacturing practice (GMP) conditions are needed, and at our hospital such a facility is being built.

The hES cell characteristics of the line 181 cells were supported by the expression of the surface markers, Oct-4, SSEA-4 and Tra-1-60. In addition, after injection of the 181 cells into immunodeficient mice, teratomas containing tissue components consistent with all the three embryonic layers were found in the recipient mice, as a sign of pluripotency.

Also when cultured at high density, spontaneous differentiation occurred.

We added rLIF to the cultures in spite of earlier reports that it does not support the growth of human ES cells without feeders (Thomson *et al*., 1998).

We could not obtain any really good quality blastocysts for our stem cell cultures, because all such embryos are either

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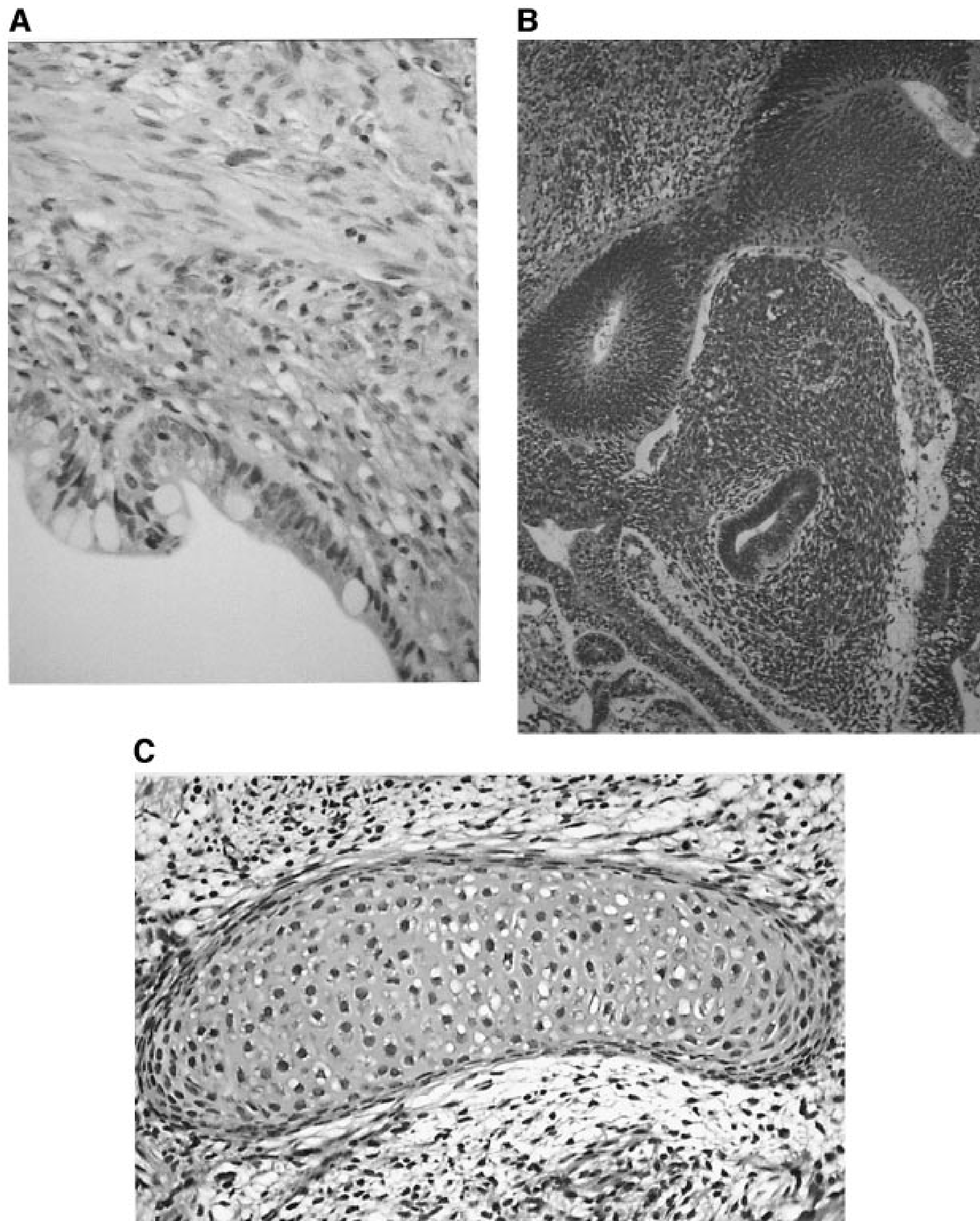

Figure 4. (**A**) Teratoma tissue demonstrating differentiation to intestinal tissue consistent with endodermal differentiation. Cylindrical epithelial cells and goblet cells (g) are shown. Mesodermal differentiation is demonstrated by connective tissue stroma (s) and smooth muscle cell layer of the intestine (m). Orginal magnification ×100. (**B**) Teratoma tissue containing components characteristic of neural epithelium and neural rosettes. Original magnification ×100. (**C**) Teratoma tissue with differentiation to cartilage consistent with mesodermal differentiation. Original magnification ×100.

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transferred to the subject or frozen for the couple's infertility treatment in the future. None of our blastocysts had a good cell number in the ICM. Nevertheless, we obtained cell lines from ICM with low numbers of cells. The line HS207 was actually derived from a blastocyst with very few cells in the ICM, although it did not grow as well as the line HS181, and its pluripotency has not been verified. Nevertheless, this is encouraging when thinking of an hES cell bank with a large enough number of different genotypes for cell transplantation in the future. Creating good quality embryos for research might be an excellent option, but it bears ethical problems (McLaren, 2001), which may be avoided by using supernumerary low-quality embryos.

We intend to use this method to establish new ES cell lines for the new cell bank supported by the Swedish Medical Research Council and Juvenile Diabetes Research Foundation, to provide cells for academic collaborators.

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