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Biochemical and Biophysical Research Communications 309 (2003) 104–113
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Generation of cloned calves and transgenic chimeric embryos from bovine embryonic stem-like cells

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Received 30 July 2003

Abstract

Bovine embryonic stem-like cells (ES-like cells) appear to maintain a normal diploid karyotype indefinitely during culture in vitro and to express marker proteins that are characteristic of ES cells from mice, namely, alkaline phosphatase (AP), stage-specific embryonic antigen-1 (SSEA-1), STAT-3, and Oct 4. After proliferation of undifferentiated ES-like cells in vitro, some bovine ES-like cells differentiated to neural precursor cells, which were cultured in the presence of basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF). In addition, calves were successfully cloned using ES-like cells and the frequency of term pregnancies for blastocysts derived from ES-like cells was higher than those of early pregnancies and maintained pregnancies after nuclear transplantation (NT) with bovine somatic cells. Successful cloning from bovine ES-like cells should allow the introduction into cattle of specific genetic characteristics of biomedical and/or agricultural importance.

Keywords: Bovine; ES-like cells; In vitro differentiation; Cloning; Nuclear transplantation

Embryonic stem (ES) cells are pluripotent cells derived from preimplantation embryos [1,2]. These ES cells can be maintained in culture indefinitely as undifferentiated cells but can differentiate into various types of cells under certain conditions [3–6]. ES cells also exhibit totipotency and cloned animals have been generated from undifferentiated ES cells at higher frequencies than they have been from somatic cells by transfer of their nuclei to enucleated oocytes.

Wakayama and co-workers [7–9] reported that late-passaged ES cells (at the 8th–9th or 22nd–32th passages) can be used to produce transgenic clonal mice. Thus, cloning with ES cells offers the possibility of creating, in a single generation, mutant animals that would otherwise take years to obtain by breeding livestock. In spite of the success of cloning with cells from cultured inner cell masses (ICMs) [10], differentiated embryonic cells [11,12], and fetal [12–15] and adult somatic cells [12,16–19], all reports of cloning of mammals other than mice have indicated that cells from primary or early passaged cultures are required as donors of nuclei. In general, it is unlikely that such "primary" cells will be used for the routine manipulation of genomes because of the length of time required for selection of transfected clones, growth of colonies, and genetic testing before nuclear transfer (NT) [20–22]. Moreover, the potential of such cells for differentiation in vitro and for tissue replacement is quite restricted [7]. Thus, there is a need for lines of karyotypically stable and pluripotent ES cells with nuclei that can direct the development of complete and viable embryos when transferred to enucleated oocytes.

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doi:10.1016/S0006-291X(03)01536-5

In cattle, which are important from both an agricultural and a biotechnological perspective, ES-like cells have been used as a source of donor nuclei for NT [23–25]. To date, however, cloned embryos derived from ES-like cells have failed to develop for more than 60 days after NT, presumably because of incomplete placental development [24,25].

In mice, ES cells provide one of the most useful models for the transfer of exogenous DNA to the animal and they allow precise genetic modifications by gene targeting [26]. Such gene targeting in livestock has been reported, although the animals have been cloned by NT techniques from cultured somatic cells [20–22,27]. The generation of immortalized stem cells that would allow genetic manipulation is also important if we are to develop a system for the introduction of targeted mutations into bovines. However, there are very few reports describing the pluripotency of transfected embryo-derived cell lines.

We report here the characterization of lines of bovine pluripotent ES-like cells and the production of cloned calves from ES-like cells. We also describe the somatic differentiation of ES-like cells in vitro to neural progenitor cells and the production of transgenic (Tg) chimeric embryos that harbored a gene for enhanced green fluorescent protein (EGFP). These results suggest the possibility of gene manipulations of bovine ES-like cells by homologous recombination to generate the targeted cloned calves.

Materials and methods

Embryos, blastocysts, and ES-like cells. Embryos from Japanese black and Holstein–Friesian cows were collected nonsurgically seven days after the onset of estrus [23] and stored in liquid nitrogen for 2–3 years prior to use, as described elsewhere [28]. Frozen and thawed bovine blastocysts were used for microsurgical removal, by dissection, of ICMs. The morphologically intact ICMs were transferred to minimum essential medium α (MEMα; Life Technologies, Rockville, MD), supplemented with 10% FBS, 0.1 mM of 2-mercaptoethanol, human leukemia inhibitory factor (LIF) at 10 ng/ml (Sigma Chemical, St. Louis, MO), human epidermal growth factor (EGF; 10 ng/ml; Sigma), penicillin (100 IU/ml), and streptomycin (50 μg/ml) on a feeder layer of mitotically inactivated murine STO cells [23–25] and cultured in a humidified atmosphere of 5% CO_2 in air at 38.6 °C. The first passage was performed when colonies of cells with the morphology of ES-like cells had reached approximately 500 μm in diameter, seven days after the start of culture. In some experiments, colonies of cells were transferred to standard medium plus LIF (10 ng/ml) for induction of differentiation or without LIF for induction of the formation of embryoid bodies (EBs) in the absence of a feeder layer.

Markers of undifferentiated or neurally differentiated ES-like cells. Bovine ES-like cells [lines W1, W2, and W3 from Japanese black cows; and line H-1 from Holstein–Friesian cows; passage 15 (P15) to P20] were seeded at 1×10^4 cells per well in four-well dishes (Nalge Nunc International, Roskilde, Denmark) without a feeder layer and cultured for five days in standard medium plus LIF (10 ng/ml). Cells were then fixed and treated with monoclonal antibodies against mouse SSEA-1 (5 μg/ml; Cosmo Bio, Tokyo, Japan) or against mouse STAT-3 [29,30] (10 μg/ml; Sigma) or against Oct 4 [31] (5 μg/ml; Sigma) and then with fluorescein isothiocyanate-conjugated (FITC-conjugated) second antibodies raised in rabbit against mouse IgM or IgG (1:100; Sigma). Alkaline phosphatase (AP) activity was determined as described previously [32,33]. To initiate differentiation, ES-like cells (P14–P18) were cultured in serum-free MEMα supplemented with human bFGF (10 ng/ml; Sigma), human EGF (10 ng/ml; Sigma), and human PDGF (10 ng/ml; Sigma) for 7–10 days [3,30]. After removal of growth factors, cells were cultured for a further 7–14 days for induction of differentiation. Differentiated cells were examined for immunoreactivity with antibodies against mouse glial fibrillary acidic protein (GFAP; Sigma) and against mouse β-tubulin III (Sigma) with FITC-conjugated rabbit antibodies against mouse IgG (1:100; Sigma) as second antibodies.

Nuclear transfer, activation of oocytes, and culture of embryos. Nuclear transfer (NT) was performed as described previously [34]. Cumulus-oocyte complexes (COCs) were aspirated from follicles of 2–8 mm in diameter with an 18-gauge needle into a disposable 10-ml syringe. Ten bovine COCs were incubated in separate 100-μl aliquots of IVMD-101 medium (Research Institute for Functional Peptides, Yamagata, Japan) at 39 °C in a humidified atmosphere of 5% CO_2 in air for 22 h. The oocytes were enucleated by pushing out the first polar body and the metaphase II plate, plus a small amount of the cytoplasm that surrounded them, with a glass pipette into TALP (mTALP) medium [35] supplemented with 5 μg/ml cytochalasin B (Sigma). Bovine ES-like W3 cells were cultured in MEMα supplemented with 10% FBS, LIF (10 ng/ml), EGF (10 ng/ml), penicillin (100 IU/ml), and streptomycin (50 μg/ml) for 2 days. The density of dishes of ES-like W3 cells used for injection was approximately 60–80% of the density of confluent cells. After insertion of donor ES-like cells into the perivitelline space of oocytes, cells and cytoplasts were fused electrically by two pulses of 20 V/150 mm of direct current for 50 μs in an Electro Cell Fusion System from Bex (Tokyo, Japan) in fusion medium [10,34]. Reconstituted oocytes were activated by incubation in modified TALP (mTALP) medium [35] supplemented with 0.1% bovine serum albumin (BSA) and 10 mg/ml cycloheximide (CH; Sigma) for 5 h. On day 1 (day 0 being the day of NT), the resultant embryos were transferred to dishes of mTALP supplemented with 3% calf serum and cultured at 38.6 °C. On day 6 or day 7, the embryos that had developed into normal blastocysts were counted.

Formation of chimeric embryos. Bovine ES-like cells (W3, P11) and primary bovine fetal fibroblasts were transfected, in the presence of a lipid-based transfection reagent (Effectene, Qiagen, Qiagen GmbH, Hilden, Germany), with DNA fragments derived from pCX-Neo-EGFP [36] and incubated for 2 days. G418-resistant cloned cells (concentration of G418, 400 μg/ml) were transferred to fresh culture plates for the generation of EGFP-expressing lines of transgenic cells. Recipient embryos for generation of chimeric embryos were produced by in vitro fertilization (IVF) as described previously [37]. Ten to fifteen EGFP-positive ES-like cells or EFGP-positive fibroblasts were injected into the perivitelline space of embryos at the 8- to 16-cell stage (day 3 after IVF) with the aid of a micromanipulator, as described elsewhere [38,39]. The expression of EGFP in embryos was examined by fluorescence microscopy.

Embryo transfer and analysis of parentage. On day 6 or day 7, cloned embryos that had developed to normal blastocysts were transferred non-surgically to recipient cows. Genomes of cloned calves, recipient cows, and nuclear-donor ES cells at the 19th passage were examined for the presence of specific microsatellite DNA sequences using 14 sets of bovine primers that had been provided by the Livestock Technology Association of Japan (Shirakawa, Japan) [40]. To study the parentage of bovine clones, polymerase chain reactions (PCRs) were performed essentially as described previously [16]. Then an aliquot of each reaction mixture, combined with a set of internal size markers (TS 369, Takara, Kyoto, Japan), was subjected to electrophoretic separation of fragments and analysis with an automated fluorescent DNA sequencer (ABI 377; Applied Biosystems, Tokyo, Japan). Data were then analyzed with Genescan version 3.1 and Genotyper version 2.1 (Applied Biosystems).

Reverse transcription-polymerase chain reaction (RT-PCR) and analysis by PCR of the expression of genes for marker proteins. Total RNA (10 μg) was isolated from ES cells with Trizol Reagent (Invitrogen BV, Groningen, The Netherlands) and then the reverse transcriptase SuperScript II (Invitrogen BV) was used to synthesize cDNA according to the protocol from the manufacturer. Genomic DNA was prepared by the standard method, as described elsewhere [16–18]. The reaction mixture for PCR contained cDNA (equivalent to 2.5 μg of total RNA) or genomic DNA (0.3 μg), specific primers (5 pmol each), 50 mM dNTPs, 1× buffer for KOD Dash, and 0.5 U KOD Dash (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 1 min. This cycle was repeated 40 times with final extension for 10 min at 72 °C. The following primers were used for PCR: primers specific for SRY DNA [A, sense 5′-GCCATTCTTCGAGGAGGCACAGA-3′ (nt 402 to nt 426) and antisense 5′-TATCGACCTCGTCGG AAGGC-3′ (nt 485 to nt 466), and B, sense 5′-CGACCCATGAATGCATTC ATGGTG-3′ (nt 265 to nt 288) and antisense 5′-TATCGA CCTCGTCGGAAGGC-3′ (nt 487 to nt 466)]; for Oct 4 mRNA [A, sense 5′-AAGAACATGTGTAAGCTGCGGCC-3′ (nt 1025 to nt 1047) and antisense 5′-TCTGGGCTCTCCCATGCATTCAAACT GA-3′ (nt 1522 to nt 1495), and B, sense 5′-AGTCCCAGGACATCA AAGCTCTGCAGAA-3′ (nt 846 to nt 873) and antisense 5′-TCTG GGCTCTCCCATGCATTCAAACTGA-3′ (nt 1522 to nt 1495)], for STAT-3 mRNA [sense, 5′-TCTGGCTAGACAATATCATCGACCT TG-3′ (nt 1908 to nt 1934) and antisense 5′-TACCTGAAGACCAA GTTCATCTG-3′ (nt 2356 to nt 2334)]; for nestin mRNA [A, sense 5′-TCTGATGGGTTTGCTGATGAGGAAGA-3′ (nt 4985 to nt 5011) and antisense 5′-CCCTCTGCCTGTTCTAGTCCATTCTCCAT-3′ (nt 5448 to nt 5420), and B, sense 5′-GTACTGCAGCCTGCATGCT GGGATCAG-3′ (nt 4945 to nt 4971) and antisense 5′-TCTCCTGTG CATCTAGGCCCAAGCCAG-3′ (nt 5629 to nt 5655)]; for GFAP mRNA [A, sense 5′-TGCGCGGCACGAACGAGTCCCT-3′ (nt 918 to nt 939) and antisense 5′-CGAGAAACCAGCCTGGACAAGTCC-3′ (nt 1213 to nt 1190), and B, sense 5′-CTCCAGGAGCAGCTGGCC CAGCA-3′ (nt 651 to nt 673) and antisense 5′-GCCCTGGACA TCGAGATCGCCACCTACAGGAA-3′ (nt 1121 to nt 1091)]; for β-tubulin III mRNA [sense 5′-CAGAGCAAGAACAGCAGCTA CTT-3′ (nt 999 to nt 1021) and antisense 5′-GTGAACTCCATCTC GTCCATGCCCTC-3′ (nt 1226 to nt 1201)]; and for glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA [sense 5′-GGGCTTG GCTTCGGTGACAACACCAAGGCGGC-3′ (nt 646 to nt 677); and antisense 5′-CGAGCAAAGGCCTCTGCCACCTTGCGGTT-3′ (nt 836 to nt 808)]. Amplifications yielded products of 84, 221, 498, 677, 447, 463, 711, 295, 472, 228, and 190 bp, respectively.

Results and discussion

Isolation of lines of bovine ES-like cells

Four (27%) ICMs from a total of 15 frozen embryos became attached to the feeder layer of murine STO cells and began to spread after six or seven days in culture. The ICMs grew as individual colonies composed of tightly packed compact cells. We removed and separated the growing colonies mechanically from the outer flattened cells and treated each colony with trypsin to dissociate the cells. After replating, these cells again formed compact colonies of small cells (Fig. 1A). Four lines of ES-like cells (W1, W2, W3, and H1) were established and each line maintained a diploid karyotype (W1 and W3, 58+XY; W2 and H1, 58+XX) up to the 15th–20th passage. The results coincided with the results of the sexing of bovine ES-like cells with Y chromosome-specific repeated DNA, namely, sex-determining region Y (SRY), by PCR (data not shown). The various lines of bovine ES-like cells were cryopreserved after various numbers of passages. These cells have been successfully thawed, without loss of developmental ability, after preservation for more than three years. Thus, it appears that these lines of ES-like cells remain genotypically and phenotypically stable for many generations.

Leukemia inhibitory factor (LIF) inhibits the differentiation and supports the proliferation of undifferentiated murine stem cells [28]. However, we observed the frequent spontaneous differentiation of bovine ES-like cells into cells that resembled either fibroblasts or epithelial cells in the absence of a feeder layer, when the medium was supplemented with human LIF (data not shown). When bovine ES-like cells were cultured in the absence of a feeder layer, they multiplied rapidly for several days and formed aggregates that increased in size with time in culture. From 7 to 14 days after the start of culture, a distinct ring or outer layer of large endoderm cells formed around the cell aggregates. These cystic, EBs (Fig. 1B) were composed of two layers of cells with heterogeneous cellular particles within the cavity. Endoderm-like cells were located on the external surface of the cystic EBs and ectoderm-like cells were located on the inner surface. The cells of bovine EBs were able to differentiate into epithelial, fibroblastic and neuron-like cells upon reattachment to the surface of a dish (data not shown). Thus, maintenance of bovine ES-like cells in an undifferentiated state seemed to be strictly dependent on the presence of a feeder layer. In the presence of LIF, the bovine ES-like cells differentiated in vitro when they were cultured without a feeder layer. This characteristic was similar to that of human ES cells [4].

Expression of marker proteins by bovine ES-like cells

We examined the bovine ES-like cells for markers of cells in an undifferentiated state. We detected alkaline phosphatase (AP) activity in the bovine ES-like cells, as shown in Fig. 1C. This activity has been found in pluripotent stem cells of the mouse [28,30] and human [4,41] and in stem-like cells of the sheep [11], pig [15], and horse [42], but it has not previously been detected in bovine stem-like cells [23]. We are unable, at present, to explain the discrepancy, in terms of alkaline phosphatase activity, between our ES-like cells and the stem-like cells described previously. Furthermore, our bovine ES-like cells expressed a cell-surface marker protein that is characteristic of undifferentiated mouse ES cells, namely, SSEA-1 (Fig. 1D) [31]. Fluorescence-activated cell sorting (FACS) analysis indicated that more than 35% of bovine ES-like cells were strongly positive for immunoreactivity with antibodies against mouse

SSEA-1 (Fig. 1F) but none of the bovine ES-like cells expressed SSEA-3 or SSEA-4 (data not shown). These results were different from those reported by Mitalipova et al. [43]. We do not know the reason for this discrepancy. Bovine ES-like cells differed from lines of human stem cells, which do not express SSEA-1 but express

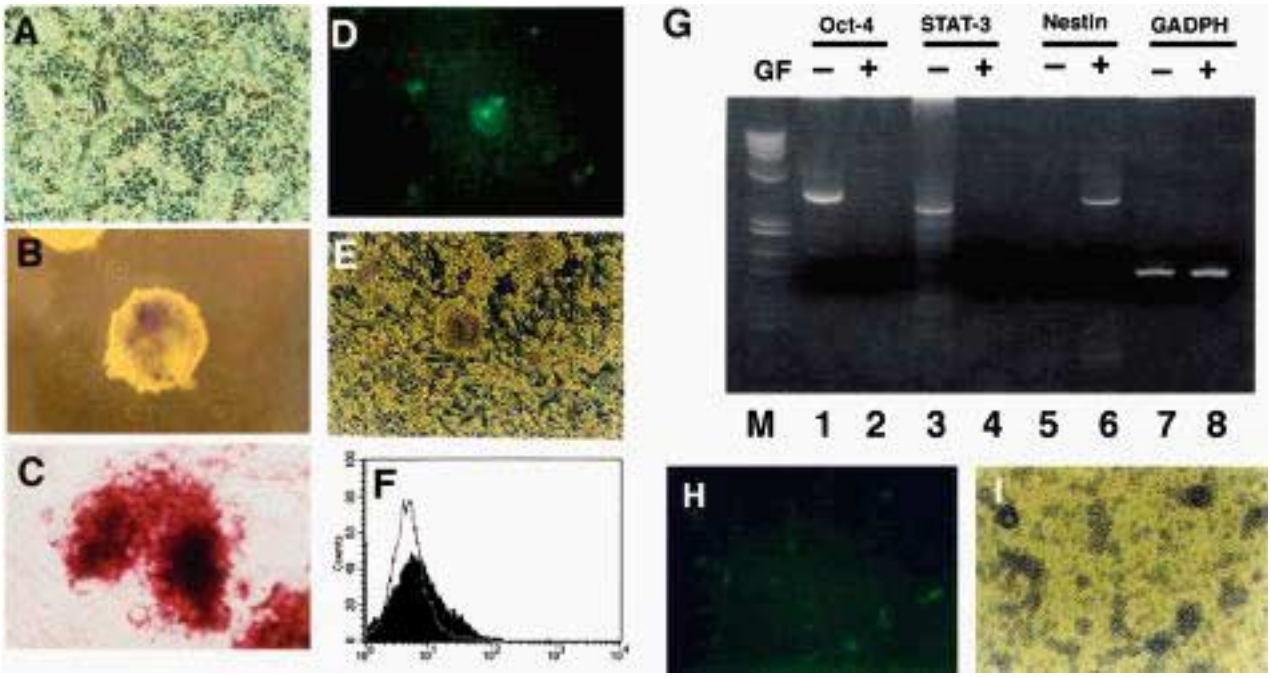

Fig. 1. Characterization of bovine ES-like cells. (A) Phase-contrast image of confluent colonies of ES-like cells after culture for six days in the absence of a feeder layer but in the presence of human LIF. Differentiated epithelial-like cells are visible among other cells in the culture. (B) Embryoid bodies (W3 cells), two weeks after the start of suspension culture. (C) Expression of alkaline phosphatase in W3 cells. (D,E) Immunofluorescence staining of bovine ES-like cells with SSEA-1-specific antibodies. Aggregates of round cells (E) were immunostained (D). (F) Flow-cytometric profiles of bovine ES-like cells after immunostaining with antibodies against SSEA-1 (indicated in black) and with rabbit IgM (indicated by a line). (G) Analysis of the expression of genes for Oct 4, STAT-3, nestin, and GAPDH by RT-PCR. GF indicates the inclusion of a mixture of growth factors (bFGF, EGF, and PDGF) in the medium for seven days. (H,I) Immunofluorescence staining of bovine ES-like cells with antibodies specific for STAT-3. Colonies of round cells (I) were immunostained (H). Magnification: B and C, 200×; A, D, E, H and I, 100×. M: Molecular size markers (*Hae*III digest of ϕ X174).

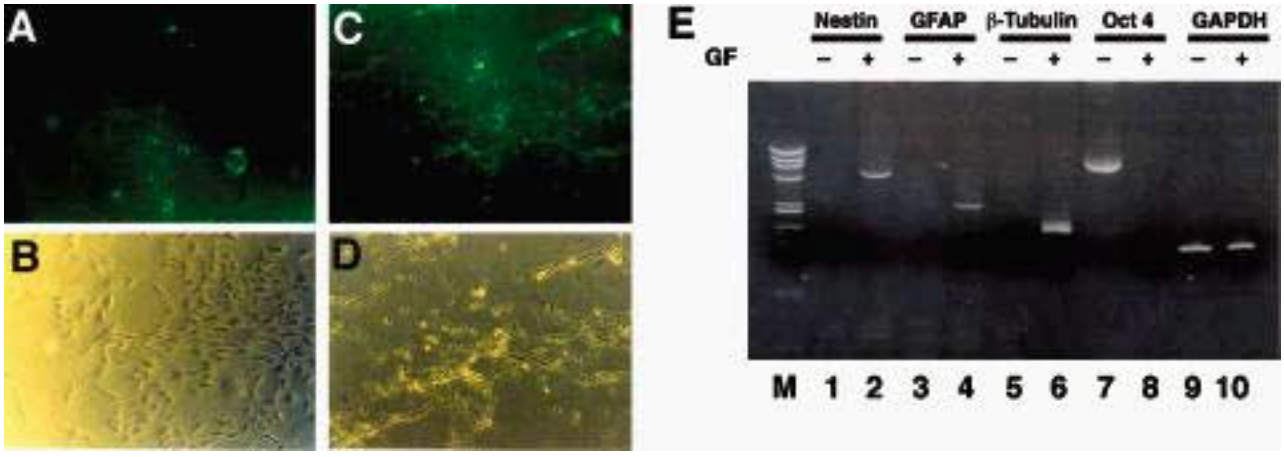

Fig. 2. Morphological differentiation and expression of markers by neural precursors derived from ES-like cells. Immunostaining with antibodies against GFAP (A,B) and β-tubulin III (C,D). Magnification: 100×. (E) The expression of marker genes in neural precursors derived from bovine ES-like cells. RNA from ES-like cells (lanes 1, 3, 5, 7, and 9) and from ES-like cells treated with a mixture (GF) of bFGF, EGF, and PDGF for seven days (lanes 2, 4, 8, and 10) and 10 days (lane 6) was analyzed by RT-PCR as described in Materials and methods. M, Molecular size markers (*Hae*III digest of ϕ X174).

either SSEA-3 or SSEA-4 [4]. We used the ES-like W3 cells that had been sorted using antibodies against SSEA-1 for subsequent studies of cloned calves and for the culture of chimeric embryos in vitro.

We examined the expression of the transcription factor Oct 4 in undifferentiated and differentiated bovine ES-like cells, after growth both in the absence of a feeder layer and in medium supplemented with LIF, by reverse transcription and the polymerase chain reaction (RT-PCR). We detected a fragment of amplified Oct 4 DNA of 498 bp in our analysis of RNA from undifferentiated bovine ES-like cells but not from differentiated ES-like cells. Thus, Oct 4 was expressed in our bovine ES-like cells, as it is in human ES cells [30] and in equine ES-like cells (Fig. 1G) [42]. Bovine ES-like cells also expressed STAT-3 (Figs. 1G and H), which is a transcription factor that plays a central role in the maintenance of the pluripotency of murine ES cells [29]. The expression of STAT-3 by undifferentiated ES-like cells was confirmed by RT-PCR, which yielded an amplified DNA fragment of 447 bp that was derived from STAT-3 mRNA. Differentiated ES-like cells did not express STAT-3 mRNA (Fig. 1G), as confirmed by immunostaining with antibodies against mouse STAT-3 (Figs. 1H and I). By contrast, we detected an amplified DNA fragment derived from nestin mRNA in the analysis of differentiated ES-like cells but not of undifferentiated ES-like cells (Fig. 1G). Both Oct 4 and STAT-3 are essential for the maintenance of the stem cell phenotype in mice, in conjunction with activation of LIF signaling pathways [29,30]. However, as described above, we were able to induce the differentiation of bovine ES-like cells in the presence of LIF, in the absence of feeder layer. Moreover, we also detected the formation of EBs from bovine ES-like cells in the absence of both a feeder layer and LIF. The signals that maintain bovine ES-like cells in an undifferentiated state remain to be identified and they will be the focus of our future investigations.

Differentiation of neural precursor cells from bovine ES-like cells in vitro

We cultured four lines of bovine ES-like cells in defined medium to examine their potential for differentiation to neural precursor cells. After 7–10 days in culture in the presence of basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF), ES-like cells developed into bipolar fibrous cells that were immunopositive for nestin (data not shown). Upon removal of growth factors, ES-like cells seemed to differentiate still further and, within 7–14 days in vitro, their morphology resembled that of glial cells, astrocytes or neurons. Many of these cells (more than 65%) were immunopositive for the astrocyte-specific marker glial fibrillary acidic protein (GFAP; Figs. 2A and B). Other cells (approximately 35%) were immunopositive for the neuron-specific marker β-tubulin III and had an obviously neural morphology (Figs. 2C and D). These experiments did not, of course, rule out the possibility that the antibodies that we used had failed to crossreact with the respective antigens in bovine ES-like cells. Therefore, we next performed RT-PCR to examine the expression of genes for nestin, GFAP, and β-tubulin III. We detected the expression of each of these genes in the apparently neurally differentiated ES-like cells (Fig. 2E). We detected an amplified DNA fragment of 677 bp that was derived from Oct 4 mRNA in our analysis of undifferentiated ES-like cells but not of differentiated ES-like cells (Fig. 2E). These results coincided with previous observations that ES-derived nestin-positive populations of animal cells are dominated by neural stem cells and differentiate into midbrain-type neurons [39,44]. The cocktail of growth factors (bFGF, EGF, and PDGF) that we used in the present study seemed to be suitable for the efficient conversion of bovine ES-like cells to neural cells. Since ES cells can be maintained and can proliferate in an undifferentiated state, we postulated that it should be possible to manipulate the genome of ES-like cells to produce cells that express transgenes in vitro and, thus, to generate gene-targeted animals.

Detection of the expression of EGFP in chimeric embryos in vitro

After 5 days in culture, more than 40% of the chimeric embryos (17 out of 41; prepared from embryos generated by IVF) included contributions by EGFP-expressing stem cells (Table 1). Fluorescence due to EGFP was detectable both in the ICM and in trophectodermal cells of the injected blastocysts (Figs. 3A and B). By contrast, only a few transfected fibroblasts were present in the morulae and very few cells were incorporated into the ICM area of developing blastocysts (Figs. 3C and D). Our data showed clearly that the transfected ES-like cells were able to participate in the

Table 1
Rate of production of bovine chimeric embryos[a]

Cells[b]	Recipients[c]	BCs[d] (%)	EGFP[e] (%)	DG[f] (%)
Fibroblasts	12	6 (50)	0	6 (50)
ES-like cells	41	27 (66)	17 (42)	14 (34)

[a] Bovine chimeric embryos were cultured for 4 days after injection of transfected ES-like cells.
[b] Cells that were the source of the transgene.
[c] Number of host embryos at 8- to 16-cell stage.
[d] Number of blastocysts (BCs).
[e] Number of blastocysts with a signal due to EGFP in ICM and trophectodermal cells. Expression of EGFP in BCs was monitored 7 days after IVF.
[f] Number of degenerated embryos.

formation of chimeras after injection into 8- to 16-cell bovine embryos, while fibroblasts did not have this ability. Although the detection of EGFP in the ICM and trophectoderm after injection of transfected ES-like W3 cells into host blastocysts does not demonstrate conclusively the pluripotency of ES-like cells, our results suggest that ES-like cells have a different capability for aggregation from that of somatic fibroblasts.

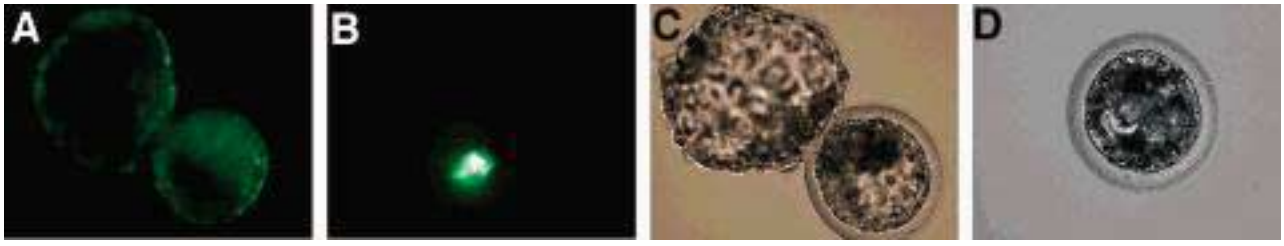

Fig. 3. Expression of EGFP in transgenic chimeric embryos. (A,C) Expression of EGFP in transgenic blastocysts four days after injection of G418-selected ES-like W3 cells that had been transfected with pCX-Neo-EGFP. Distinct expression of EGFP was apparent in both the ICM and trophectodermal cells (A). (B,D) Expression of EGFP in blastocysts four days after injection of fibroblasts transfected with pCX-Neo-EGFP. The fluorescent signal remained and did not expand at the site of injection of cells (B). Images were obtained by fluorescence microscopy with excitation at 480 nm. Magnification: (A, B) 100×; (C, D) 100×.

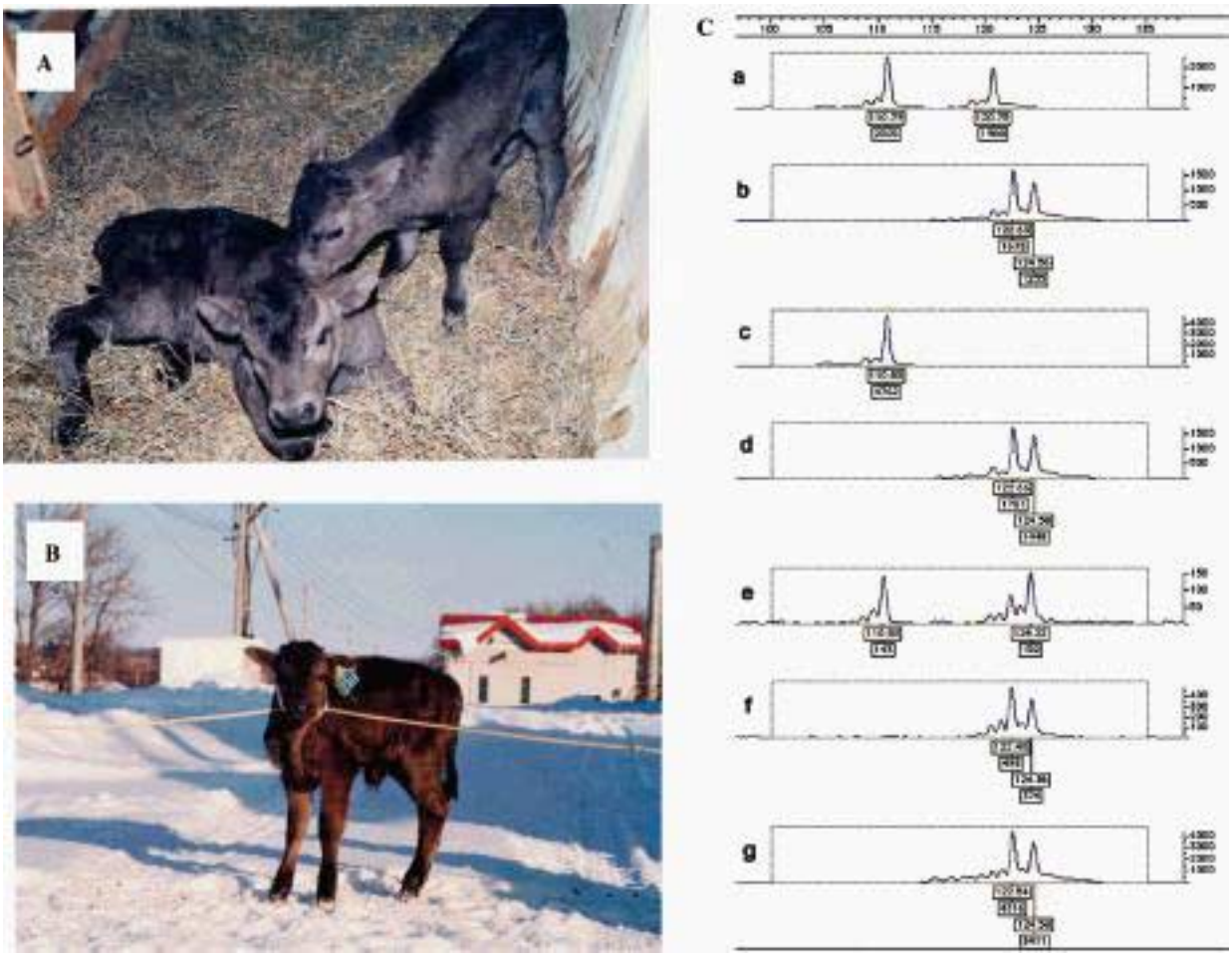

Fig. 4. Photographs of calves obtained after nuclear transfer. The calves were photographed two days (A; calves 1 and 2) and four weeks (B; calf 2) after birth. (C) Fingerprinting of DNA from cloned calves, recipient cows, and donor ES-like W3 cells. Electrophoretograms show amplified fragments of DNA (after PCR with primer set BMS 1997, obtained from the Livestock Technology Association of Japan [40]) derived from leukocytes from recipient cows 1, 2, and 3 (panels a, c, and e) and cloned calves 1, 2, and 3 (panels b, d, and f) and from donor ES-like W3 cells (panel g). Upper and right-side scales indicate the sizes of DNAs (bp) and the intensities of DNA fragments, respectively. Numbers in boxes indicate the sizes of DNAs (upper) and the intensities of DNA fragments (lower).

Production of cloned calves from bovine ES-like cells

A summary of the generation of cloned calves from bovine ES-like W3 cells which had been purified by FACS with SSEA-1-specific antibodies is shown in Table 2. The rates of the successful fusion of oocytes and the cleavage of embryos were 78% and 68%, respectively. These rates were unaffected by the number of passages of W3 cells and were similar to reported values obtained for bovine embryos that had been reconstructed from somatic cells [17,18,32]. However, the rate of formation of blastocysts from W3 cells was only 4%. This lower frequency can be attributed to the inadequate coordination of the cell cycle in W3 cells and the activation of oocytes. The optimal timing for activation in nuclear transfer (NT) experiments in bovines differs between somatic cells and blastomeres [32,39]. In the present study, we used a method for activation that has been applied to somatic cells. We activated recipient oocytes and donor cells after cell fusion since formation of blastocysts never occurred when W3 cells were fused with activated cytoplasts (unpublished data). Fusion prior to activation is associated with the exposure of the transferred nuclei of ES-like cells to cytoplasmic factors within oocytes and might, thus, facilitate genetic reprogramming. The complexity of the many steps involved in NT, which include oocyte maturation, removal of chromosomes, activation of oocytes, synchronization of cells, and culture of embryos, might explain the low rate of blastocyst formation. Among these possibilities, the synchronization of ES-like cells at a specific stage seems likely to be critical for blastocyst formation. The low rate of development to blastocysts might have been caused by the incompatibility of the cell cycle of the donor cell nucleus with that of the cytoplasts. Kasinathan et al. [45] showed that the stage of the cell cycle of the donor is important for NT, in particular during late fetal development, and that the activity of

Table 2
Development in vitro, pregnancies and births after transfer to oocytes of nuclei from ES-like cells

Passage number	No. of nuclei transferred	No. of fusions (%)[a]	No. of cells Cleaved (%)[b]	No. of BCs (%)[b]	No. of BCs transferred	No. of BCs surviving as fetuses at indicated day of gestation (%)			Pregnancystatus
						42 d	90 d	180 d	
14	54	42 (78)	31 (74)	3 (7)	3	2 (67)	0	0	Aborted (90 days)
15	45	36 (80)	24 (67)	1 (3)	1	1 (100)	1 (100)	1 (100)	Live offspring[c]
18	123	95 (77)	63 (66)	3 (3)	3	2 (67)	2 (67)	2 (67)	Live offspring[d]
Total	222	173 (78)	118 (68)	7 (4)	7	5 (71)	3 (43)	3 (43)	

[a] As a percentage of nuclei transferred.
[b] As a percentage of fusions. BCs: Blastocysts.
[c] One male calf, weighing 42 kg, was born alive by normal parturition.
[d] Two male calves, weighing 61 and 34 kg, respectively, were born alive by cesarean section.

Table 3
Analysis of parentage[a]

Marker	Dye[b]	Temp[c]	Genotype of recipient 1	Genotype of calf 1	Genotype of recipient 2	Genotype of calf 2	Genotype of recipient 3	Genotype of calf 3	Genotype of cell line
DIK024	FAM	55.0	231/247	239	231/239	239	239	239	239
BMS1987	FAM	55.0	111/121	123/125	111	123/125	111/124	123/125	123/125
DIK069	FAM	55.0	184/188	182/191	187/189	182/191	178/182	182/191	182/191
BM6026	FAM	60.0	158/173	167/169	167	167/169	167/173	167/169	167/169
LISTS093	TET	60.0	194/204	198	194	198	186/193	198	198
DIK096	TET	55.0	251	251/257	251/257	251/257	244/256	251/257	251/257
BMS607	HEX	55.0	157/159	148/163	155	148/163	163	148/163	148/163
DIK093	TET	60.0	237	230/237	230	230/237	ND	ND	230/237
BM121	TET	55.0	124/136	123/125	122	123/125	119/136	123/125	123/125
DIK067	HEX	55.0	198	199	197	199	ND	ND	199
DIK039	TET	55.0	197/204	185/190	190/196	185/190	185/189	185/190	185/190
INRA130	TET	60.0	113/115	113	111	113	102/114	113	113
BM4505	HEX	60.0	239/244	230/244	234/239	230/244	231/233	230/244	230/244
BM7246	HEX	55.0	143	118	137	118	138	ND[d]	118

[a] For each microsatellite marker, the genotype was determined in terms of the sizes (bp) of fragments [40]. Almost all satellite markers provided strong support for the genetic identity between the donor ES-like cells and cloned calves.
[b] Fluorescent dye.
[c] Annealing temperature for PCR (°C).
[d] ND, Not determined.

cells at the G1 phase of the cell cycle supports higher rates of development than that of cells at the G0 phase. This phenomenon resembles observations with murine ES cells: development to blastocysts occurred more frequently when ES donor cells were at the G1 phase [46]. Thus, it seems likely that the rate of blasotcyst formation and late fetal development might be dependent on synchronization of the donor ES cells with the activated oocytes.

In previous studies, between 2% and 3% of blastocysts developed to term after transfer to recipients [8]. Epigenetic changes, accumulated during the culture of ES cells, have been the primary cause of anomalies in clones derived from murine ES cells [8]. However, we did not observe such changes in our bovine ES-like W3 cells and 43% of pregnancies reached term (Table 2; $P > 0.05$). Seven embryos derived from ES-like cells were transferred to seven recipient cows and five fetuses (71%) were detected by ultrasonography between 42 and 47 days after transfer (Table 2; $P > 0.05$). The frequency of pregnancies (71%) and that of term pregnancies for blastocysts derived from ES-like cells were higher than those of the early pregnancies (approximately 55%) and maintained pregnancies (10–20%) that have been reported after NT with bovine somatic cells [13,18,19,25]. The calves that we obtained were first to be generated, to our knowledge, from ES-like cells in a straightforward NT protocol. Our success demonstrated the potential superiority of our method as compared with NT using nuclei from bovine somatic cells and other approaches using ES-like cells [13,47,48].

Two viable fetuses were delivered by cesarean section on day 277. One calf (no. 1) died two days after birth as a result of abdominal bleeding while the other (no. 2) remains alive. It appears normal and is 585 days old at the time of writing. One additional cloned calf (no. 3) was born by normal parturition on day 283 (Table 3; Fig. 4). Our success seems to have been greater than that of other groups, who have reported low rates of survival to term, with only 10–15% of blastocysts developing to term [13,19]. Investigations of abnormalities found in clones should contribute to decreases in the high incidence of birth defects, stillbirths, and postnatal deaths associated with in vitro generated counterpart (IVP) and NT-derived embryos [49]. The higher rate of pregnancies that we achieved with bovine ES-like cells was similar to pregnancy rates with mouse ES cells. ES cells yield consistently higher rates of live births, as compared with rates from somatic cells. A possible explanation for the higher rate of success of pregnancies in cattle might be the unique characteristics of the enucleated oocytes of Japanese black beef cows, as suggested by Yanagimachi [50]. It is very likely that ES-like cells and enucleated oocytes of Japanese black beef cows might provide some clues that will help us correct or overcome the epigenetic errors in cloned animals [50]. Further studies are needed if we are fully to understand the properties of bovine ES cells that are required for successful reprogramming, full-term development, and healthy postnatal development.

We examined the cloned calves and recipient cows to confirm that the nuclei of donor ES-like cells were the source of the calves' genomes. We used 14 bovine DNA microsatellite markers (MS) to confirm the genetic identity of the cloned calves to the ES-like cells used for NT. Our analysis confirmed that the donor cell lines had been the source of the genetic materials used to produce the newborn calves (Table 3).

In conclusion, the results presented here indicate that pluripotent bovine ES-like cells can be stored without loss of the ability to differentiate into neural precursor cells. In contrast to fibroblasts, as donor cells, our bovine ES-like cells expressed stem cell markers, such as STAT-3, SSEA-1, AP, and Oct 4. Moreover, we were able to use ES-like cells as the source of nuclei for NT to produce cloned cattle with a higher frequency of pregnancies to term than that achieved with somatic cells [13,18,19,25,43,47,48]. The present study suggests that EGFP-expressing bovine ES-like cells can be incorporated into both the ICM and trophectodermal cells in developing blastocysts. These findings should facilitate targeted genetic manipulations, such as "gene knockout" by homologous recombination, and should allow production of cloned cattle in a single step after modification, as appropriate, of the genome.

Note

The sequences of cDNAs and genomic DNA encoding bovine Oct 4, STAT-3, nestin, GFAP, β-tubulin III, GAPDH, and Sry have been submitted to GenBank and have been assigned Accession Nos.: AB085759, AB085753, AB085756, AB085757, AB085755, AB085760, and AB085754, respectively.

Acknowledgments

The authors thank Drs. H. Niemann, K. Abe, A. Ogura, H. Okano, K. Itakura, R. Chiu, R. Yanagimachi, S. Nishikawa, and M. Okabe for many helpful discussion about the original manuscript and for various reagents. They also thank Ms. H. Yamashina, S. Watanabe, M. Hirose, K. Takahashi, T. Ishihara, C. Ishihara, K. Kurosaka, and S. Yamaoka for their skilled technical assistance. This work was supported by grants from the Itoh Memorial Foundation and from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (to K.K.Y.).

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