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Establishment of human embryonic stem cell lines from frozen–thawed blastocysts using STO cell feeder layers

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BACKGROUND: Recently, human embryonic stem (hES) cells have become very important resources for basic research on cell replacement therapy and other medical applications. The purpose of this study was to test whether pluripotent hES cell lines could be successfully derived from frozen–thawed embryos that were destined to be discarded after 5 years in a routine human IVF-embryo transfer programme and whether an STO cell feeder layer can be used for the culture of hES cells. METHODS: Donated frozen embryos (blastocysts or pronuclear) were thawed, and recovered or *in vitro* developed blastocysts were immunosurgically treated. All inner cell masses were cultured continuously on an STO cell feeder layer and then presumed hES cell colonies were characterized. RESULTS: Seven and two cell lines were established from frozen–thawed blastocysts (7/20, 35.0%) and pronuclear stage embryos (2/20, 10.0%), respectively. The doubling time of hES cells on the immortal STO cell feeder layer was ~36 h, similar to that of cells grown using fresh mouse embryonic fibroblast (MEF) feeder conditions. Subcultured hES cell colonies showed strong positive immunostaining for alkaline phosphatase, stage-specific embryonic antigen-4 (SSEA-4) and tumour rejection antigen 1-60 (TRA1-60) cell surface markers. Also, the hES colonies retained normal karyotypes and Oct-4 expression in prolonged subculture. When *in vitro* differentiation of hES cells was induced by retinoic acid, three embryonic germ layer cells were identified by RT–PCR or indirect immunocytochemistry. CONCLUSIONS: This study indicates that establishment of hES cells from frozen–thawed blastocysts minimizes the ethical problem associated with the use of human embryos in research and that the STO cell feeder layer can be used for the culture of hES cells.

Key words: embryonic stem cells/frozen–thawed human blastocysts/inner cell mass/*in vitro* differentiation /STO cell

Introduction

Embryonic stem (ES) cells are derived from the inner cell mass (ICM) cells of early mammalian blastocyst. These cells are pluripotent and generally retain their long-term proliferative potential in an undifferentiated state. Also, ES cells can differentiate into derivatives of all three embryonic germ layers upon transfer to *in vitro* differentiation culture conditions or an *in vivo* environment (Thomson *et al*., 1998; Reubinoff *et al*., 2000). ES cells have become a powerful tool for *in vitro* investigation of developmental processes at both cellular and organism levels, and offer tremendous potential for clinical application as an unlimited source of cells for transplantation and tissue generation therapies. Establishment and differentiation of human ES (hES) cells under *in vitro* or *in vivo* conditions have been studied extensively (Thomson *et al*., 1998; Reubinoff *et al*., 2000, 2001; Assady *et al*., 2001; Kaufman *et al*., 2001; Kehat *et al*., 2001; Levenberg *et al*., 2002).

The primary difficulty in establishing hES cells is obtaining an embryo source without inciting ethical concerns. Infertility clinics generally maintain surplus embryos, left over after embryo transfer for pregnancy induction, and frozen for a certain period of time for future implantation. The use of frozen blastocysts that are no longer needed and would otherwise be discarded may not pose an ethical problem. Furthermore, establishment of ES cells from frozen blastocysts would be much easier than using cells in the pronuclear (PN) stage or 2- to 3-day-old frozen donated embryos.

Alternatives to mouse cells have been evaluated, but mouse embryonic fibroblasts (MEFs) are most commonly used as the feeder layer for culture of hES cells to help maintain the pluripotence of stem cells (Thomson *et al*., 1998; Reubinoff *et al*., 2000). Shamblott et al. (1998) have reported that ready-made STO fibroblast cells can also be used as feeder cells for culture of embryonic germ cells. STO cells are immortal MEFs

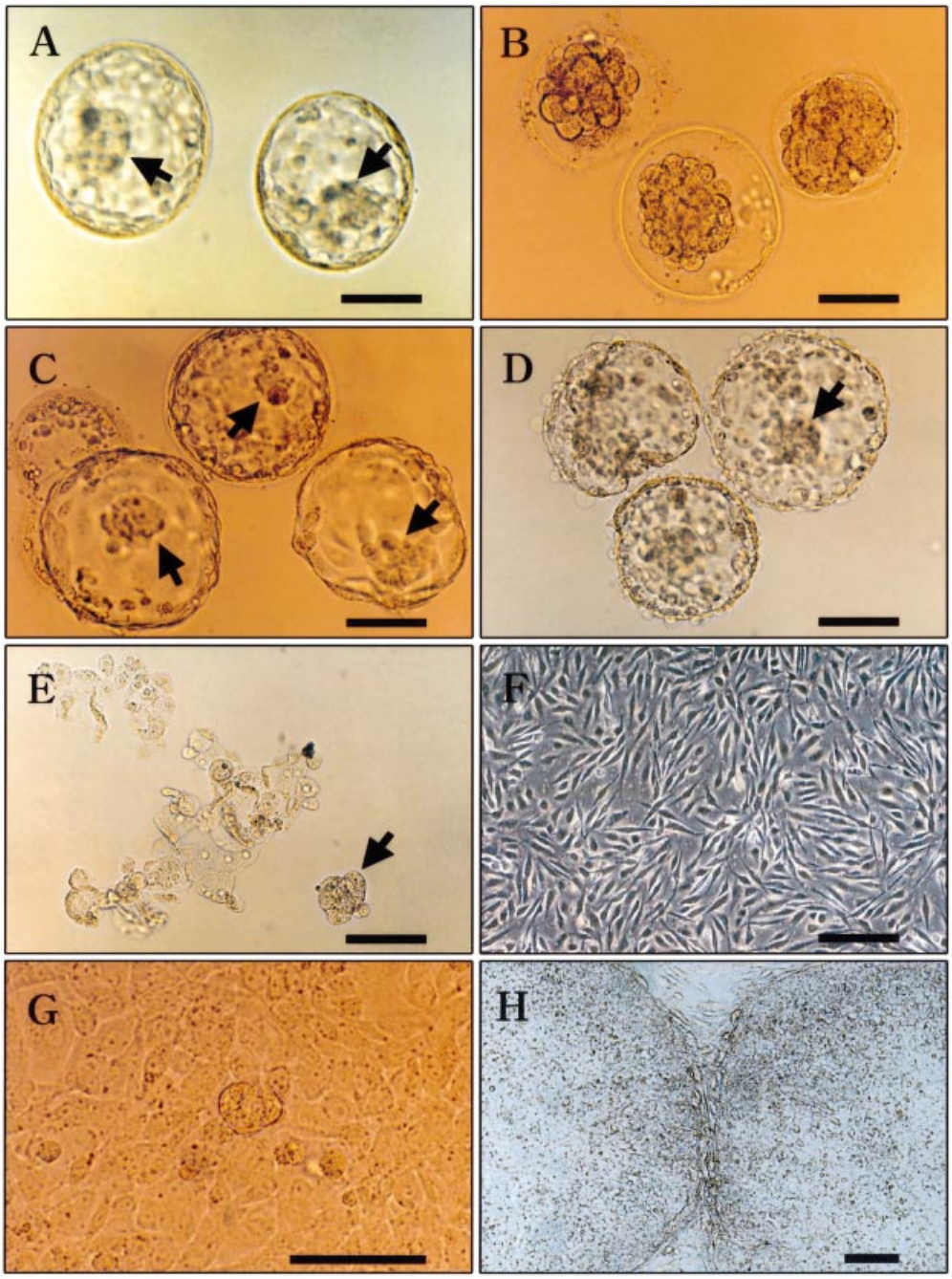

Figure 1. A serial establishment of an ES cell line (MB01) derived from frozen–thawed human blastocysts. (**A**) *In vitro* produced day 5 human blastocysts prior to freezing. Healthy ICMs are shown. (**B**) Immediately after thawing, recovered blastocysts were shrunken. (**C**) ICMs are clearly shown as a re-expanded blastocyst 25 h after thawing. (**D**) Trophectoderm cells were digested away by immunosurgery. (**E**) An ICM was isolated from the frozen–thawed blastocyst by additional mechanical pipetting after immunosurgery. (**F**) STO cells used as a feeder cell layer. (**G**) Plating of ICM cells onto mitotically incactivated STO feeder cells. (**H**) Expanded hES cell colonies at 25 culture passages. Boundary lines were touched between colonies. All arrows indicate ICM cells. Scale bar = 100 μm.

that produce leukaemia inhibitory factor (LIF) and are more easily maintained than MEFs for the preparation of feeder layers.

In this study, we examined whether hES cell lines can be successfully derived from frozen–thawed embryos, that would have been discarded from a routine human IVF-embryo transfer programme, using an STO cell feeder layer. In addition, we differentiated *in vitro* an hES cell line into specific cells representing three embryonic germ layers.

Materials and methods

Derivation and culture of hES cells

hES cells were derived from frozen–thawed zygotes or blastocysts that were destined to be discarded after 5 years in a routine human IVF-embryo transfer programme with the patient's consent. For development into the blastocyst stage (Figure 1A), embryos were cultured in modified CR1aa medium (Rosenkrans *et al*., 1993) containing 20% human follicular fluid (hFF). Immunosurgery on human blastocysts to derive hES cell lines was carried out as follows; zona pellucida was

Table I. PCR primers used to detect gene expression in hES cells

Gene	Sence	Antisense	Product size (bp)	Relationship	Category
NF-H	TGAACACAGACGCTATGCGCTCAG	CACCTTTATGTGAGTGGACACAGAG	400	Brain; neural	Ectoderm
Keratin	AGGAAATCATCTCAGGAGGAAGGGC	AAAGCACAGATCTTCGGGAGCTACC	780	Skin	Ectoderm
Enolase	TGATTCAAGTCGCCTGATGATCCCC	TGCGTCCAGCAAAGATTGCCTTGTC	490	Muscle	Mesoderm
cAcT	TCTATGAGGGCTACGCTTTG	CCTGACTGGAAGGTAGATGG	630	Heart	Mesoderm
Amylase	GCTGGGCTCAGTATTCCCCAAATAC	GACGACAATCTCTGACCTGAGTAGC	490	Pancreas	Endoderm
β-Actin	CGCACCACTGGCATTGTCAT	TTCTCCTTGATGTCACGCAC	200		Housekeeping

removed with 0.25% pronase (Sigma, Saint Louis, MO), the ICM cells were isolated by treatment with rabbit anti-human cell antibody (1:20, 30 min) and then exposed to guinea pig complement (Sigma, 1:10, 2 min) (Figure 1D and E). Subsequently, the recovered ICM was plated on a 10 μg/ml mitomycin C (Sigma) mitotically inactivated STO cell (ATCC CRL-1503, 250 000 cells/1.77 cm^2, no. 3653, Becton Dickinson, NJ) feeder layer (Figure 1F and G). Basic culture medium for hES cell maintenance consisted of knockout-Dulbecco's modified Eagle's medium (KO-DMEM; Gibco, Grand Island, NY) supplemented with 20% defined fetal bovine serum (FBS; Hyclone, Logan, UT), 1 mM glutamine, 0.1 mM β-mercaptoethanol, 1% ribonucleosides and 1% non-essential amino acids (NEAAs). For the initial culture of ICM cells, 2000 U of LIF (Chemicon, Temecula, CA) and 4 ng/ml of human basic fibroblast growth factor (bFGF; KOMA Biotech, Inc.) were added.

Five to 8 days after primary plating, the ICM cell clump was removed mechanically with a micropipette and replated on a fresh STO cell feeder layer. Expanded colonies (Figure 1H) were dissociated by treatment with 0.04% collagenase (Sigma) and/or mechanical slicing using a 28 gauge needle. The resulting colonies were propagated further in clumps of 100–200 hES cells on a STO cell feeder layer about every 7 days. During the 10 passages that hES cells underwent, 20% of defined FBS was exchanged with 20% of serum replacement (Hyclone) in basic hES culture medium with 4 ng/ml bFGF but without LIF.

Assessment of hES cell growth on the STO cell feeder layer

The growth of hES cells on the STO cell feeder layer was examined from day 1 to day 5 after plating the hES cell clumps. Cell doubling of several selected colonies (n = 10) was evaluated daily by counting along a major axis under the same magnification (×40 or ×60) with an inverted microscope (Olympus).

Stem cell marker staining

To assess alkaline phosphatase (AP) activity, hES cell colonies (>15 passages) were fixed in 4% formaldehyde for 15 min and then stained with Fast red TR/naphthol AS-MX for 15 min (Sigma). To detect the human stem cell markers stage-specific embryonic antigen (SSEA)-3 or SSEA-4, ES colonies were fixed with 90% acetone in H_2O at room temperature for 15 min. To detect SSEA-1, tumour rejection antigen (TRA) 1-60 or TRA1-81, cells were fixed with 100% ethanol 4°C for 15 min. The monoclonal antibodies against stem cell markers (SSEA-1, MC-480; SSEA-3, MC-631; and SSEA-4, MC-813-70) were supplied by Developmental Studies Hybridoma Bank (Iowa City, IA). Also, monoclonal antibodies against TRA1-60 and TRA1-81 were donated by Peter Andrews, University of Sheffield. Antibody localization was detected using a rabbit anti-mouse antibody conjugated to fluorescein isothiocyanate (FITC; Jackson Immunoresearch Lab, Inc., Baltimore, PA).

Oct-4 expression measured by indirect immunostaining

Oct-4 expression was assessed in undifferentiated hES cells with an H-134 antibody (Santa Cruz, CA). ES colonies were fixed in a 4% paraformaldehyde solution at 4°C for 10 min. Antibody localization was determined with a goat anti-rabbit antibody labelled with FITC

Chromosome analysis

For chromosome analysis, hES cells were cultured in feeder-free Matrigel (Becton Dickinson, 1:20)-coated plates for 4–6 days. After treatment with 5% colcemid (Gibco), harvested ES cells were stained using a standard G-banding technique. Karyotyping was analysed using a Cytovision program (Applied Imaging Co.).

In vitro differentiation

Embryoid bodies (EBs) were prepared in a bacteriological dish (no. 1007, Becton Dickinson) for 5 days following 0.04% collagenase treatment and mechanical dissection of hES cell colonies. To initiate differentiation, EBs were treated with 1 μM retinoic acid (RA; Sigma) for 1 week and plated onto a 0.1% gelatin-coated dish in differentiation medium (KO-DMEM containing 1 mM glutamine, 0.1 mM β-mercaptoethanol, 1% NEAA and 10% FBS) for 3 weeks.

RT–PCR analysis

Expression of specific genes in undifferentiated hES cells was analysed by RT–PCR analysis with primers for three embryonic germ layer cells in day 5 EBs and in RA-treated plus day 14 differentiated ES cells. Total RNA from samples was extracted using a TRI reagent kit (Sigma) according to the manufacturer's instructions. cDNA was synthesized from ~1 μg of total RNA using SuperScript II reverse transcriptase (Invitrogen, Grand Island, NY). cDNA samples were subjected to PCR amplification with primers selective for human neurofilament heavy chain (NF-H, 400 bp), keratin (780 bp), enolase (490 bp), cAct (630 bp) and amylase (490 bp) genes. Also, as a control for mRNA quality, β-actin (200 bp) was assayed using the same RT–PCR method. The PCR primers are described in Table I. The PCR products were size fractionated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. Final analysis was obtained in an image analyser (Biorad).

Indirect immunocytochemistry

In vitro differentiation was assessed by indirect immunocytochemistry. Muscle-specific actin staining was assessed by fixing cells in methanol/acetone (1:1) at 4°C for 15 min, and neuronal cell staining was assessed by fixing cells in 4% paraformaldehyde at 4°C for 15 min. Monoclonal antibodies against muscle actin (1:100, Sigma), β-tubulin III (1:200, Sigma), oligodendrocytes (O4; 1:50, Chemicon), and tyrosine hydroxylase (TH; 1:1000, Chemicon), and polyclonal antibodies against glial fibrillary acid protein (GFAP; 1:500, DAKO, Denmark) and glutamic acid decarboxylase (GAD; 1:4000, Chemicon) were examined. Primary antibody reactions were per-

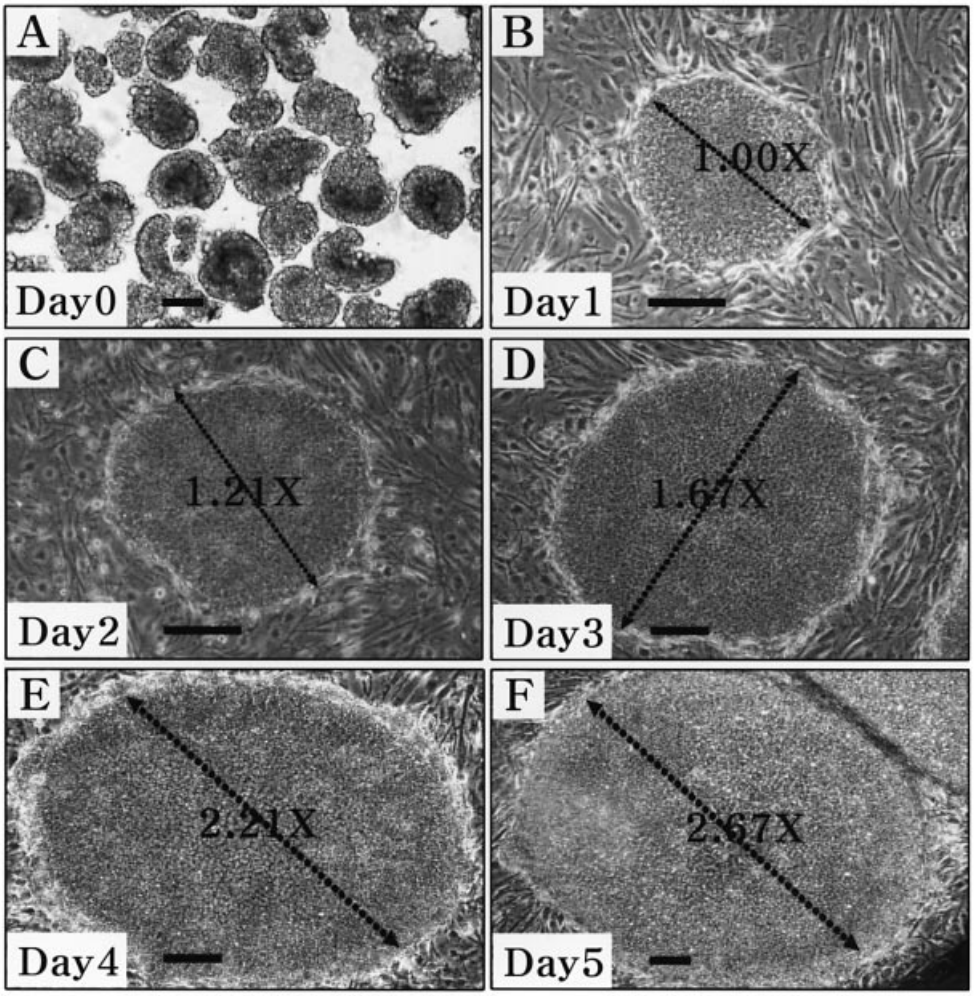

Figure 2. Phase contrast imaging of cultured hES cells (MB03) on a mitotically inactivated STO cell feeder layer. (**A**) Mechanically dissociated hES cell clumps before subculture on an STO cell feeder layer. (**B**) hES cell clump stably positioned on the STO feeder layer on day 1 after plating. (**C–F**) A gradually growing hES cell colony. Growing speed is indicated as doubling over the dotted line from day 1 to day 5 (B–F) after plating of hES cell clumps. In STO cell feeder conditions, hES cell doubling time was calculated as ~36 h. Scale bars = 100 μm.

Table II. Human ES cell lines derived from frozen–thawed embryos

Embryo stage[a]	No. of frozen–thawed embryos	No.(%) of surviving embryos	No.(%) of blastocysts	No. (%) of immunosurgery-treated blastocysts[b]	No. (%) of colonies formed from ICM cells[c]	No. (%) of ES cell lines
PN	20	18 (90.0)	9 (45.0)	6 (30.0)	3 (15.0)	2 (10.0)
Blastocyst	20	15 (75.0)	15	14 (70.0)	11 (55.0)	7 (35.0)

[a]Thawed embryos were donated with patient's consent.
[b]Very poor quality embryos were excluded at immunosurgery.
[c]Colonies formed from ICM cells 5–10 days after initial plating.

formed at 4°C overnight. Antibody localization was determined by staining with a rabbit anti-mouse antibody conjugated to FITC or a goat anti-rabbit antibody labelled with tetramethylrhodamine isothiocyanate (TRITC; 1:200, Jackson Immunoresearch Laboratories Inc.) at room temperature for 1 h.

Results

We established nine hES cell lines [Maria Biotech (MB) 01–MB09] from frozen–thawed embryos. Detailed information about the derivation of hES cells from frozen–thawed embryos is indicated in Table II. Using immunosurgery, two hES cell lines were established from 20 frozen–thawed PN stage embryos (10.0%) while seven hES cell lines were derived from 20 frozen–thawed blastocysts (35.0%). Thus, blastocysts are an efficient source for establishment of hES cell lines. ICM cells (Figure 1A, C–E and G) must be healthy to establish a good quality hES cell line. As shown in Figure 1, pluripotent hES cell lines can be generated by using STO cells as a feeder layer (Figure 1H).

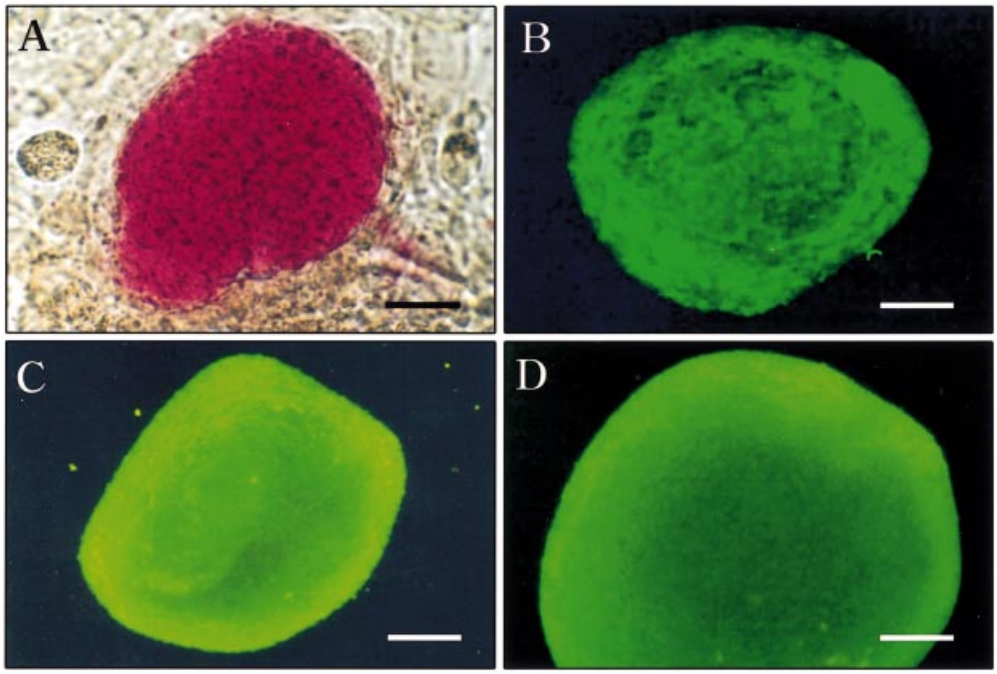

Figure 3. Positive marker expression of hES cells (MB02). hES cell colony stained histochemically for alkaline phosphatase (**A**). hES cell colony immunostained with anti-SSEA-4 (**B**), anti-TRA1-60 (**C**) and anti-Oct-4 (**D**), respectively. Scale bars = 100 μm.

Cultures were passaged by dissecting large hES colonies into a number of small sized clumps with collagenase treatment and mechanical slicing (Figure 2A). As shown in Figure 2, a small hES colony increasingly expanded its territory. The growth of hES cells on the STO cell feeder layer, determined by counting cells along the major axis under the same magnification, was 2.67 doublings over 4 days. Thus, doubling time of hES cells on the STO cell feeder layer was ~36 h (96 h/2.67 = 35.955 h), which is similar to the doubling time observed with the more commonly used fresh MEFs for the culture of hES cells.

hES cells were characterized by indirect immunocytochemistry. All ES cell lines stained positively for AP (Figure 3A), SSEA-3, TRA1-81 (data not shown), SSEA-4 and TRA1-60 (Figure 3B and C) but not for SSEA-1 (data not shown), which was examined at 15–20 passages or >30 passages. In addition, Oct-4, another gene characteristic of undifferentiated hES cells, was expressed (Figure 3D). However, after >15 passages of continuous culture, karyotype analysis revealed that five hES cell lines are normal XY lines (MB01, MB04, MB06, MB07 and MB09) and four hES cell lines have normal XX karyotype (MB02, MB03, MB05 and MB08) (Figure 4).

The expression of several cell-specific genes of the three embryonic germ layer cells was assessed in undifferentiated hES cells, day 5 EBs and RA-treated plus 2-week differentiated ES (RA) cells under differentiation conditions using RT–PCR. As shown in Figure 5, RA-treated, differentiated ES cells expressed cAct as well as NF-H, keratin, enolase and amylase. In particular, keratin, enolase and amylase genes were commonly expressed at high levels in both EBs and RA-treated ES cells. On the other hand, keratin and enolase were also expressed in undifferentiated hES cells. The housekeeping gene β-actin served as an internal control.

We examined *in vitro* differentiation patterns in RA-treated and 3-week differentiated hES cells using indirect immunocytochemistry. Figure 6 shows phase contrast imaging of spontaneous contracting cardiomyocytes (Figure 6A) and typical neurons (Figure 6B) derived from hES cells. Muscle actin (Figure 6C) and several neuronal cell markers (Figure 6D–H) were positively immunostained in *in vitro* differentiated hES cells. In addition, the functional neuronal markers GAD (Figure 6G) and TH (Figure 6H) were expressed in differentiated hES cells.

Discussion

In this study, we established nine hES cell lines from frozen–thawed zygotes and blastocysts that were destined to be discarded with patient consent after 5 years in a routine IVF-embryo transfer programme. Three hES cell lines (MB01–MB03) have been registered with the US National Institutes of Health (NIH).

In the human IVF-embryo transfer programme, surplus embryos left over after embryo transfer to induce pregnancy are maintained frozen for certain periods of time for future implantation. The use of frozen embryos, that are no longer needed and would otherwise be discarded, with patient's consent may minimize ethical problems related to the use of human embryos for research. A recently developed culture medium (G1/G2 or P1) has been useful in the development of blastocysts from human zygotes (Gardner *et al*., 1998; Plachot *et al*., 2000). In this study, we used modified CR1aa medium containing 20% hFF to grow human embryos to the blastocyst stage. Our results indicate that frozen–thawed blastocysts are more advantageous than frozen–thawed PN stage embryos for the establishment of ES cells.

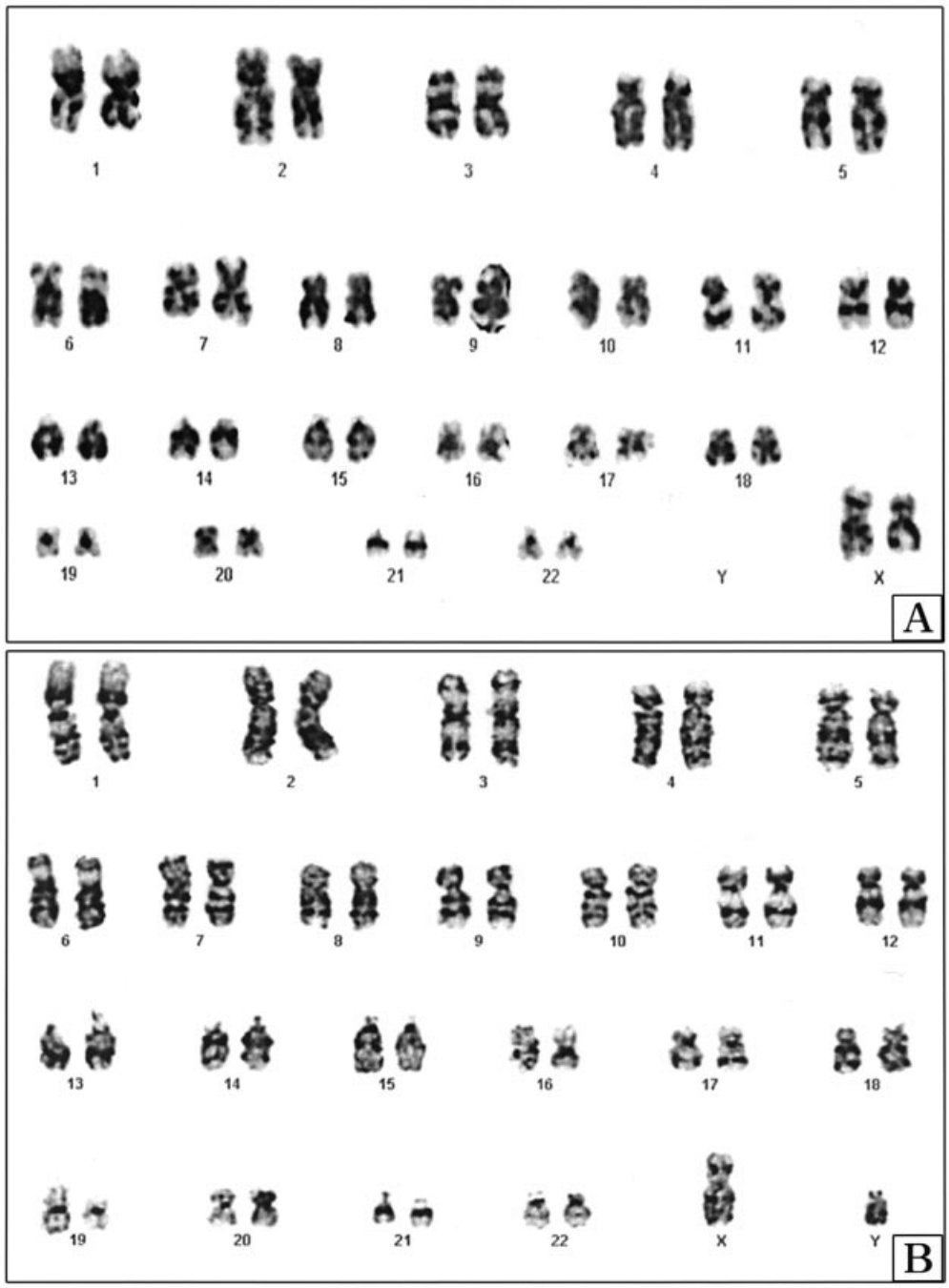

Figure 4. Normal karyotypes (**A**) 46, XX (MB02); (**B**) 46, XY(MB07) of hES cell lines.

To date, all human ES cell lines have been developed using a layer of so-called feeder cells from mice or human cells that supply components necessary to sustain human stem cells. In this study, we used STO cells as a feeder layer instead of MEF feeders (Thomson *et al*., 1998; Reubinoff *et al*., 2000) or human skin fibroblasts (Hovatta *et al*., 2003) for the culture of hES cells to help maintain them as pluripotent stem cells. STO cells are ready-made immortal MEFs that are maintained as easily as other cell lines. Xu et al. (2001) reported that the frequency of spontaneous differentiation of hES cells is higher when grown under feeder-free conditions using conditioned medium (CM) from STO cells than when grown under feeder-free conditions using CM from MEFs. To circumvent this problem, we prepared an STO cell feeder layer from two to five passages after thawing. Our results indicate that the doubling time of hES cells on the STO cell feeder layer was ~36 h, similar to the doubling time of cells grown on MEF feeder cells. Therefore, well maintained STO cell feeders and serum replacement added KO-DMEM medium sufficiently maintain pluripotency and minimize spontaneous differentiation in hES cells cultured for a long duration (>350 doublings). As in other reports (Thomson *et al*., 1998; Reubinoff *et al*., 2000), our

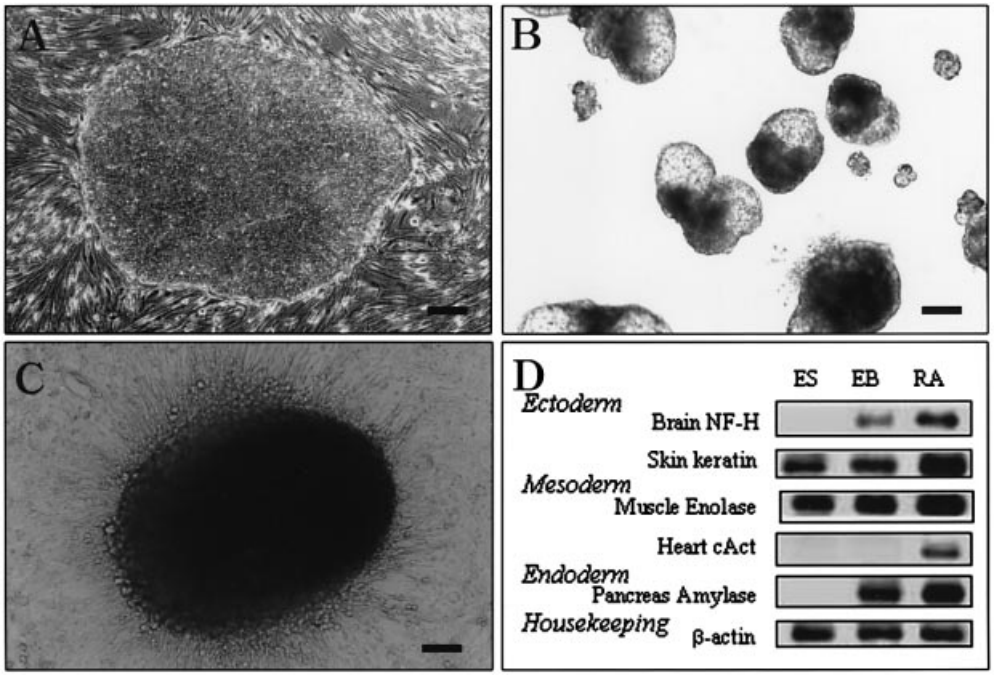

Figure 5. Expression of cell-specific genes in hES cells (MB03). Undifferentiated hES cells (**A**), day 5 embryoid bodies (**B**), and differentiated hES cells (**C**) 10 days after treatment with RA (1×10^{-6} μM). The genes were categorized into three embryonic germ layers and analysed by RT–PCR (**D**). Scale bars = 100 μm.

established hES cells grew in tight colonies composed of cells with high nuclear to cytoplasm ratios and prominent nucleoli, as indicated in Figures 1 and 2.

Human ES cells express a series of surface antigens as well as Oc-t , have a normal karyotype and exhibit remarkable long-term proliferative potential. The SSEAs 1, 3 and 4 are globoseries glycolipids. Similar to other reports (Andrews *et al*., 1996; Thomson and Marshall, 1998; Thomson *et al*., 1998; Reubinoff *et al*., 2000), our hES cells express SSEA-3 and SSEA-4 (the epitope recognized by the latter is more readily detected than that in the former) but not SSEA-1. Also, our hES cells express TRA1-60, TRA1-81 (the epitope recognized by the former is more readily detected than by the latter) and AP activity. Cell fate during development depends upon transcription factors that act as molecular switches to activate or repress specific gene expression programmes (Niwa *et al*., 2000). Oct-4 (also called Oct-3) is a mammalian POU transcription factor expressed in early embryo cells and germ cells (Botquin *et al*., 1998). Oct-4 activity is an essential characteristic of pluripotential founder cell populations in mammalian embryo (Nichols *et al*., 1998). Oct-4 has been detected *in vitro* only in undifferentiated embryonal carcinoma cells, ES cells and embryonic germ cells (Yeom *et al*., 1996). Oct-4 expression was confirmed in the undifferentiated hES cells by indirect immunostaining.

However, stem cells can either continue to grow in a pattern of prolonged self-renewal or differentiate. This fate choice is highly regulated by intrinsic signals and the external micro-environment (Odorico *et al*., 2001). Shuldiner et al. (2000) reported that none of the growth factors evaluated exclusively directs hES cell differentiation. Also, they demonstrated that pluripotent stem cells express a wide range of receptors for growth factors, and multiple human cell types may be enriched *in vitro* by specific factors. Among the additive growing factors, RA was identified as a morphogenic and teratogenic compound and as a signalling molecule influencing gene expression in a complex manner via a family of RA receptors (Rohwedel *et al*., 1999). *In vitro* differentiation of ES cells was initially confirmed morphologically and, later, characteristics specific to ES cells were reconfirmed by RT–PCR or indirect immunocytochemistry. Early heart development is known to be sensitive to RA concentrations. RA increased the concentrations of α-actin and α-actinin in the cytoplasmic and cytoskeletal fractions of cells at all stages of development (Aranega *et al*., 1999). However, in this study, the production of cardiomyocytes was limited, rather than frequent and transient. Differentiation into muscle actin was frequently observed upon addition of RA to culture conditions. After hES cells differentiated *in vitro* into three embryonic germ layer cells, RT–PCR analysis identified expression of brain neurofilament, skin keratin, muscle enolase, heart cardiac actin and pancreatic amylase genes. However, keratin and enolase gene expression was also detected in undifferentiated hES cells. This pattern was similar to that reported by Shuldiner et al. (2000). *In vitro* differentiation of hES cells into muscle, neuronal and glial cells was confirmed by indirect immunostaining with monoclonal or polyclonal antibodies. Also, some neurons express the functional neuronal markers GAD and TH, suggesting that these cells may be glutamatergic and dopaminergic neurons, respectively. In addition, when hES cells were injected intramuscularly into 5- to 7-week-old SCID mice (ICR strain), teratoma formation with various differentiated cells (glandular, blood vessel, cartilage, epithelial, etc.) was confirmed histologically (data not shown). Therefore, our

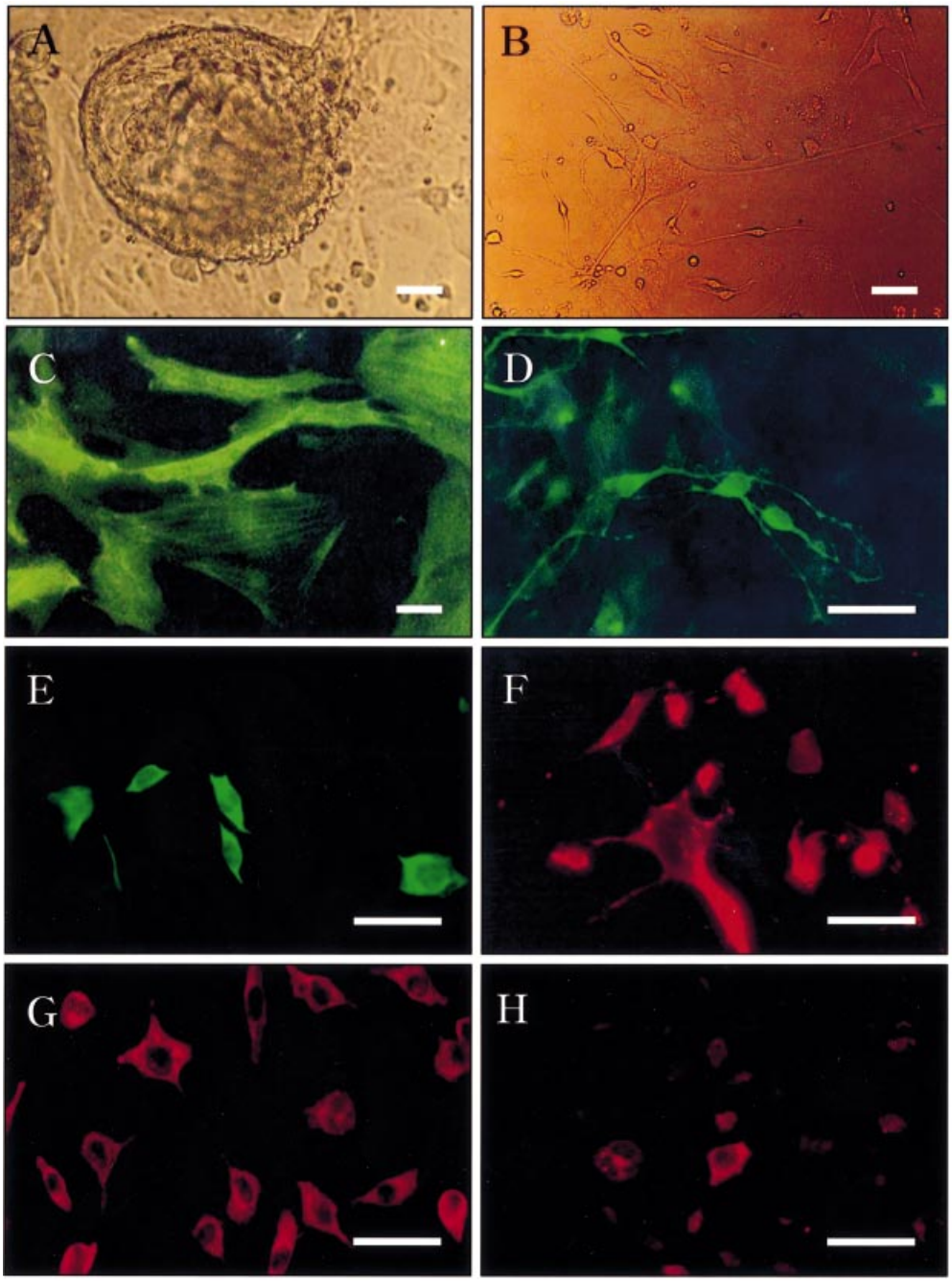

Figure 6. *In vitro* differentiated hES cells (MB01) after treatment with RA (1×10^{-6} μM). Phase morphology of cardiomyocytes and neural cells (**A** and **B**). Positive indirect immunocytochemistry identified muscle actin (**C**) as a muscle cell marker, β-tubulin III as a neuronal marker (**D**), glial fibrillary acid protein as an astrocyte marker (**E**), O4 as an oligodendrocyte marker (**F**), and glutamic acid decarboxylase (**G**) and tyrosine hydroxylase (**H**) as functional neuronal markers. Scale bars = 100 μm.

hES cells were able to differentiate into cell types of all germ layers both *in vitro* and *in vivo*.

hES cell lines will serve as unique models in developmental reseach, toxicology screening and cell therapy (Thomson *et al*., 1998; Reubinoff *et al*., 2000). However, the use of human embryos for development of ES cells is currently a controversial ethical and political problem in many countries. In Korea, the use of frozen–thawed embryos destined to be discarded after 5 years in a routine human IVF-embryo transfer programme is legal. We believe we are the first to establish hES cell lines on an STO cell feeder layer. Additional studies underway are focused on the reproducibility of our method and improvement of protocols in order to obtain larger populations of specific differentiated cells.

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