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Isolation of embryonic stem-like cells from equine blastocysts and their differentiation in vitro[1]

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Abstract **Embryonic stem (ES) cells are pluripotent cells with the potential capacity to generate any type of cell. We describe here the isolation of pluripotent ES-like cells from equine blastocysts that have been frozen and thawed. Our two lines of ES-like cells (E-1 and E-2) appear to maintain a normal diploid karyotype indefinitely in culture in vitro and to express markers that are characteristic of ES cells from mice, namely, alkaline phosphatase, stage-specific embryonic antigen-1, STAT-3 and Oct 4. After culture of equine ES-like cells in vitro for more than 17 passages, some ES-like cells differentiated to neural precursor cells in the presence of basic fibroblast growth factor (bFGF), epidermal growth factor and platelet-derived growth factor. We also developed a protocol that resulted in the differentiation of ES-like cells in vitro to hematopoietic and endothelial cell lineages in response to bFGF, stem cell factor and oncostatin M. Our observations set the stage for future developments that may allow the use of equine ES-like cells for the treatment of neurological and hematopoietic disorders.**

Key words: Equine stem cell; Differentiation in vitro; Neural precursor cell; Hematopoietic precursor cell; Endothelial precursor cell

1. Introduction

One of the goals of animal biotechnology is the application of current techniques of genetic engineering to the production of farm animals with desired traits. At present, foreign genes can be introduced into the murine genome via embryonic stem (ES) cells and the use of homologous recombination techniques [1]. In this system, cultured murine ES cells can be used as a tool for the addition or deletion of genes at specific sites in the genome. Moreover, ES cells have the stable developmental capacity to form derivatives of all three layers of embryonic germ cells, and successful differentiation in vitro to neurons, hematopoietic cells and cardiac muscle has been reported [2–4].

The isolation of pluripotent lines of human ES cells from blastocysts has also been reported [5,6]. ES cells have been used as indefinite donor cells for the production of transgenic clonal offspring [7–9]. In view of the probable significant contribution of ES cells to efforts to manipulate the genome, to generate transgenic clonal offspring, and to replace various tissues and organs, it appears appropriate to attempt to adapt this technology to the genetic improvement of farm animals. With respect to species other than mice and man, sheep [10], pig [10], rabbit [11], bovine [12,13], mink [14], hamster [15], rat [16], and monkey [17] have all been reported as sources of putative ES cells. However, neither production of chimeric animals with traits that are transmitted through the germ line nor satisfactory demonstration of the essential traits of 'ES cells' has yet been reported.

The method used for the isolation of murine ES cells [18] has been adapted to other species with some modifications. However, attempts at the primary culture of cells of the inner cell mass (ICM) of various species on a feeder layer of embryo-derived mouse STO cells or a primary culture of mouse embryonic fibroblasts in the presence of leukemia inhibitory factor (LIF) have seldom been successful. A combination of growth factors might be required for the proliferation of these pluripotent cells, as has been demonstrated in the case of cultures of mouse primordial germ cells [19]. Among studies of domestic animals, investigations of the development in vitro of equine embryos at the preimplantation stage have been quite limited [20–22]. Isolation of equine ES cells would put horses in the front lines of animal biotechnology and the modification of animal genomes.

We report here the successful isolation of immortalized equine ES-like cells with a normal karyotype from frozen and thawed blastocysts that were cultured on a feeder layer derived from bovine umbilical fibroblasts. These cells exhibited some of the essential features of ES cells, expressing the transcription factor STAT-3 [23], Oct 4 [24], the cell surface marker known as stage-specific embryonic antigen-1 (SSEA-1)

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[1] The DNA sequences of equine cDNAs and genomic DNA for Oct 4, STAT-3, nestin, GFAP, β-tubulin III, GATA-4, Flk-1, GAPDH and SRY have been submitted to GenBank and have been assigned accession numbers AB086363, AB086364, AB086361, AB086362, AB086411, AB086360, AB086358, AB086359, and AB085758, respectively.

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[25], and alkaline phosphatase [26]. We confirmed the capacity of these ES-like cells for somatic differentiation in vitro to neural progenitor cells and to endothelial or hematopoietic lineages.

2. Materials and methods

2.1. Recovery of equine blastocysts and freezing and thawing for subsequent propagation of cells

We used four Hokkaido native ponies as the source of embryos. We flushed their uteri 26 times in all and, as a result, we obtained a total of 14 blastocysts on 6–7 days after natural ovulation as described elsewhere [27,28]. After washing in Dulbecco's phosphate-buffered saline (DPBS; Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Groningen, The Netherlands), blastocysts were equilibrated for 10 min in a 10% (v/v) solution of glycerol in DPBS supplemented with 20% newborn calf serum (Mitsubishi-Kasei, Tokyo, Japan). The blastocysts were then put into individual plastic straws (0.25 ml). Each straw was held for 15 min at −5°C and then cooled to −30°C at a rate of 0.5°C/min in a programmable freezer. Finally, straws were plunged into liquid nitrogen and stored for up to 1 year prior to use. Blastocysts were thawed by immersion of straws for 20 s in a water bath at 37°C. Then blastocysts were incubated with DPBS plus 10% FBS that contained 6% glycerol and 0.3 M sucrose for 5 min, and with DPBS plus 10% FBS that contained 3% glycerol and 0.3 M sucrose for another 5 min, with DPBS plus 10% FBS that contained 0.3 M sucrose for 5 min and then, finally, they were washed with DPBS plus 10% FBS. These blastocysts were used for microsurgical dissection of the ICM.

2.2. Preparation of a feeder layer

Biopsy samples of bovine umbilical cord were collected from a newborn calf and washed thoroughly in PBS (Sigma) supplemented with penicillin (100 IU/ml) and streptomycin (50 μg/ml), to remove toxic agents and possible contaminants. Samples were cut into small pieces of approximately 1–4 mm^3 with fine scalpel blades in a sterile dish. Several of these small pieces of tissue were placed in a tissue culture dish (diameter 6 cm) and cultured in α-minimum essential medium (αMEM; Life Technologies, Rockville, MD, USA), supplemented with 10% FBS, penicillin (100 IU/ml) and streptomycin (50 μg/ml), in a humidified atmosphere of 5% CO_2 in air at 38.6°C. Umbilical cord-derived fibroblasts grew to confluence and were then subcultured two or three times before use as a feeder layer.

2.3. Isolation and proliferation of ES cells

Three ICMs were isolated microsurgically from three morphologically intact, frozen and thawed equine blastocysts. They were placed in αMEM supplemented with 10% FBS, 0.1 mM 2-mercaptoethanol (2-ME), human LIF (10 ng/ml; Sigma), penicillin (100 IU/ml) and streptomycin (50 μg/ml) with a feeder layer of mitotically inactivated umbilical cord-derived fibroblasts in four-well culture plates (Nalge Nunc International, Roskilde, Denmark) in a humidified atmosphere of 5% CO_2 in air at 38.6°C. After 3–5 days, growing colonies whose morphology was typical of ES cells were dispersed by brief treatment with a mixture of 0.25% trypsin–EDTA (Life Technologies). The resultant dissociated cells were replated on a fresh feeder layer and cultured under the same conditions as described above. After 10–14 days, cells with ES-like morphology [25] that had become confluent were picked up and replated on a fresh feeder layer (passage 1 or P1 cells). P1 cells were then subjected to repeated passages at 6- to 7-day intervals. Cultures were examined daily and medium was replaced every second day.

In some experiments, colonies of cells were transferred to standard culture medium (αMEM supplemented with 10% FBS, 0.1 mM 2-ME, penicillin and streptomycin) plus human LIF (10 ng/ml) in culture plates for induction of differentiation or to standard culture medium without LIF for induction of the formation of embryoid bodies (EBs) in the absence of a feeder layer. Karyotype analysis (Giemsa staining) was performed as described previously [10,29].

2.4. Characterization of stem cells

For analysis of the expression of SSEA-1, we plated equine ES cells at 2×10^5 cells per well in four-well dishes and cultured them for 5 days in standard medium with LIF. Then cells were harvested and incubated with horse immunoglobulin (IgG; Sigma) and mouse monoclonal antibodies against mouse SSEA-1 (IgM; Kyowa Medex, Tokyo, Japan) and subsequently with fluorescein isothiocyanate-conjugated (FITC-conjugated) second antibodies raised in rabbit against mouse IgM or IgG (Fujisama Pharmacia, Tokyo, Japan). Expression of SSEA-1 was examined by flow cytometry in a cell sorter (FACScan; Becton-Dickinson, Mountain View, CA, USA).

To evaluate the expression of STAT-3, we plated frozen and thawed equine ES cells (P17) at 1×10^4 cells per well in four-well dishes without a feeder layer and cultured them in the presence of LIF for 4 days in standard medium. Cells were fixed in 4% paraformaldehyde and immunostained with mouse STAT3-specific antibody (10 μg/ml; Sigma) and with FITC-conjugated rabbit antibodies against mouse IgG as second antibodies (1:100; Sigma). Images were obtained by fluorescence microscopy (TE300; Nikon, Tokyo, Japan). Alkaline phosphatase activity was examined as described previously [26].

2.5. Differentiation to neural precursor cells in vitro

To initiate differentiation, we cultured dissociated ES cells (P18) at 1×10^4 cells per well in serum-free standard medium with mouse basic fibroblast growth factor (bFGF, 10 ng/ml; Sigma), mouse epidermal growth factor (EGF, 10 ng/ml; Sigma) and mouse platelet-derived growth factor (PDGF, 10 ng/ml; Sigma) for 7 days [2,30]. Then we analyzed cells for the expression of nestin using a mouse nestin-specific antibody (Chemicon International, Temecula, CA, USA) and FITC-conjugated rabbit antibodies against mouse IgG as second antibodies (Jackson Laboratories, West Grove, PA, USA). Upon removal of growth factors, cells were allowed to undergo differentiation for 7–14 days. Differentiated cells were examined for immunoreactivity with antibodies against an astrocyte-specific marker, mouse glial fibrillary acidic protein (GFAP; Sigma), and against a neuron-specific marker, mouse β-tubulin III (Sigma). Cells were fixed and analyzed as described above.

2.6. Differentiation in vitro to hematopoietic and endothelial precursor cells

Dissociated ES cells (P18) were cultured at 1×10^4 cells per well in standard medium with 10% FBS, stem cell factor (SCF, 10 ng/ml; Sigma), bFGF (10 ng/ml; Sigma) and oncostatin M (OSM, 10 ng/ml; Sigma). After 10 days, cobblestone-like and endothelial-like cells were examined for immunoreactivity with an antibody against a marker of hematopoietic progenitor cells (mouse CD45; Sigma) and FITC-conjugated second antibody (PharMingen, San Diego, CA, USA).

Endothelial-like cells were cultured for an additional 7–10 days in medium without growth factors. These cells were then analyzed for the expression of markers of endothelial precursor cells with antibodies against mouse CD31, CD34 and fetal liver kinase-1 (Flk-1) (all purchased from Sigma) and FITC-conjugated rabbit antibodies against mouse IgG as second antibodies (PharMingen). Cells were fixed and analyzed for the expression of each marker as described above.

2.7. Reverse transcription-polymerase chain reaction (RT-PCR) and analysis by PCR of the expression of marker genes

Total RNA (10 μg) was isolated from ES cells with Trizol-Ls reagent (Invitrogen), and SuperScript II (Invitrogen) reverse transcriptase was used to synthesize cDNA according to the protocol from Invitrogen. Genomic DNA was prepared by the standard method as described elsewhere [29]. The reaction mixture for PCR contained cDNA (equivalent to 25 μg of total RNA) or genomic DNA (0.5 μg), specific primers (5 pmol each), 50 mM dNTPs, 1×buffer for KOD Dash and 0.5 U of KOD Dash polymerase (Toyobo, Osaka, Japan). Each thermal cycle for amplification included incubation at 94°C for 15 s, at 55°C for 30 s and at 72°C for 2 min. The cycle was repeated 40 times with final extension for 10 min at 72°C. Primers specific for SRY DNA [combination 1, sense 5′-gCCATTCTTCgAggAggCACAgA-3′ (nt 402–425) and antisense 5′-TATCgACCTCgTCggAAggC-3′ (nt 466–486)] and [combination 2, sense 5′-TggTgTggTCTCgTgATCAggCgCAAgg-3′ (nt 284–314) and antisense 5′-TCTgTgCCTCCTCgAAgAATggC-3′ (nt 402–425)], for Oct 4 mRNA [sense 5′-TCCCAggACATCAAAgCTCTgCAgA-3′ (nt 848–873) and antisense 5′-TCTgggCTCTCCCATgCATTCAAACTgA-3′ (nt 1495–1524)], for STAT-3 mRNA [sense, 5′-TCTggCTAgACAATATCATCgACCTTg-3′ (nt 1908–1935) and antisense 5′-TTATTTCCAAACTG-

CATCAATgAATCT-3′ (nt 2413–2439)], for GFAP mRNA [sense 5′-gCCCTggACATCgAgATCgCCACCTACAgg-3′ (nt 1091–1120) and antisense 5′-ATgTTCCTCTTgAggTggCCTTCTgACAC-3′ (nt 1214–1244)], for β-tubulin III mRNA [sense 5′-CAgAgCAAgAACAg-CAgCTACTT-3′ (nt 999–1022) and antisense 5′-gTgAACTCCA-TCTCgTCCATgCCCTC-3′ (nt 1201–1227)], for GATA-4 mRNA [sense 5′-CTCTggAggCgAgATgggACggg-3′ (nt 971–994) and antisense 5′-gAgCggTCATgTAgAggCCggCAggCATT-3′ (nt 1452–1481)], for Flk-1 mRNA [sense 5′-CTgCCTACCTCACCTgTTTCCTgTA-Tgg-3′ (nt 3833–3861); and antisense 5′-ggATATCTTgAAATgT-TTTTACACTCAC-3′ (nt 4003–4031)], and for GAPDH mRNA [sense 5′-gggCTTggCTTCggTgACAACACCAAggCggC-3′ (nt 646–678) and antisense 5′-CgAgCAAAggCCTCTgCCACCTTgCggTT-3′ (nt 808–837)] were used for PCR, yielding products of 83, 140, 674, 531, 151, 228, 510, 197, and 191 bp, respectively.

3. Results and discussion

3.1. Derivation of cell lines E-1 and E-2

Three ICMs were isolated by microsurgery from frozen and thawed equine blastocysts. They were plated on a feeder layer of bovine umbilical cord fibroblasts and they began to spread out after 3–5 days in culture (Fig. 1A). Two of the three ICMs grew as individual colonies composed of tightly packed compact cells. The growing colonies were mechanically dislodged from outer flattened cells and treated with trypsin to dissociate cells. After replating, these dissociated cells formed compact colonies of small cells, as commonly observed in the case of murine ES cells (Fig. 1B) [25]. We established two lines of ES-like cells, E-1 and E-2, and these cells had a diploid XY karyotype (62+XY) [29] up to the 15th–20th and 11th–15th passages, respectively (Fig. 1C). The results were confirmed by sexing equine ES-like cells with a Y chromosome-specific DNA probe, namely, sex-determining region Y (SRY) [31], by PCR (Fig. 1D). The homogeneity in terms of chromosome number and the diploidy of the cells suggested that these lines should remain genotypically and phenotypically stable in vitro for many generations. Both lines of equine ES-like cells were successfully cryopreserved after any number of passages examined after 3–8 months of continuous development. Cells have been successfully thawed without loss of the potential for proliferation after preservation for more than 12 months

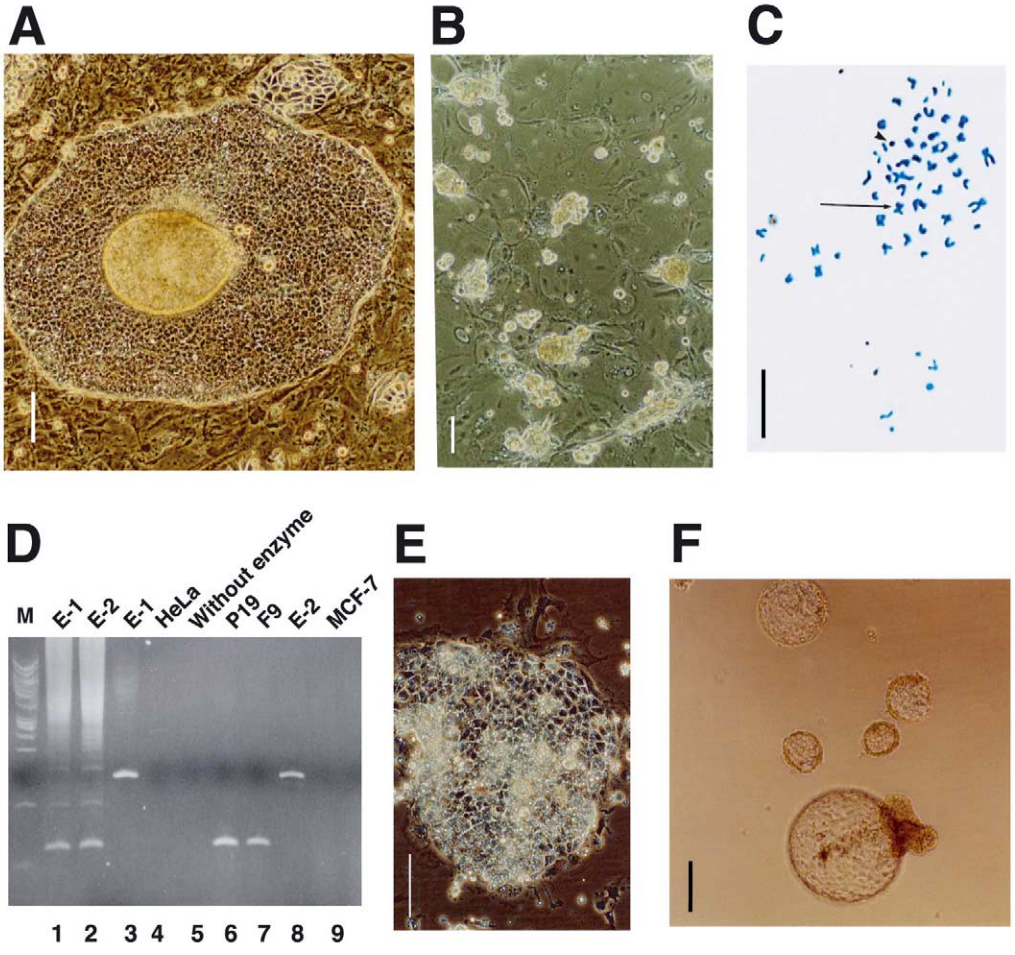

Fig. 1. Characterization of equine ES-like cells. A: ICM after 5 days in culture (E-1 line). B: Colonies of ES-like cells (E-1 line). C: A metaphase spread of ES-like cells (E-2 line) that included an X chromosome (arrow) and a Y chromosome (arrowhead). Female and male karyotypes were identified by fluorescence in situ hybridization using X chromosome- and Y chromosome-staining probes (gifts from Animal Research Institute, Shirakawa, Fukushima, Japan). D: Amplification by PCR of sex-determining region Y (SRY) from equine ES-like cells. Lane 1, E-1 line; lane 2, E-2 line; lane 3, E-1 line; lane 4, HeLa cells; lane 6, P19 cells; lane 7, F9 cells; lane 8, E-2 line; lane 9, MCF-7 cells; lane 5, E-1 line without KOD Dash polymerase. Lanes 1, 2, 4, 5, 6, 7, 9, amplification with combination 1 primer sets for SRY-1; lanes 3 and 8, amplification with combination 2 primer sets for SRY-1. M, Molecular markers (*Hae*III digest of ΦX174). E: ES-like cells (E-1 line) undergoing partial differentiation, after culture for 5 days in the absence of a feeder layer of bovine cells but in the presence of LIF (10 ng/ml). F: EBs, 2 weeks after the start of suspension culture (E-2 line). Magnification: A, B and F, ×200; E, ×100; C, ×600.

[27,28]. E-1 cells have been cultured for 56 passages in vitro and E-2 cells have been cultured for 38 passages. Strelchenko et al. [32] reported a limit of approximately 60 divisions for most non-immortalized cells. Therefore, we can distinguish immortalized cell lines from non-immortalized cell lines on the basis of the ability of cells to undergo more than 60 divisions. Since our lines of equine ES-like cells have divided a minimum of 392 and 266 times, respectively, these cells appear to be immortal and to remain in an undifferentiated state indefinitely in vitro. By contrast to murine ES cells, equine ES-like cells differentiated to epithelial-like cells in the absence of a feeder layer, provided the medium was supplemented with LIF (Fig. 1E). When equine ES-like cells were cultured as large clumps of cells (more than 100 cells per aggregate) in the absence of a feeder layer, they multiplied rapidly for several days after transfer to fresh medium and formed tight aggregates that increased in size with time in culture. When such cultures were maintained for 2–4 weeks, the cell aggregates developed into cystic EBs (Fig. 1F). These cystic EBs were composed of two layers of cells with heterogeneous cellular particles within the cavity (Fig. 1F, see largest EB). Endoderm-like cells were located on the external surface of the cystic EBs and ectoderm-like cells were located on the internal surface. The phenotype of equine EBs resembled that of mouse and bovine EBs [13,25]. The cells of equine EBs were able to differentiate into epithelial, fibroblastic and neuron-like cells when re-attached to the surface of a dish (data not shown). Thus, the equine ES-like cells seemed to be strictly dependent on a feeder layer for maintenance of an undifferentiated state.

3.2. Expression of markers by equine ES-like cells

We examined the two lines of ES-like cells for the expression of markers that indicate an undifferentiated state. We

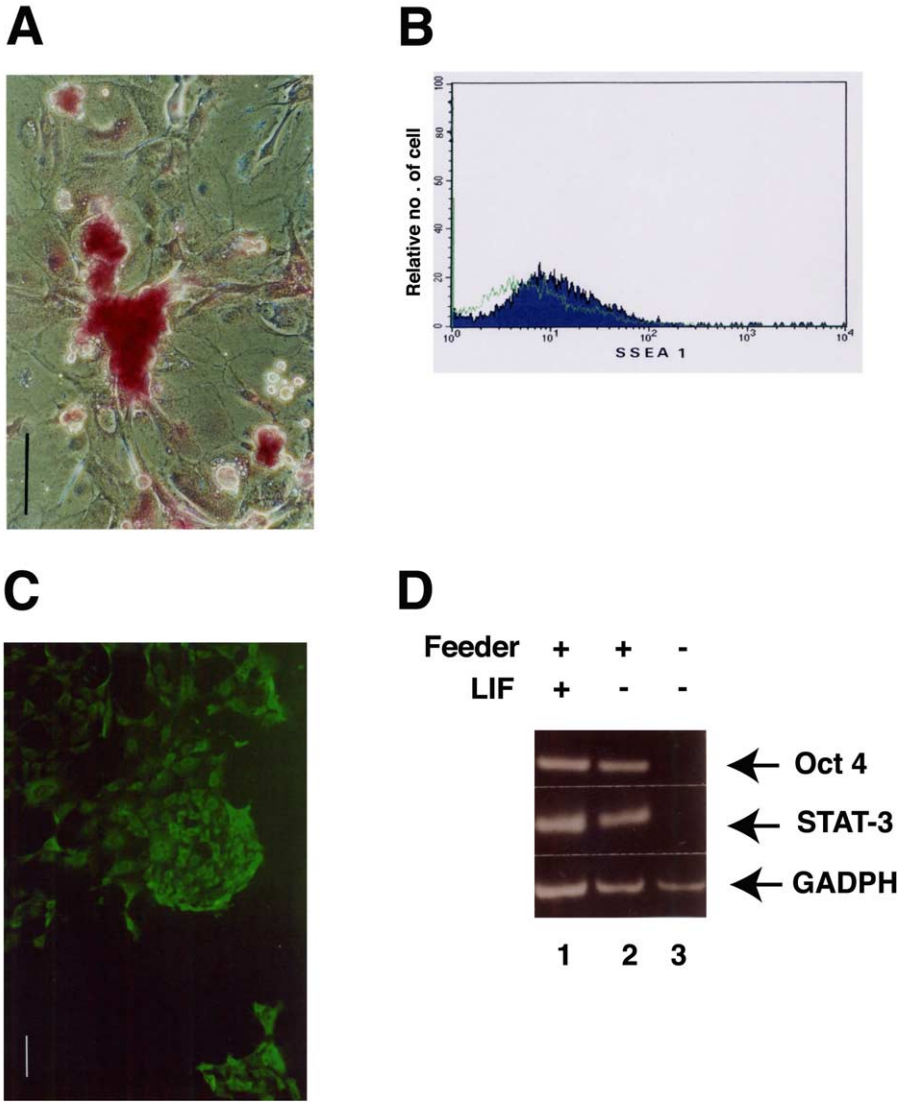

Fig. 2. Expression of molecular markers of undifferentiated equine ES-like cells. A: Alkaline phosphatase, as visualized by the method of Talbot et al. ([26]; E-2 line). B: Flow-cytometric profiles of cells (E-1 line) after immunostaining with antibodies against SSEA-1 (indicated in blue) and with equine IgG (indicated in green). Close to 40% of cells were immunopositive for SSEA-1. C: Immunostaining of ES-like cells with STAT-3-specific antibody (E-1 line). D: Analysis of the expression of genes for Oct 4, STAT-3 and GAPDH by RT-PCR (E-1 line). Total RNA was extracted from equine ES-like cells (E-1 line) that had been cultured on a feeder layer (lanes 2) and in the presence of LIF (lane 1) and in the absence of both LIF and a feeder layer (lane 3). Magnification: A and C, ×100.

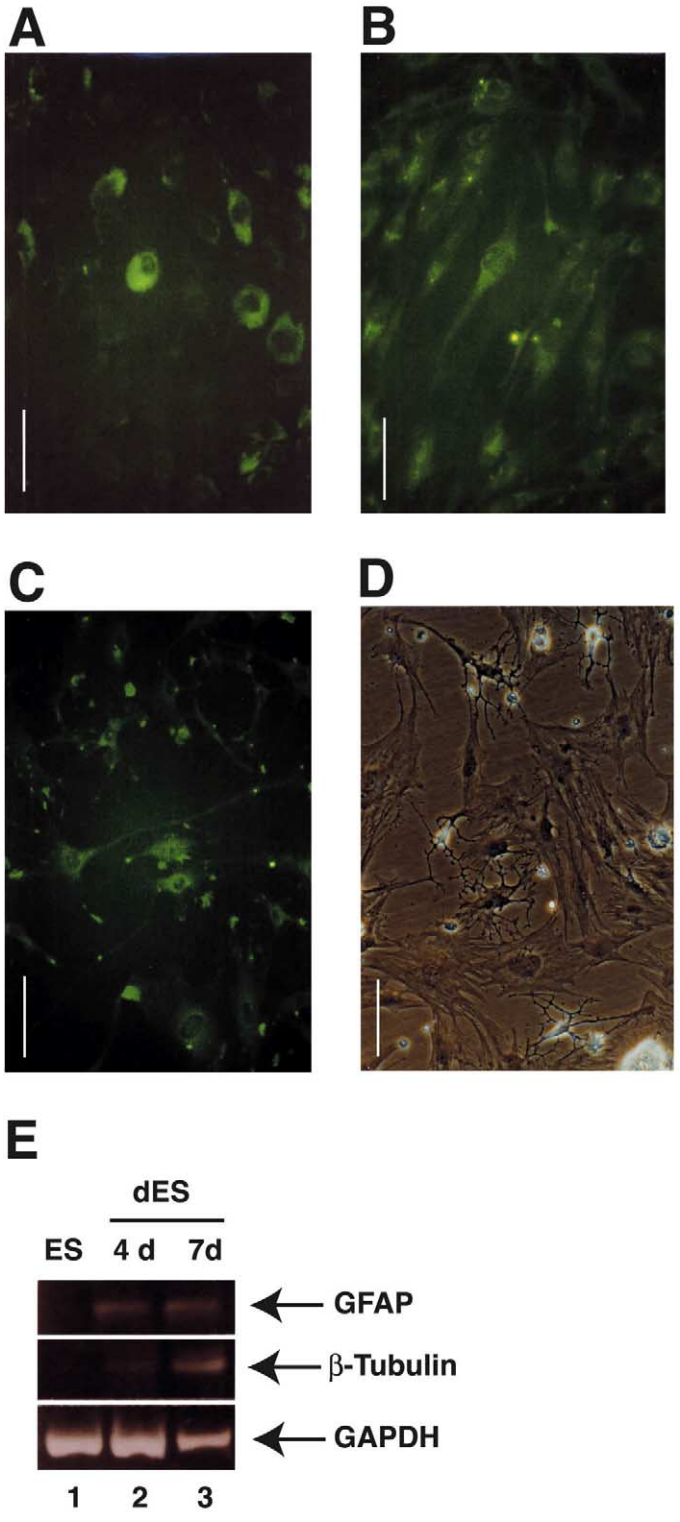

Fig. 3. Morphological differentiation and expression of markers by ES-like cell-derived neural precursors. ES-like cells were cultured in serum-free αMEM plus 10% FBS with bFGF, EGF and PDGF for 7 days and then analyzed for markers of neural precursor cells. A: Immunostaining with the nestin-specific antibody. B: Immunostaining with antibody against the astrocyte-specific antigen GFAP. C: Immunostaining with antibody against the neuron-specific antigen β-tubulin III. D: Phase contrast micrograph showing the extensive outgrowth of neural structures from ES-like cells. Magnification: A–D, ×200. E: Analysis of the expression of marker genes in equine ES cell-derived neural precursors. Total RNA from ES-like cells (lane 1) and from ES-like cells that had been treated with bFGF, EGF and PDGF for 4 days (dES; lane 2) and 7 days (dES; lane 3) was analyzed by RT-PCR for the expression of genes for GFAP, β-tubulin III and GAPDH.

detected tissue-non-specific alkaline phosphatase activity [26] in both lines of equine ES-like cells (Fig. 2A). Such activity has been demonstrated in pluripotent stem cells of mouse [25] and human [5,6] origin, as well as in stem-like cells of sheep [10] and pig [10], but not in bovine stem-like cells [13]. The equine ES-like cells expressed the cell surface marker SSEA-1, which is characteristic of undifferentiated mouse ES cells [25]. Fluorescence-activated cell sorting (FACS) analysis indicated that close to 40% of our equine ES-like cells were strongly positive for immunostaining with an antibody raised against mouse SSEA-1 (Fig. 2B). The equine ES-like cells differed from human stem cells, which do not express SSEA-1 but do express SSEA-3 and SSEA-4 [5,6]. The equine ES-like cells did not express either SSEA-3 or SSEA-4 (data not shown).

Equine ES-like cells expressed the STAT-3 antigen (Fig. 2C) [23]. We examined the expression of STAT-3 in undifferentiated and differentiated equine ES-like cells, in the absence of a feeder layer and in medium without LIF, by RT-PCR. We detected an amplified DNA fragment of 531 bp in the analysis of RNA from undifferentiated equine ES-like cells but not from differentiated ES-like cells (upper panel in Fig. 2D). This fragment was of the size expected for the cDNA of STAT-3, which is a transcription factor that plays a central role in the maintenance of the pluripotent phenotype of murine ES cells [23]. A reduction in the level of active STAT-3 caused by withdrawal of or reduction in the level of LIF is detrimental to the self-renewal of mouse ES cells because the inhibition by LIF of differentiation-signaling pathways in ES cells involves STAT-3 [23].

It has been demonstrated that Oct 4, a protein that is primarily expressed in pluripotent lineages and is regarded as having a role in the development of germ cells [24,33,34], is widely expressed in early embryos. Therefore, we examined the expression of Oct 4 in undifferentiated and differentiated equine ES-like cells and confirmed the expression of Oct 4 in undifferentiated ES-like cells by RT-PCR, detecting an amplified DNA fragment of 674 bp that was derived from RNA from undifferentiated equine ES-like cells but not from differentiated ES-like cells (upper panel in Fig. 2D). Thus, Oct 4 was expressed in our equine ES-like cells, as it is in human ES cells [6]. Maintenance of murine ES cells in an undifferentiated state requires the cooperative actions of Oct 4 and STAT-3. Thus, Oct 4 and STAT-3 are essential for maintenance of the stem cell phenotype, in conjunction with activation of the LIF signaling pathway [23,24,33].

The feeder layer that is required to support the serial cultivation of mouse ES cells can be replaced by LIF. However, such was not the case for equine ES-like cells in the undifferentiated state. We noted that the morphology of equine ES-like cells in the undifferentiated state resembled that of murine ES cells. However, the presence of LIF was not required for maintenance of equine ES-like cells in an undifferentiated state. By contrast, cultivation of ES-like cells on a feeder layer was necessary to maintain equine ES-like cells in an undifferentiated state. Such differences between the two systems and the signals that maintain equine ES-like cells in an undifferentiated state remain to be clarified. In contrast to murine ES cells, human ES cells do not rely on LIF for inhibition of differentiation [5,6], and we found the same to be true for our equine ES-like cells. The markers, mentioned above, that are characteristic of ES cells should be useful for the selection of clonal ES or ES-like cells from other mammals.

3.3. Differentiation of neural precursor cells from equine ES-like cells in vitro

Interest in the therapeutic potential of ES cells has been stimulated by the finding that human ES cells can serve as a source of precursors to neural cells [5,6]. To examine the ability of equine ES-like cells to differentiate to neural precursor cells, we cultured ES-like cells in a defined medium that contained a mixture of growth factors. After 7 days in culture, equine ES-like cells developed into bipolar fibrous cells that were immunopositive for nestin (Fig. 3A) [30]. Upon removal of growth factors, the bipolar fibrous cells differentiated to astrocytes or neurons within 7–14 days, becoming immunopositive for the astrocyte-specific marker GFAP (Fig. 3B) [30]. Some cells also expressed the neuron-specific marker β-tubulin III (Fig. 3C) [30] and had an obviously neural morphology with the extensive outgrowth of fibers (Fig. 3D). We performed RT-PCR to examine the transcripts of the genes for GFAP and β-tubulin III and detected the expression of these genes in the neurally differentiated ES-like cells (Fig. 3E). In the present study, we developed a new method for induction of the differentiation of neural precursors from ES-like cells, using a combination of bFGF, EGF and PDGF. Our method does not involve treatment with retinoic acid [30], formation of EBs [6,30] or feeder layer-derived induction activity [35]. Our results are in harmony with the observations that neural stem cells can be isolated and cultured in vitro from the adult subventricular zone [36] and other regions of the brain in the presence of EGF and bFGF [36]. Culture medium containing bFGF and PDGF promotes the proliferation of glial precursor cells [2]. We used both growth factors together to induce the differentiation of equine ES-like cells to neural and glial precursor cells.

3.4. Differentiation of precursors to hematopoietic and endothelial cells

Murine ES cells that have been induced to differentiate in vitro can give rise to hematopoietic precursors and endothelium in the presence [3,4] and in the absence [37] of the formation of EBs. To examine the ability of equine ES-like cells to generate hematopoietic precursors, we incubated ES-like cells in the presence of bFGF, SCF and OSM [38]. We found two different types of cell colony in the same culture wells after 10 days of culture. One type of colony consisted of non-adherent, rounded, cobblestone-like cells and the other consisted of adherent endothelial-like cells (Fig. 4A). Formation of cobblestone areas is a typical feature of hematopoietic stem cells [38]. The clusters of cobblestone cells were immunopositive for CD45 [37,38], a marker of hematopoietic progenitor cells (Fig. 4B). By contrast, endothelial-like cells were only minimally immunopositive (Fig. 4B). After a further 7–10 days in culture, adherent cells cultured in the absence of growth factors were positive for immunostaining with various markers of endothelial cells, namely, Flk-1 [37], CD31 and CD34 (Fig. 4C–E) [37,38]. We used RT-PCR to detect transcripts of genes for GATA-4 and Flk-1 and confirmed the expression of these genes in the ES cell-derived hematopoietic precursor cells (Fig. 4F) [37,38].

The ES cells that were proliferating in two dimensions were able to differentiate to hematopoietic cells and, thus, it ap-

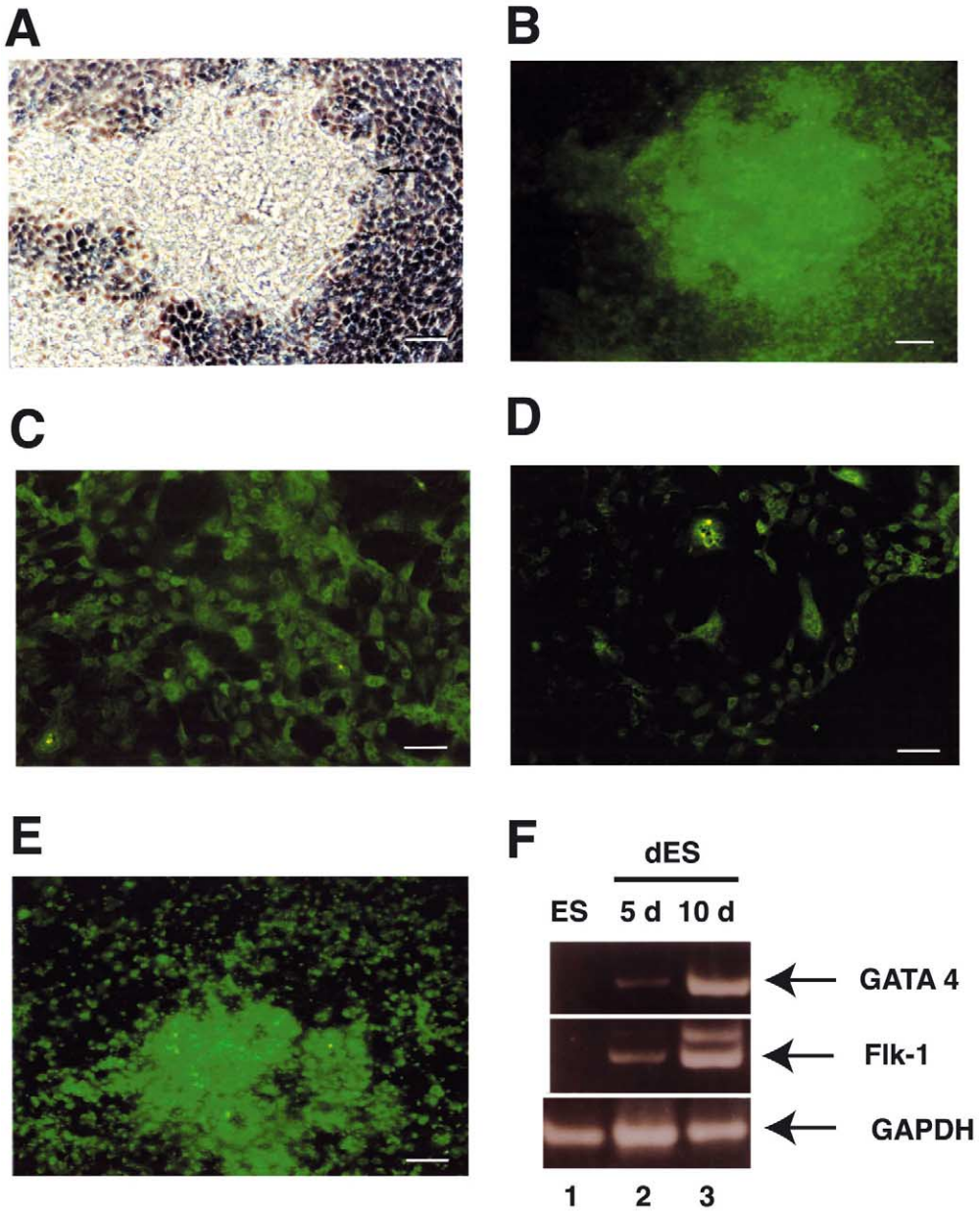

Fig. 4. Morphological differentiation and expression of markers by ES-like cell-derived hematopoietic precursors. ES-like cells were cultured in αMEM plus 10% FBS, SCF, bFGF and OSM for 10 days. A: Phase contrast micrograph of cobblestone-like cells and adherent endothelial-like cells. B: Immunostaining of the same culture as in A with CD45-specific antibody. Round cobblestone-like cells were immunopositive. ES-like cells that had differentiated to endothelial precursors were immunostained with antibodies specific for CD31, CD34, and Flk-1, as shown in panels C, D, and E, respectively. Magnification: A–E, ×100. F: Expression of genes for markers of hematopoietic and endothelial differentiation in undifferentiated ES-like cells (ES) and differentiated ES-like cells (dES). RNA from ES-like cells (lane 1) and from ES-like cells treated with SCF, bFGF and OSM for 5 days (lane 2) and 10 days (lane 3) was analyzed by RT-PCR for expression of genes for GATA-4, Flk-1 and GAPDH.

peared that neither a three-dimensional EB structure nor a layer of feeder cells was required for this process, as was also the case for the differentiation of equine ES-like cells to neural precursor cells.

In this study, we demonstrated that immortalized pluripotent equine ES-like cells can be induced to generate both neural and hematopoietic cells in vitro. However, here we cannot report the chimerism of equine ES-like cells and their germline transmission. The production of chimeric equines with traits that are transmitted through the germ line is now being undertaken to confirm the competence of totipotency and pluripotency of the ES cells.

Equine ES-like cells should be useful as an unlimited and valuable source of cells for gene targeting and the generation of transgenic clonal offspring and also as a powerful tool for studies of the mechanisms of activation of growth factors.

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