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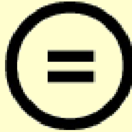
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Master's Thesis

Establishment of Bovine Embryonic Stem Cell Lines from *In Vitro* Produced Embryos

Department of Biology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

Eun Ji Noh

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Establishment of Bovine Embryonic Stem Cell Lines from *In Vitro* Produced Embryos

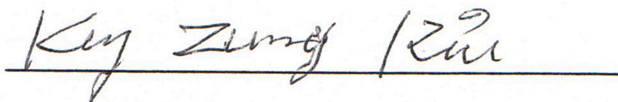
Eun Ji Noh

(Supervised by Professor Se Pill Park)

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science

February, 2012

This thesis has been examined and approved



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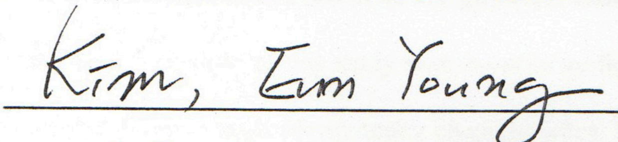

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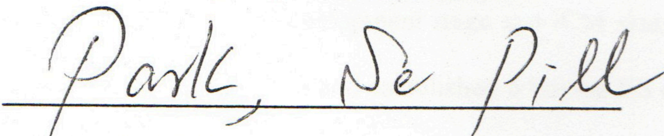

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Establishment of Bovine Embryonic Stem Cell Lines from *In Vitro* Produced Embryos

Eun Ji Noh

Department of Biotechnology

Graduate School of Jeju National University

(Directed by Professor Se Pill Park)

ABSTRACT

Bovine embryonic stem (ES) cells are powerful tool for the agricultural and biomedical applications, and the aim of this study was to optimize the generation condition of bovine ES cells and to determine their plutipotency chatacteristics. From total 126 blastocysts produced *in vitro*, classified by their development stage and ICM size, 21 primary bovine ES cell-like colonies were formed (16.7%) and established 6 bovine ES cell lines (28.6%, 6/21; Hatched x 4, Hatching x 1 and expanded x 1) maintained for more than 12 passage (> 90 days). Mechanically isolated over medium sized ICM of hatched blastocyst has high potency to form into ES cell growth on the 10 ul mouse embryonic feeder cell drop covered with oil. These cells exhibit typical ES cell morphology and express pluripotency markers through the immunocytochemistry, RT-PCR and real-time RT-PCR, including Oct4, stage-specific embryonic antigens 1, Nanog, Tumor rejection antigen-1-81, Rex1 and alkaline phosphatase.

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Also, RT-PCR results demonstrated expression of genes representative of the three embryonic germ layers ectodermal (Pax6 and DBH), mesodermal (CMP and Enolase) endodermal (a-FP and albumin) markers in spontaneous differentiation. In addition, bovine ES cell lines were directed differentiated easily into neuronal (Map2 and Tuj1) and atrial cells (GFAP). Bovine ES cell lines had normal karyotype having a chromosome count of 58+XY (JNU-ibES-05). This result demonstrated that bovine ES cell line can be efficiently established using healthy hatched blastocysts produced *in vitro* in the our designed optimized bovine ES cell culture condition.

Keywords: bovine, ES cell, pluripotency, differentiation, feeder cell drop

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INTRODUCTION

Embryonic stem (ES) cells were derived from inner cell mass (ICM) cells of preimplantation embryos (1, 2). These cells are pluripotent and should retain their long-term proliferative potential in an undifferentiated state (3). Also, ES cells can differentiate into derivatives of all three embryonic germ layers when they were transferred to an *in vitro* differentiation culture condition or into an *in vivo* environment (4, 5, 6). Since the first ES cell line was isolated from mouse in 1981 (7), stable ES cell lines have been established in many species including of human (8). ES cells derived from farm animals could provide a powerful tool for studies on early embryonic development (9) gene targeting (10), cloning (11) and regenerative medicine (12). Because of their potential use for targeted gene manipulation, availability of ES cells in livestock species could have enormous agricultural, pharmaceutical and biomedical applications.

However, ES cell lines from domestic animals has been relatively less studied than that of murine ES cells due to several shortcoming such as longer generation time and difficulties in culture (13, 14). The derivation and maintenance of ES cell line from bovine blastocysts is perhaps the most difficult and challenging among all mammals. To date, suitable conditions for preventing spontaneous differentiation of bovine ES cells were not determined, such as ICM isolation or subculture technique, feeder cell type, ES cell culture medium and growth factors. Also, there were conflicting or variable results for the pluripotent marker expression in bovine ES cell (15, 16, 17), unlikely in mouse (18) or human (8). In addition, there have been several reports on derivation of bovine ES-like cells, but no stable bovine ES cell lines are available yet (19, 20).

In this study, using *in vitro* produced bovine embryos classified by development stage and ICM size, we designed an minimized feeder cell drop and culture medium to enhance the generation rate and to extend the maintenance term of bovine ES cells, and we attempted to determine the typical bovine ES cell morphology, ES cell marker expression and their

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pluripotency characteristics in spontaneous or directed differentiation condition *in vitro*.

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Materials and Methods

All chemicals and reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) unless otherwise stated.

1. Production of bovine *in vitro* matured (IVM), *in vitro* fertilized (IVF) and *in vitro* cultured (IVC) embryos

Bovine ovaries were obtained from a slaughterhouse and transported to the laboratory within 2 hours in 0.9% saline at 35℃. Cumulus oocyte complexes (COCs) were aspirated from visible follicles 2 to 6 mm in size using an 18 gauge needle attached to a 10 ml disposable syringe. The medium used for COCs collection was HEPES-buffered Tyrode′s medium (TL-HEPES). Sets of ten COCs were *in vitro* matured in TCM199 (Gibco, Grand Island, NY, USA) supplemented with 10% FBS, 0.2 mM sodium pyruvate, 1 µg/ml follicle-stimulating hormone (Folltropin™, Bioniche Animal Health, Belleville, On, Canada), 1 µg/ml estradiol-17β, 1 mM EGF under mineral oil at 38.8℃ in 5% CO_2, 5% O_2, and 90% N_2 incubator. After incubation for 22-24 h in the IVM medium, the COCs were used for IVF using highly motile sperm recovered from frozen-thawed bull semen, which had been separated on a discontinuous Percoll (GE Healthcare, Bio-Sciences AB, Uppsala) column. Matured COCs were transferred to 44 µl drops (10 COCs per drop) of fertilization medium containing TL- stock supplemented with 6 mg/ml fatty acid free-bovine serum albumin (FAF-BSA), 0.2mM pyruvate, and 25 µg/ml gentamycin. Sperm were counted by hemacytometer and diluted at a concentration of 1 x 10^6 cells/ml. 2 µl each of sperm, heparin (2 µg/ml) and PHE containing 18.2 M penicillamine, 9.1 M hypotaurine and 1.8 M epinephrine were added in a 44 µl IVF drop. Fertilization was assessed by the cleavage rate

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(≥ 2-cell) after 44 ± 2 h of incubation. For IVC, cleaved embryos were incubated in CR1aa medium containing 3 mg/ml of FAF-BSA until embryonic day 4 and then they were transferred into CR1aa medium containing 10% FBS until embryonic day 9.

2. Preparation of mouse embryonic fibroblast (MEF) feeder cells

For MEF cell preparation, uteri isolated from 13.5 day pregnant CDF1 mice were washed with phosphate-buffered saline (PBS). The head and visceral tissues were removed from isolated embryos, and the bodies were washed with fresh PBS, minced using a pair of scissors, transferred into a solution of 0.25 mM trypsin/1 mM EDTA, and incubated at 37°C in a shaking incubator for 30 min. After trypsinization, the cells were dissociated by pipetting up and down, and were then collected by centrifugation. The pelleted cells were washed twice in culture medium and the resuspended cells were cultured on 100-mm dishes ($\geq 1 \times 10^6$) in a 5% CO_2 incubator at 37°C in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) containing 10% defined-Fetal Bovine Serum (FBS, Hyclone, Logan, UT, USA), 50 units/ml of penicillin and 50 μg/ml of streptomycin. The 3rd to 5th passages cultured and 10 mg/ml Mitomycin-C treated MEF cells were used for the feeder cells, they were seeded at a density of 1.6×10^5cells/ml in a 2-well dish with several numbers of 20 μl drops , and covered using mineral oil.

3. Derivation and culture of IVF-bovine embryonic stem cell (ibES)

The inner cell mass (ICM) of bovine IVM/IVF/IVC blastocysts was isolated mechanically using 28 G needles attached to 0.5 ml syringes (BD, Sparks, MD, USA). Isolated ICMs were classified by embryo development stage and size (small, medium and large) as parameter for the experiment of ibES cell establishment. An ICM cells were placed

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onto each MEF feeder cell drop, cultured in DMEM (Gibco, Grand Island, NY, USA) containing of 20% fetal bovine serum (FBS, Hyclone, South Logan, Utah, USA), 0.55 mM ß-mercaptoethanol (Gibco), 1% nonessential amino acids (Sigma), 4 ng/ml basic fibroblast growth factor (bFGF, Koma Biotech. Inc.), 1 ng/ml Insulin-like growth factor (IGF, Bio-Research Product Inc.), 10 ng/ml epidermal growth factor (EGF) and 500 ng/ml Human noggin (PeproTech Inc.). The medium was changed every 2 days. After 5-8 days, the ICM-derived outgrowths were separated by mechanical dissociation and replated on mitomycin C-treated MEF feeder cell drop To passage the putative ES cells, individual colonies with a uniform and undifferentiated morphology (a small cytoplasmic/nuclear volume ratio, form tight, rounded and multi layer clumps) were selected using a micropipette, mechanically dissociated into 2-3 clumps and then they were placed on MEF feeder cell drop. The established ibES cells were passaged every 7-10 days after replating.

4. Alkaline phosphatase (AP) staining and immunocytochemistry

To detect AP activity, ibES cell colonies were fixed in 4% paraformaldehyde (PFA) in 0.1 M PBS for 2 min and then treated with Fast Red Violet/Naphthol AS-BI phosphate mixed solution for 15 min (Millipore, Billerica, MA, USA). For immunocytochemistry, ibES cell colony or *in vitro* differentiated cells were fixed in 4% PFA for 20 min at 4°C and then permeabilized with 0.2% Triton X-100 for 10 min at RT. To block any non-specific binding, the cells were incubated with 10% normal goat serum (VECTOR, Burlingame, CA, USA) for 1 h, and then incubated with primary antibody at 4°C overnight. The primary antibodies used were anti-stage-specific embryonic antigen 1 (SSEA-1, Santa Cruz, CA, USA, 1:20), anti-SSEA-4 (Santa Cruz, 1:20), anti-tumor rejection antigen (TRA) 1-81 (Santa Cruz, 1:20), anti-Nanog (Santa Cruz, 1:20), anti-Oct-4 (Santa Cruz, 1:250), anti-Map2 (Chemicon, 1:1,000), anti-Tuj1 (Chemicon, 1:1,000) and anti-GFAP (Chemicon, 1:1,000). The secondary

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antibodies used were Alexa Fluor 488 conjugated goat anti- mouse IgG or IgM (for SSEA-1, SSEA-4, TRA1-81 and Oct-4), Alexa Fluor 594 conjugated goat anti-rabbit IgG (for Nanog), goat anti-mouse tetramethylrhodamine isothiocyanate (TRITC, Jackson Laboratories, Bar Harbor, Maine, USA, for Tuj1) or goat anti-rabbit TRITC (for Map2 and GFAP) at a dilution of 1:200. Nuclei were stained with 5 μg/ml of 4'-6-diamidino-2-phenylindole (DAPI). Cells were observed and photographed with an inverted Olympus IX-71 (Japan) microscope equipped for epifluorescence.

5. mRNA Extraction and RT-PCR

For the RT-PCR analysis of bovine ES cell marker expression or *in vitro* differentiation ability, we examined the six ibES cell lines or selected two ibES cell lines, respectively. Total RNA was extracted from several colonies using Dynabeads mRNA DIRECT Micro Kit (Dynabeads mRNA purification kit; Dynal, Oslo, Normay) according to the manufacture's instruction. Briefly, ES colonies and differentiation cells were suspended in 100 μl lysis/binding buffer (100 mM Tris-HCl, pH 7.5, 500 mM LiCl, 10 mM EDTA with pH 8.0, 0.1% LiDS, 5 mM DTT) and vortexed at room temperature for 5 min to lysis cells. A 50 μl aliquot of an oligo (dT) 25 magnetic bead suspension was added to samples, and the samples were incubated at room temperature for 5 min. The hybridized mRNA and oligo (dT) beads were washed twice with washing buffer A (10 mM Tris-HCl, pH 7.5, 0.15 M LiCl, 1 mM EDTA, 1% LiDS) and once with washing buffer B (10 mM Tris-HCl, pH 7.5, 0.15 M LiCl, 1 mM EDTA). mRNA samples were eluted from beads in 15 μl double-distilled DEPC treated water. cDNA was synthesized from about 1 μg total RNA using SuperScript II reverse transcriptase (Invitrogen). cDNA samples were subjected to PCR amplification with selective primers described in Table 1. The PCR products were size fractionated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. Final analysis was

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obtained in image analyzer (Biorad).

6. Real time RT-PCR Quantification

After messenger RNA was extracted, cDNA was synthesized using an oligo (dT) 12–18 primer and Superscript reverse transcriptase (Invitrogen). Real-time RT-PCR (Bio-Rad, Chromo4) was performed using the primer sets shown in Table 1. In all experiments, β-actin mRNA served as an internal standard. The threshold cycle (Ct) value represents the cycle number at which the sample fluorescence rises statistically above background noise. To monitor the reactions, we followed the protocol provided with the DyNAmo SYBR green qPCR kit which contains a modified Tbr DNA polymerase, SYBR Green, optimized PCR buffer, 5 mM $MgCl_2$ and a dNTP mix that includes dUTP (Finnzyme Oy, Espoo, Finland). The PCR protocol used a denaturation step at 95°C for 15 min, followed by amplification and quantification cycles that were repeated 40 times at 94°C for 30 sec, at 50 or 56°C for 1 min, and at 72°C for 1 min using a single fluorescence measurement and a melting curve program of 65–95°C with a heating rate of 0.2°C/sec and continuous fluorescence measurement. Samples were then cooled to 12°C. Fluorescence of SYBR Green was measured after the extension step during the PCR reactions. PCR products were analyzed by generating a melting curve. Since the melting curve of a product is sequence-specific, it can be used to distinguish non-specific from specific PCR products. To do this it is necessary to determine the crossing points (CP) for each transcript. The CP is defined as the point at which fluorescence rises appreciably above background noise. Gene expression was quantified by the 2-ddCt method [9].

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Table1. Primer sequences to detect bovine ES and differentiation markers

Name	Gene Bank accession No.	Primer sequences	Length (bp)	Specificity
bOct4	NM-174580	CTCTTTGGAAAGGTGTTCAG GTCTCTGCCTTGCATATCTC	155 bp	ES
bSSEA1	NM-174736	TACTACCGCCAGCTGAGTCAATAC ATATAATCCAGGTGCTGCGAGTT	149 bp	
bALP	NM-173987	ATGACAAAGCTTATATGGCACTGAC CAAAAGAGAAGACATGAGAGTGGTC	132 bp	
bRex1	NM-001192308	GTCCTTACAGAGTTTAAAGAAGCCC CTTCCTGGTTAGAAGAGCAAAACTT	131 bp	
bPax6	NM-001040645	CAGGTATGGTTTTCTAATCGAAGGG ACTGCTGCTGATGGGAATATGACTA	108 bp	Ectoderm
bDβH	NM-180995	TGAGATCGTGTCCAGGTTGGAA TTTGCCCCCACTGATGTTCA	100 bp	
bCMP	NM-001143866	AATTCCTTCACTGTGTCCAGCG TTGGCTTCTTGGCAGCATC	110 bp	Mesoderm
bEnolase	NM-174049	AAGTTTGGTGCGAACGCCAT ATTGAAAGCCGGAACTGGGA	141 bp	
bα-FP	NM-001034262	AAATTTGGACCCCGGACCTT GCACGTTTCCTTTGCAGCAT	139 bp	Endoderm
bAlbumin	NM-180992	AACACAAGCCCAAGGCAACA TTTGGACCCTCCACAGCAAA	121 bp	
bβ-actin	NM-173979	GTCATCACCATCGGCSSTGA GGATGTCGACGTCACACTTC	111bp	House Keeping

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7. *In vitro* differentiation of ibES cells

For spontaneous differentiation, ibES colonies recovered at a clump state were suspension cultured in feeder free conditions in ES cell culture medium without bFGF and Noggin for 2 weeks. Embryoid bodies (EB) were then plated on 0.1% gelatin-coated culture dishes and cultured in the same medium for an additional 7 days. During differentiation, the medium was replaced every 2 days. For the directed neuronal cell differentiation, day 7 EBs were dissociated with TrypLE (Invitrogen) solution and single cells or clumps were cultured onto 0.1% gelatin-coated, 2-well plates in N2 medium, consisting of DMEM/F12 supplemented with 0.01% BSA, 20 nM progesterone, 100 μM putrescine, 25 μg/ml insulin, 50 μg/ml transferrin, and 30 nM sodium selenite. Immunocytochemistry was performed after an additional 8 days of culture.

8. Karyotyping of ibES cells

After treatment in 5% Colcemid (Gibco) for 4 h, ibES cells were dissociated using TrypLE solution, hydrated in hypotonic KCl for 15 min, washed and fixed in methanol and acetic acid (BDH, Bristol, UK) in a ratio of 3:1. The fixed cells were dropped onto clean slides at RT. The slides were stained with a freshly made staining solution containing 3 ml of Leishman stain in 17 ml Gurrs buffer (Invitrogen) for 8 min. A coverslip was mounted on the slides with Histomount (National Diagnostics, Atlanta, GA, USA) and slides were viewed using a light microscope under oil immersion optics (Nicon C1) at 1000X magnification.

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Results

1. Establishment of IVF-bovine embryonic stem (ibES) cell line

Through the bovine IVM/IVF/IVC system, at day9 after IVF, as shown in Table 2, embryos at different stages were obtained [expanded blastocysts, 28.3% (45/159); hatching blastocyst, 32.1% (51/159); hatched blastocyst, 39.6% (63/159)]. To establish bovine ES cells, from total 126 blastocysts that including of hatched (n=43), hatching (n=41), or fully expanded (n=42) produced at day 9 post IVF (Fig.1A), ICM cells were recovered mechanically. The isolated ICM cells were classified by size into small (40-90um, n=15), medium (90-140um, n=56) and large (140-190um, n=55) (Fig.1B-D). Primary ES-like colonies were appeared at 4 to7 days after plating of ICM cells on mitomycin-C treated MEF feeder cell drop (Fig.1E). The bovine ES cells had a high nucleus cytoplasm ratio, and formed domed colony that were densely packed with obvious border within 10 days after plating, sub-cultured on new MEF feeder cell drop after dissecting into several numbers of small clumps mechanically using needles (Fig.1F). As shown in Table 3, the rates of bovine ES cell-like colonies derived from expanded, hatching and hatched blastocysts were 11.9% (5/42), 14.6% (6/41) and 23.3% (10/43), respectively. Also, the rates of ES cell-like colonies derived from ICM size small, medium and large were 13.3% (2/15), 19.6% (11/56) and 14.5% (8/55), respectively. Totally, 21 bovine ES cell-like colonies were obtained. From these ES cell-like colonies, 6 bovine ES cell lines were established (total, 6/21, 28.6%; expanded blastocyst, 1/5, 20.0%; hatching blastocyst, 1/6, 16.7%; hatched blastocyst, 4/10, 40.0%). These bovine ES cell lines were maintained for more than 12 passages (>90 days) and named JNU-ibES-01 ~06.

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Table 2. Development of Bovine IVM/IVF/IVC embryos

No. Oocytes	No. (%) of cleaved at day 2	No. (%) of developed at day9	No. (%) of embryos develop to		
			Expanded blastocyst	Hatching blastocyst	Hatched blastocyst
518	413 (79.7)	159 (38.5)	45 (28.3)	51 (32.1)	63 (39.6)

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Table 3. Establishment of bovine embryonic stem cell lines from *in vitro* fertilized embryos

Development stage	No.(%) of bovine ES cell-like colony				No. (%) of established bovine ES cell line	Name
	ICM Size			SUM		
	Small	Medium	Large			
Expanded blastocyst	1/7 (14.2)	2/18 (11.1)	2/17 (11.7)	5/42 (11.9)	1/5 (20.0)	JNU-ibES-01
Hatching blastocyst	1/6 (16.7)	2/14 (14.3)	3/21 (14.3)	6/41 (14.6)	1/6 (16.7)	JNU-ibES-02
Hatched blastocyst	0/2 (0.0)	7/24 (29.2)	3/17 (17.6)	10/43 (23.3)	4/10 (40.0)	JNU-ibES-03~06
SUM	2/15 (13.3)	11/56 (19.6)	8/55 (14.5)	21/126 (16.7)	6/21(28.6)	

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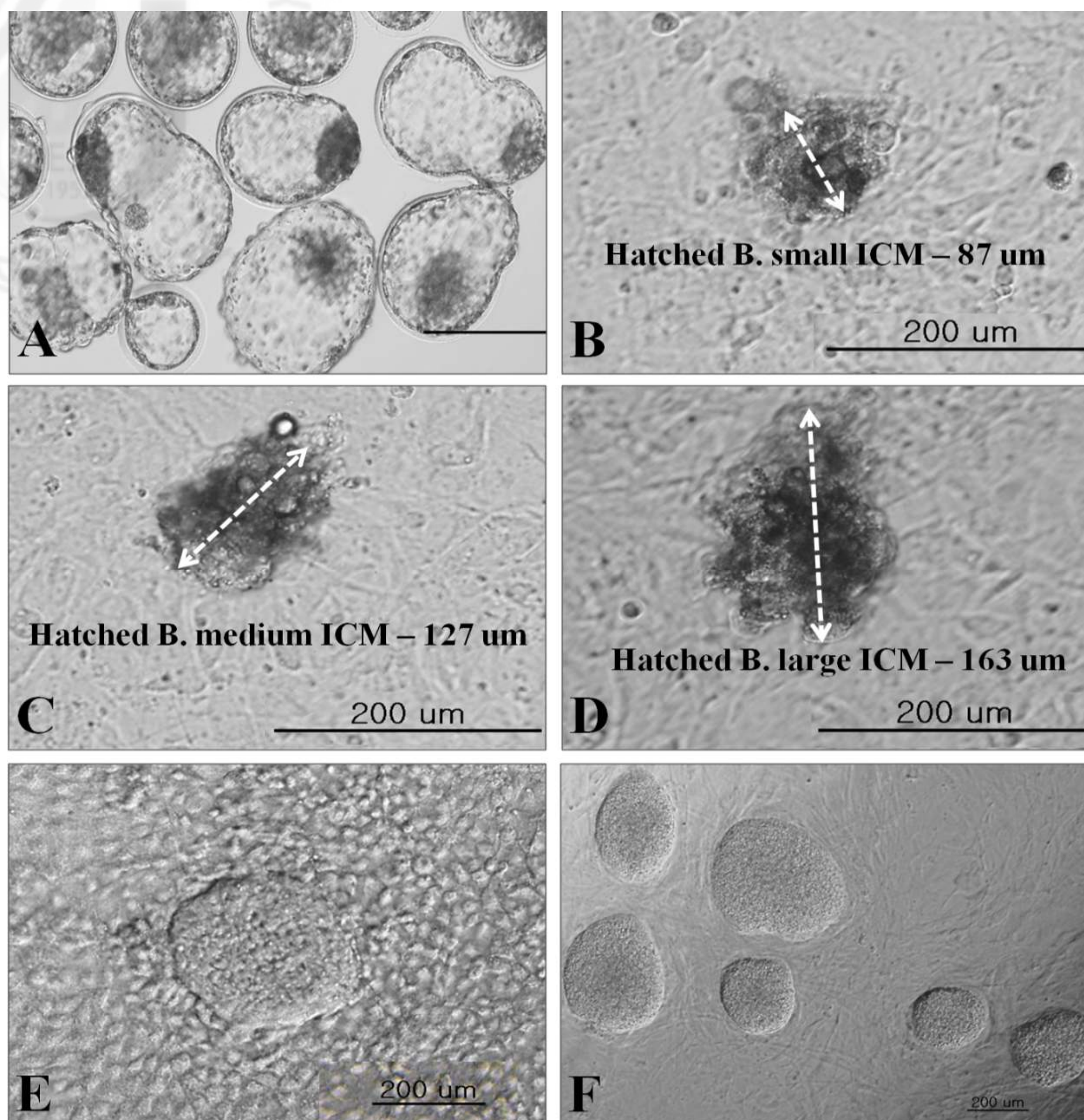

Fig 1. Morphology of a series course of establishment of bovine embryonic stem cell lines from IVF embryos. A. *In vitro* produced bovine day 9 hatching or hatched blastocysts. ICM cells were classified into small (87 μm, B), medium (127 μm, C) and large sized (163 μm, D). E. Bovine ES like-cell colony (a small cytoplasmic/nuclear volume ratio, form tight, rounded and multi layer clumps) was formed on MEF feeder cell layer at 5 day after plating. F. Multiplied bovine ES JNU-ibES-05 cell colonies.

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2. Characterization of bovine ES cell lines

In characterization of bovine ES cell colonies, we confirmed the pluripotency markers such as alkaline phosphatase (Fig 2A), Oct4 (Fig. 2B), SSEA-1 (Fig. 2C), Nanog (Fig. 2D) and TRA-1-81 (Fig. 2F) were all highly expressed, while SSEA-4 (Fig. 2E) expression was not. Also, RT-PCR (Fig. 3A) analysis revealed that the four stem cell marker genes (Oct4, SSEA 1, ALP and Rex 1) were expressed in all JNU-ibES cell lines, although the expression levels were variable among ibES cell lines. However, in the relative mRNA expression levels of four stem cell marker genes (Oct4, SSEA 1, ALP and Rex 1) among JNU-ibES cell lines, as shown in Fig. 3B, there were differences ($P < 0.05$). In Oct4 and ALP expression, the levels of JNU-ibES-02~06 were significantly higher than that of JNU-ibES-01, in Rex1 expression, the levels of JNU-ibES-03~06 were higher than those of JNU-ibES-01~02, and finally in SSEA 1 expression, the levels of JNU-ibES-03, -05 and -06 were higher than those of JNU-ibES-01, -02 and -04.

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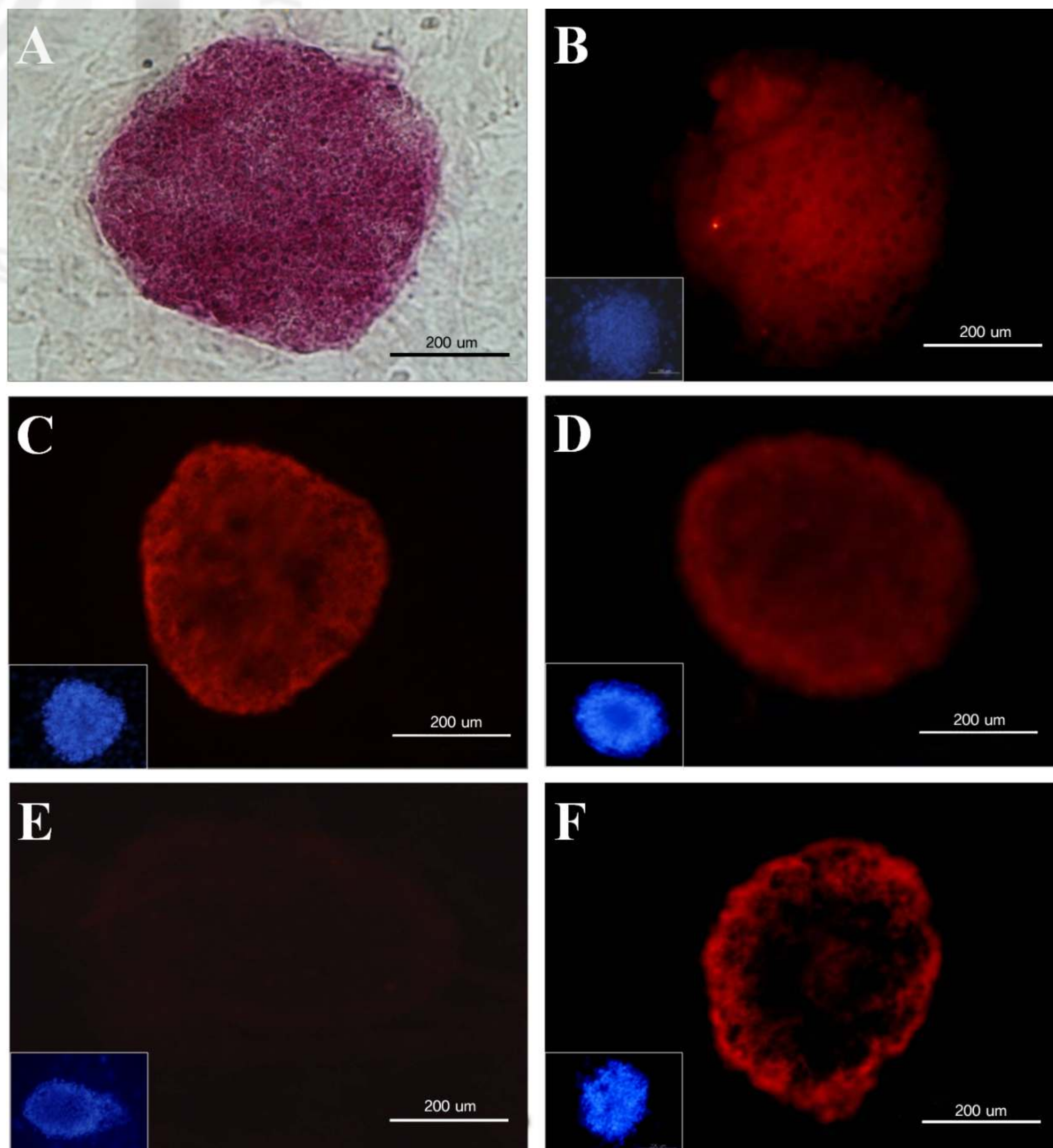

Fig 2. Characterization of pluripotency marker expression in bovine ES cell colony. Alkaline phosphatase (A). Oct4 (B), stage-specific embryonic antigen (SSEA)-1 (C), Nanog (D) and tumor rejection antigen (TRA)-1-81 (F) expressions were all high in JNU-ibES-06, while SSEA-4 (E) expression was not.

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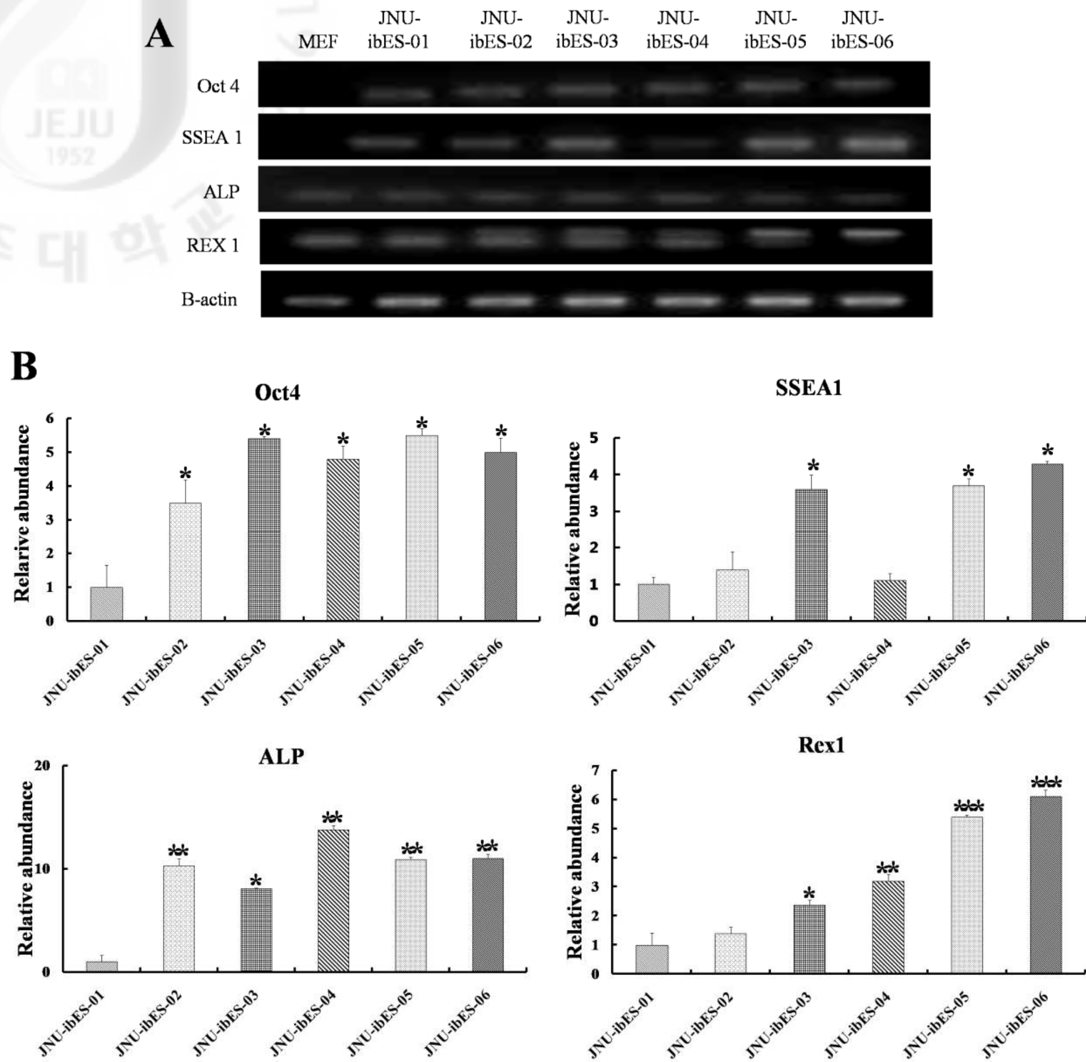

Fig 3. Gene expression analysis of bovine pluripotent ES cell markers (Oct 4, SSEA 1, ALP and Rex1) of six JNU-ibES cell lines by RT-PCR (A) and real-time RT-PCR quantification (B).

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3. *In vitro* differentiation of the bovine ES cell lines

To determine the pluripotent characteristics, spontaneous or directed *in vitro* differentiation of bovine ES cells were examined. In spontaneous differentiation, embryoid bodies (EBs, Fig. 4A) cultured in 0.1% gelatin coated culture plate for 2 weeks, were differentiated into various types of cells. (Fig.4B). In RT-PCR analysis of spontaneous differentiated JNU-ibES-05 and JNU-ibES-06 cells, the examined three germ layered marker gene (ectoderm, Pax6 and DBH; mesoderm, CMP and Enolase; endoderm, αFP and Albumin) expressions were confirmed, while those were not expressed in the same undifferentiated ibES cells (Fig. 4C). Also, when the directed differentiation ability of bovine ES cells (JNU-ibES-05) into neuronal cells (Fig. 5A) were examined, microtubule associated protein (Map) 2 (Fig. 5B), glial fibrillary acidic protein (GFAP) (Fig. 5C) and neuron-specific class Ⅲ beta-tubulin (Tuj1) (Fig. 5D) expressions were detected by immunocytochemistry.

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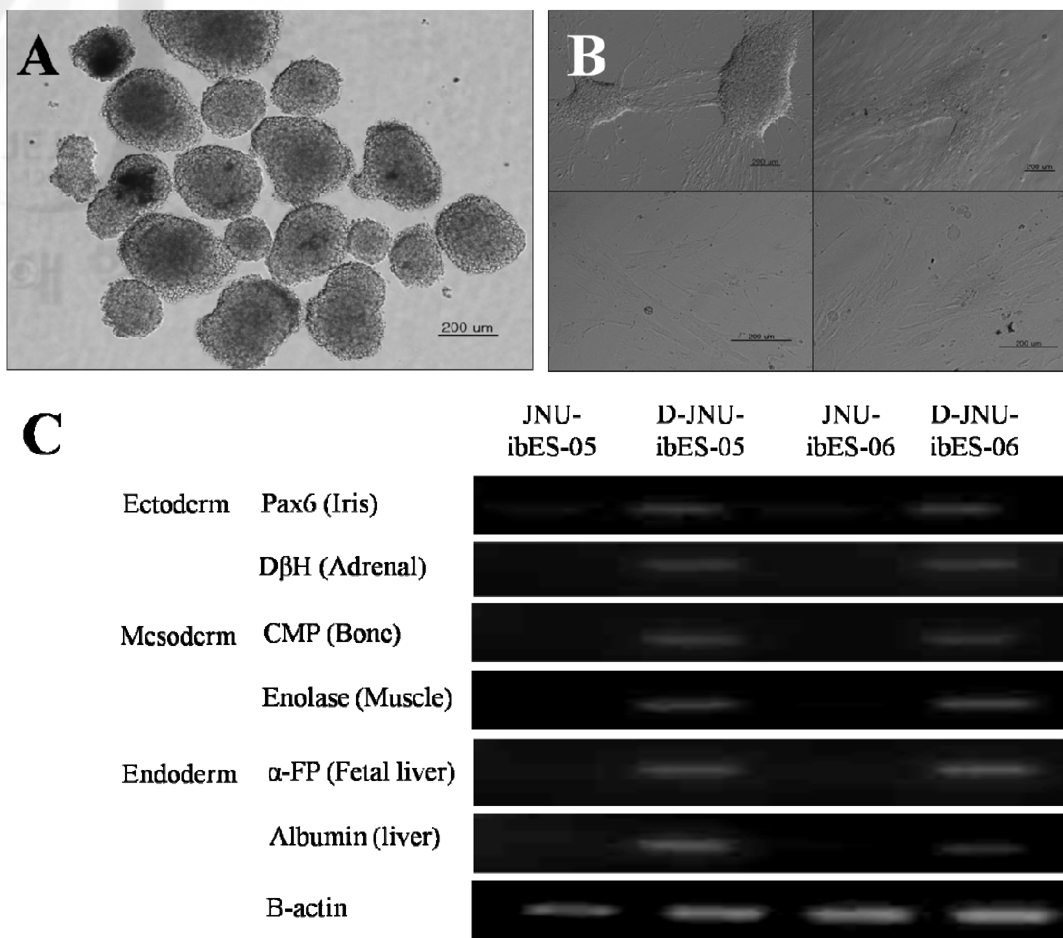

Fig 4. Spontaneous differentiation of JNU-ibES cell (A) Suspension cultured bovine ES cell in differentiation medium for 10 days. (B) In spontaneous differentiation condition, various types of cells were appeared. (C) RT-PCR analysis of three germ layered marker expression in spontaneously differentiated JNU-ibES-05 and JNU-ibES-06 cell line. Ectodermal markers (Pax6, DBH). Endodermal markers (Albumin, alpha-FP). Mesodermal markers (Enolase, CMP). House Keeping gene, β-actin, was used to normalize the samples for the cDNA content.

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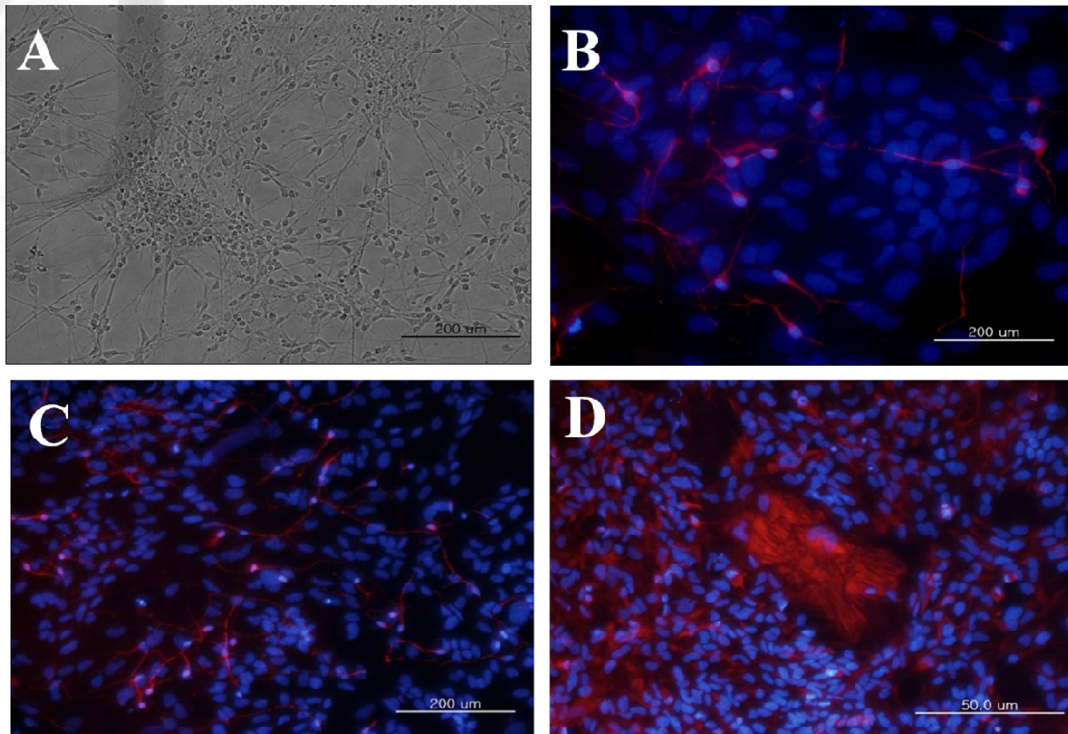

Fig 5 Directed differentiation of JNU-ibES-05 cell into neuronal cell. In directed neuronal cell differentiation of bovine ES cells (A), immunocytochemical results presented microtubule associated protein 2 (B), neuron-specific class Ⅲ beta-tubulin (C) and glial fibrillary acidic protein (D).

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4. Karyotyping of bovine ES cell lines

JNU-iBES-05 cells were confirmed as a normal karyotype having a choromosome count of 58+ XY (Fig. 6).

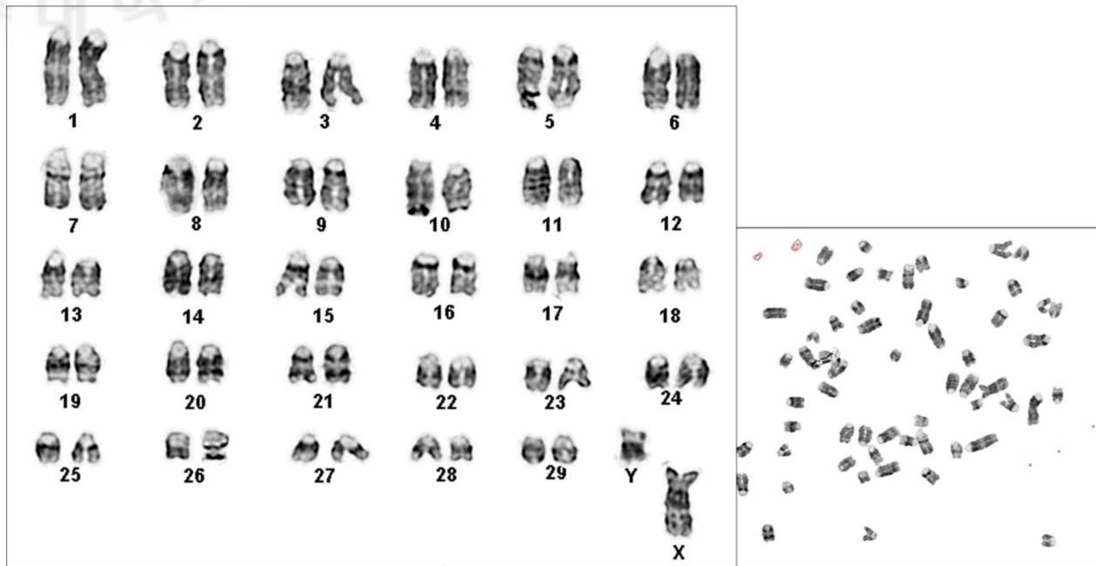

Fig 6. Karyotyping analysis of JNU-ibES-05 cells showed a normal XY line (58+ XY).

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DISCUSSION

This study demonstrates that bovine ES cell line can be efficiently generated in our developed new ES cell culture condition, including of minimized MEF feeder cell drop and oil cover, using *in vitro* produced day 9 hatched blastocyst, and these ibES cells presented typical ES cell morphology, ES cell marker expression and *in vitro* pluripotency characteristics. Our established 6 JNU (Jeju National University)-ibES cells showed a clear form of colonies, distinct boundaries, and were maintained with more proliferative potential (> 3 months) in 10 ul MEF cell drop culture than in other large scale culture (21, 22, 23). This is the first trial of minimized micro-drop culture method for the generation of bovine ES cell.

Until now, there was no report on the unlimited expanding bovine ES cell generation. The major reason of the limited proliferative potential of bovine ES cell may be the absence of optimized feeder cell environment. In general, as feeder cells, MEF or STO cells were commonly used. MEF and STO cells were known have particular function for the establishment and maintenance of mammalian pluripotent cells, including mouse ES cells, human embryonic germ cells, and hES cells (24, 25, 26). Those feeder cells presented the expression of various cytokine (LIF, IL, TNF-a), growth factors (IGFBP, FGF, VEGF, HGF) and ECM (laminin, collagen and fibronectin) (27). Most normal cells require adhesion to the ECM for survival, migration and growth, and the high concentration of ECM materials are very helpful for the ES cell growth of colony type. However, those two mouse feeder cells are incomplete as the bovine ES cell culture environment because of their different body temperature (37.0 °C vs. 39.0 °C). High temp affects negatively on the bioactivity of feeder cell. Therefore, the development of the same species bovine feeder cell is needed for the stable culture of ibES cells. Until now, many results of bovine ES cell generation were obtained from the use of mouse feeder cell, and the efficiency was very low (0.5 ~ 8.0%) (28,

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29, 22).

In this study, we reduced the negative effect of MEF feeder cell in the bovine ICM cell growth using minimized MEF feeder cell drop, and thus the generation rates of bovine ES cell-like colony and ES cell line could increase into 16.7% and 28.6%, respectively. Especially, hatched blastocysts were very efficient to generate bovine ES cell (23.3% and 40.0%, respectively). Among established ES cell lines, four has been made from day 9 hatched blastocysts and their pluripotent markers expression was also high compared to other development stage embryos. This result might be originated from the more ICM cell numbers of fast developed hatched blastocysts than those of slow developed expanded or hatching blastocysts. And the result was the same as the reports of Verma et al (30) and Anand et al (31). Also, we employed the mechanical method for the ICM cell isolation and subculture, different from ineffective enzyme dissolution using Tryple or protease (31, 22). Thus, in this study, the efficient generation of ibES cell was resulted from the use of healthy ICM cells and minimized ES cell culture environment. Six JNU-ibES cell lines has been maintained for more than 90 days, subcultured more than 12 passages and successfully frozen.

Unlike mES or hES cells (7, 8), the results of stemness ES cell marker expression of ibES cells were inconsistent or conflicted among the researchers (28, 32, 16). Mitalipova et al (28) and Satio et al (32) reported that both expressions of the stage-specific embryonic antigens (SSEA)1 and SSEA4 in bovine ES cells, while other reported no expression of SSEA1 (16). Through the replicated immunocytochemistry, we confirmed the expression of alkaline phosphate, Oct4, SSEA1, Nanog and tumor rejection antigen (TRA)-1-81 but no expression of SSEA4 in ibES cells. However, the expression of SSEA1 was proved by the results of RT-PCR and semi-quantitative RT-PCR. In addition, each ibES cell lines presented the different relative gene expression. The activity of Oct4 is essential for the identity of the pluripotential founder cell population in the mammalian embryo (33). Oct4 or

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ALP expressions were high in 5 ibES cell lines (JNU-ibES02~06) except of JNU-ibES01 (P<.05). SSEA1 expression was high in three ibES cell lines (JNU-ibES03, 05 and 06) (P<.05). Finally, Rex1, which encodes an acidic zinc finger protein, is expressed high level in four ibES cell lines (JNU-ibES03~06) (P<.05).

However, stem cells have the ability to choose between prolonged self-renewal and differentiation. This fate choice is highly regulated by intrinsic signals and external microenvironment (34). In study on differentiation of ES cells, Schuldiner et al., (35) reported that none of the growth factors directs differentiation exclusively to one cell type. In this study, EBs derived from ibES cell were differentiated into three germs layered multi-typed cells such as ectodermal cell [iris (Pax6) and adrenal (DbH)], mesodermal cell [bone (CMP) and muscle (Enolase)] and ectodermal cell [fetal liver (a-FP) and liver (albumin)]. And bovine ES cells can be directed differentiated into neuronal (Map2 and Tuj1) and glial (GFAP) cells in neuron cell culture medium. Moreover, the results of karyotyping of JNU-ibES-05 were normal. In this study, JNU-ibES cells presented clear pluripotency characteristics *in vitro*. These all data will be important source for the improvement of ibES cell culture condition.

The generation of robust bovine pluripotent stem cell lines may allow for the complex genetic manipulations, including gene knock-in and knock out technology, applicable to industry. Further studies are being focused on redefining and improving protocols for the efficient ibES cell generation through the receptor assay or homologous feeder cell development to produce prolonged proliferated ibES cell line or their enriched populations of specific differentiated cells.

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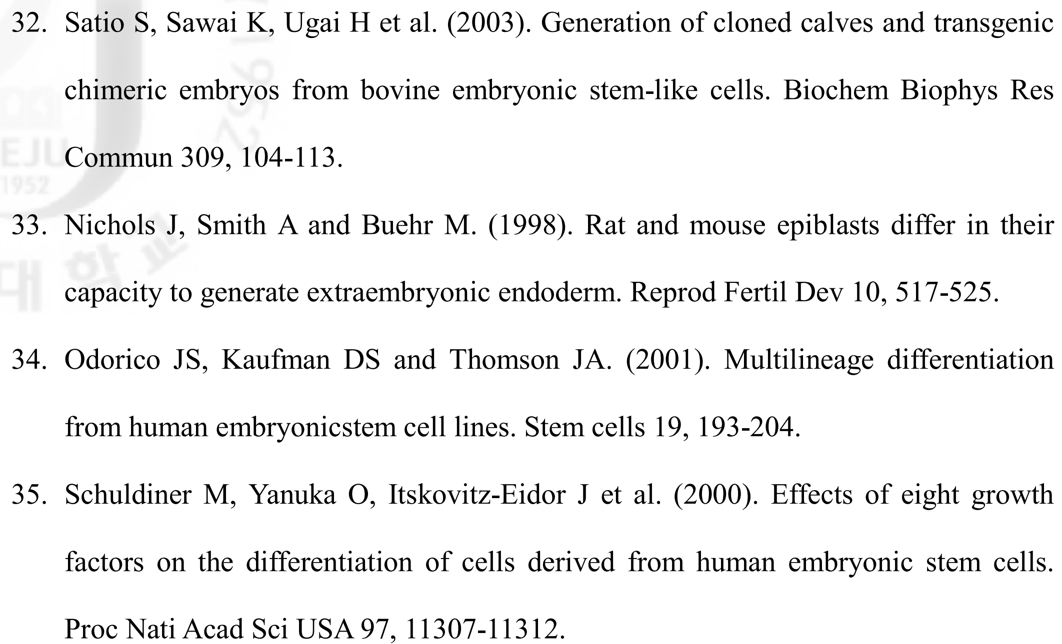

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체외 생산된 배아로부터 소 배아줄기세포주 확립

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(국문초록)

소 배아줄기세포는 농업과 의료 분야 응용에 강력한 도구가 될 수 있다. 본 연구는 소 배아줄기세포 확립 조건을 적정화하고 확립된 줄기세포의 pluripotency 특성을 조사하기 위해 실시하였다. 체외에서 생산된 총 126개의 배반포기배를 발달단계와 내부세포괴 (ICM) 크기로 구분하여 공시하였을 때, 초기 소 줄기세포-유사 콜로니 형성은 21개 (16.7%) 였으며, 이로부터 12 계대 이상 (>90 일) 유지된 6개의 소 배아줄기세포주가 확립되었다 (28.6%, 6/21; 부화배반포기배 x 4, 부화진행배반포기배 x 1, 팽창배반포기배 x 1). 중간 크기 이상의 내부세포괴를 가진 부화배반포기배로부터 ICM을 기계적으로 제거한 뒤, 10ul 드롭으로 제작된 생쥐배아지

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지세포 (MEF) 위에 옮겨 오일로 피복하여 배양하였을 때, 소 배아줄기세포 성장이 용이하다는 것을 확인하였다.

이들 세포는 전형적인 배아줄기세포의 형태를 보이며 면역세포화학염색법, RT-PCR과 real-time RT-PCR로 조사하였을 때, Oct4, stage-specific embryonic antigens 1, Nanog, Tumor rejection antigen-1-81, Rex1 and alkaline phosphatase 와 같은 pluripotency 마커가 발현되는 것을 확인하였다. 또한, 자발적 분화에서 조사된 삼배엽 분화 특성 마커, 즉 외배엽 (Pax6 and DBH), 중배엽 (CMP and Enolase) 내배엽(a-FP and albumin) 모두 발현되는 것을 공히 확인하였다. 더불어 신경유도분화에서도 신경세포 마커 (Map2 and Tuj1) 와 신경보조세포 마커 (GFAP) 를 확인할 수 있었다. 핵형분석 결과, JNU-ibES-05세포주는 60개의 역색체 (58+XY) 로 정상임을 확인하였다.

따라서, 소 배아줄기세포주는 체외에서 생산된 건강한 부화배반포기배를 이용하여 본 연구팀이 개발한 적정화된 배아줄기세포 배양환경하에서 효율적으로 확립될 수 있음을 나타낸다.

주요단어: bovine, ES cell, pluripotency, differentiation, feeder cell drop

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짧지 않은 석사 과정을 마무리하며 지난 시간들을 돌이켜보니 많은 아쉬움과 후회가 남습니다. 석사 과정 기간 동안에 배울 것이 아직도 많지만 이렇게 결실을 맺게 되어 감개무량합니다. 우선 바쁘신 와중에도 부족한 저를 이뻐해주시고, 이끌어주시고 다독여주시며 논문이 완성되기까지 많은 지도와 격려를 해주신 박세필 교수님께 진심으로 감사드립니다. 제주도와 서울 연구소를 일주일에 한번은 꼭 가셔야 될 뿐만 아니라 연구소에서 진행되는 많은 일을 하셔야 함에 무엇보다 교수님의 건강이 걱정됩니다. 또한 본 논문에 많은 관심을 기울여주시고 따끔한 충고와 애정 어린 조언을 해주시며 저를 이끌어주시던 김은영 소장님께도 감사드립니다. 더불어 귀중한 시간을 내주시어 이 논문을 심사해주신 류기중 교수님께 고개 숙여 감사드립니다. 그리고 실험실 선배로서, 인생의 선배로서 많은 가르침을 주었던 박효영 박사님과, 같이 석사과정을 밟으며 기쁠 때 같이 웃고 힘들 때 서로 위로해주던 박민지 선생님과 알게 모르게 도움도 많이 주고 여기까지 올 수 있게 힘을 많이 준 나의 언니 노은형 선생님에게도 감사의 말씀 전합니다. 또 정말 착하고 학사과정에 있음에도 열심히 배우고 있는 후배 좌익전, 손여진, 김경훈, 이귀염에게도 감사의 말을 전합니다. 조그마한 투정에도 늘 격려해주고 힘내라고 응원해준 친구 양윤실, 최현, 홍혜진, 한구슬, 유한수, 고민희, 김민아에게도 감사의 말을 전합니다.

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누구보다 따뜻한 사랑과 관심을 주시며 늘 저의 걱정뿐이신 제 인생의 든든한 후원자 아버지, 어머니 진심으로 감사하고 사랑합니다. 또 타지에서 생활하는 두 누나들을 대신해 든든한 모습으로 아들역할을 잘 해내고 있는 막내 현진이에게도 감사의 말을 전합니다. 뒤에서 묵묵히 저를 위해 늘 기도해주시는 할머니에게도 큰 감사의 말씀 전합니다. 일일이 언급하지 못하지만, 제 주위에 너무나도 감사한 분들이 많다는 것을 새삼 느낍니다. 끝이 아니라 이제 시작이라는 마음으로 사회의 한 구성원으로서 저의 역할에 충실하고자 합니다. 사랑하는 가족에게, 넓게는 동시대의 사람들에게 행복을 선사하도록 하고 끝까지 유종의 미를 거둘 수 있도록 더욱 노력하겠습니다.

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