치의과학박사 학위논문

Generation and analyses of bovine embryo-derived pluripotent stem cells

소 배아유래 전분화능 줄기세포의 생산 및 분석

2017년 8월

서울대학교 대학원

치의과학과 종양 및 발달생물학 전공

김 대 환

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지도교수: 노 상 호

이 논문을 치의과학 박사학위논문으로 제출함

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ABSTRACT

Generation and analyses of bovine embryo-derived pluripotent stem cells

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Embryonic stem cells (ESCs) have the capacity of self-renewal and differentiation into all somatic cell types. Because of these two features, ESCs are used in various studies on humans and animals. For a long time, there have been efforts to generate embryonic stem cells in cattle; however, genuine bovine ESCs have not been reported yet. In this study, bovine ESCs were generated using small molecules, and the properties of these cells were analyzed by various molecular biology techniques. In this study, small molecules that were suitable for the production of ESCs were selected, and the cultured cells were analyzed with the criteria for pluripotent stem cells including proliferation, pluripotency markers and differentiation abilities. Transcriptome analysis was performed with a microarray technique, and the

differentially expressed genes were verified with Real-time PCR. The signaling pathways for pluripotency were analyzed by KEGG assay. The generated cells showed a monolayer flat shape and were positive for alkaline phosphatase staining. In addition, the cultured cells were able to proliferate and were maintained in a culture for over 50 passages with the normal karyotype and pluripotency. Although it was defined as embryo-derived stem-like cells (eSLCs) because of abnormal *in vivo* differentiation, the population of pluripotent cells expressing only the Oct4 or Nanog gene without Cdx2 expression was confirmed in the eSLCs. As a result of the treatment with thiazovivin to improve the pluripotency, it was confirmed that the attachment rate of the blastocysts and the outgrowth rate increased significantly in the thiazovivin group compared with that of the control. Moreover, the expression of pluripotency related genes and E-cadherin genes were also increased in the thiazovivin group. However, it still failed to improve the potential of *in vivo* differentiation. Microarray analysis was conducted to analyze the limitations of the eSLCs. Tumor-related gene analysis showed that the expression of oncogenes decreased, while the expression of tumor suppressor genes increased. These results may be deeply related to the limitation of teratoma formation in the eSLCs. In addition, the TGFβ, WNT and LIF signaling pathways were found to be closely related to the maintenance of pluripotency in bovine. Different from the other two signaling pathways, the LIF signaling pathway was revealed as the inactive form. Thus, it is thought that the reactivation of the LIF signaling pathway may be the key to obtain true pluripotency in bovine. In conclusion, it demonstrated that bovine embryo-derived cells were generated

using three inhibitors. In addition, while analyzing the characteristics of the cells, the limitations for retaining pluripotency in bovine were investigated. These results can help to improve our understanding of bovine pluripotency and contribute to establishing true ESCs in large animals.

Key Words: Cattle, Embryo, Pluripotent stem cell, Somatic cell nuclear transfer, Small molecules, Microarray, Differently expressed gene

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REVIEW OF LITERATURE

Embryonic stem cells

Pluripotent stem cells (PSCs) have the capacity to self-renew by dividing and to develop into the three primary germ cell layers of the early embryo and therefore into more than 220 cell types in the adult body, but not extra-embryonic tissues such as the placenta.

ESCs are one of the typical PSCs and derived from the inner cell mass (ICM) of a blastocyst, an early-stage preimplantation embryo (Fig. 1A). In 1981, mouse ESCs (mESCs) were independently first derived from embryos [1]. The report revealed a new technique for culturing the mouse embryos in the uterus to allow for an increase in cell number, allowing for the derivation of ESCs from these embryos. In 1998, human ESCs (hESCs) are also derived from ICM of blastocyst [2]. Although ESCs were established from both mouse and human, they have very different culture conditions for supporting their pluripotency. Generally, the culture system for mESCs includes serum and leukemia inhibitory factor (LIF), while activin and fibroblast growth factor 2 (FGF2) were used for supporting pluripotency of hESCs (Fig. 1A). Recently, rat ESCs was also able to be maintained *in vitro* with two inhibitors [3]. It suggests that ESCs were derived from blastocysts in three different species, their pluripotency was not able to be supported by same culture strategy.

Although stem cell research has been studied for decades, culture system for authentic ESCs were established in several rodents and human [1-3]. Interestingly, the culture conditions for the ESCs are able to support

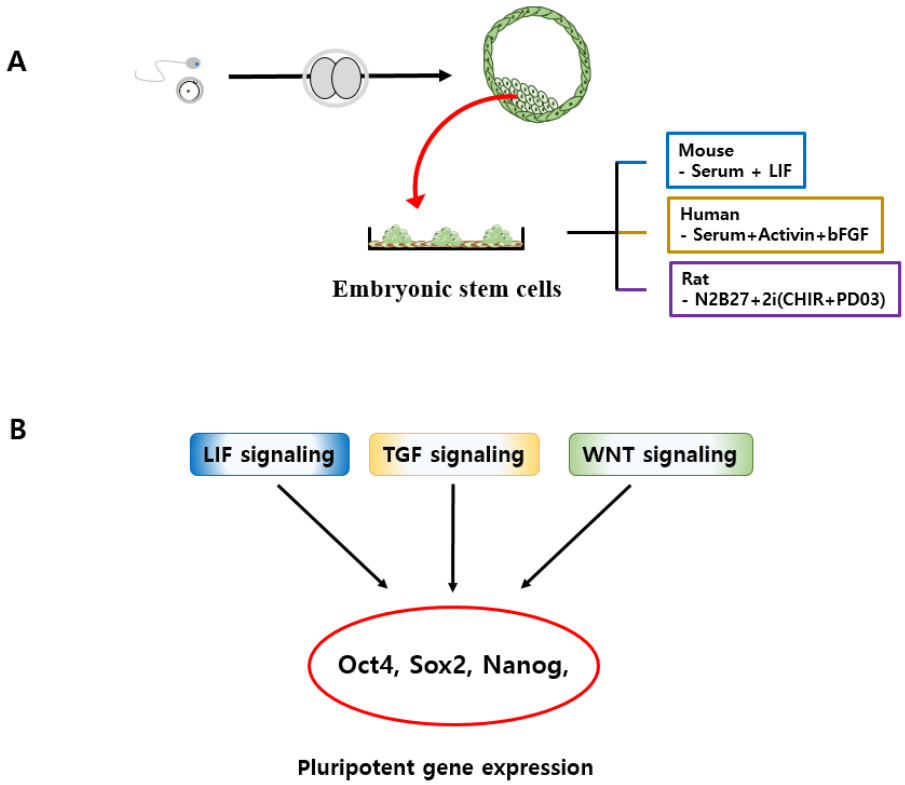


Figure 1. The establishment of embryonic stem cells (ESCs) and their pathways. (A) The generation of ESCs were usually derived from ICM of blastocyst. However, the medium conditions for ESCs were very different among species. Serum and LIF were used for mouse ESCs and Activin and bFGF were used for human ESCs. For rat ESCs, N2B27 and two inhibitors (Chir99021 and PD0325901). (B) Three different pathways, LIF, TGF and WNT, are related to pluripotency of ESCs. To maintain pluripotency in ESCs, the three pathways are able to activate pluripotent-associated core genes such as Oct4, Sox2 and Nanog.

pluripotency of induced pluripotent stem cells (iPSCs) which are devised by Takahashi and Yamanaka in 2006 [4]. In bovine, there have been many attempts to generate true ESC lines under culture condition modified either from mESCs or hESCs culture system [5-9].

However, using the culture conditions, putative ESCs were generated, because of failure to meet rigorous criteria as PSCs. It suggests that the ESC culture systems for rodents and humans are inappropriate for supporting of the pluripotency and new approaches were required for bovine ESCs such as new culture system or investigation of transcriptome.

The most important signaling pathways involved in the maintenance of pluripotency and the capacity for self-renewal in rodents and human are LIF, FGF and transforming growth factor (TGF)-β which acts through *Smad2/3/4* [10, 11] (Fig. 1B). The Wnt pathway also supports pluripotency by activating β-catenin [12]. These pathways result in the activation of three essential pluripotency-related transcription factors: *Oct4*, *Sox2* and *Nanog* (Fig. 1B). The interaction among *Oct4*, *Sox2* and *Nanog* regulates expression of pluripotent-specific target genes to maintain the pluripotent state of the cells.

Because properties such as self-renewal and multi-lineage differentiation potency [2] are common among ESCs from different species, understanding of the pluripotency control mechanism by exogenous factors in human or mouse embryonic PSC lines may be applicable in establishing large animal PSCs lines which have not been established yet. Recent study suggests that the regulation of FGF, mitogen activated protein kinase (MEK) and glycogen synthase kinase 3 (GSK3) [11, 13] by small molecule drugs promote proliferation,

differentiation and growth inhibition in a variety of cell types including undifferentiated human ESCs [14]. In addition, various combinations of small molecules are able to block or support signaling pathways involving differentiation or maintenance of ESCs [3, 15]. In particular, FGF and extracellular signal-regulated kinase (ERK) signals led ESCs into neural or mesoderm differentiation, and GSK3 signals reduced activities of *β-catenin*, adhesive molecules and cell survival rate [11, 16-19]. The application of small molecules for bovine embryo-derived cells may be useful for the generation of genuine bovine ESCs.

Although, genes involved in maintaining the pluripotent state are well characterized in mouse, the signaling pathways that regulate pluripotency in cattle have not been definitively identified. Recently, several groups published iPSCs from bovine differentiated cells, suggesting that the four factors may be also considered as key genes to retain pluripotent state in cattle [4, 20]. However, there are no studies available on successful application of shared culture condition between ESCs and iPSCs in bovine, implying that the medium for maintaining of iPSCs may be not sufficient to support pluripotency of ESCs. To understand suitable culture conditions for ESCs in bovine, it would be considered that investigations of broad-based gene expression patterns and finding of bovine specific-pluripotent genes are essential.

Somatic cell nuclear transfer

Somatic cell nuclear transfer (SCNT) is one of the powerful techniques for producing a viable embryo without fertilization between sperm and egg. The

whole process was simply presented in Fig. 2. During the process of this technique, the nucleus of a somatic cell is transferred to the cytoplasm of an enucleated egg (an egg that has had its own nucleus removed). Once inside the egg, the somatic nucleus is reprogrammed by egg cytoplasmic factors to become a zygote (fertilized egg) nucleus. The egg is allowed to develop to the blastocyst stage. It has been reported that a SCNT blastocyst can develop fully and be born as a young of sheep in 1996, showing that SCNT technique is able to be considered to produce a viable cloning embryo functionally.

In stem cell research, SCNT has become a focus of study in establishment of ESCs from therapeutic cloning embryos, which have potential regenerative medicine. A major consideration of ESCs as therapeutics is that they may trigger an immune rejection response when the ESCs are transplanted into a patient [21]. SCNT technology was originally developed for the multiplication of genetically high-valued animals, the rescue of endangered animal species, and the creation of genetically modified animals [22]. The technology has also been considered as a solution for obtaining patient-specific stem cells as it provides autologous ESCs genetically identical to the somatic cell donor [23]. Recently genuine SCNT-ESCs have been reported in human [24]. However, critical properties including immunogenicity and stability still need to be investigated [25].

In animal, SCNT-ESC technology is a useful tool for the conservation of endangered animal species and efficient production of transgenic animals [26, 27]. In addition, researches of SCNT-ESCs in large animal species like cattle can serve as a pre-clinical model for patient-specific stem cell therapies in

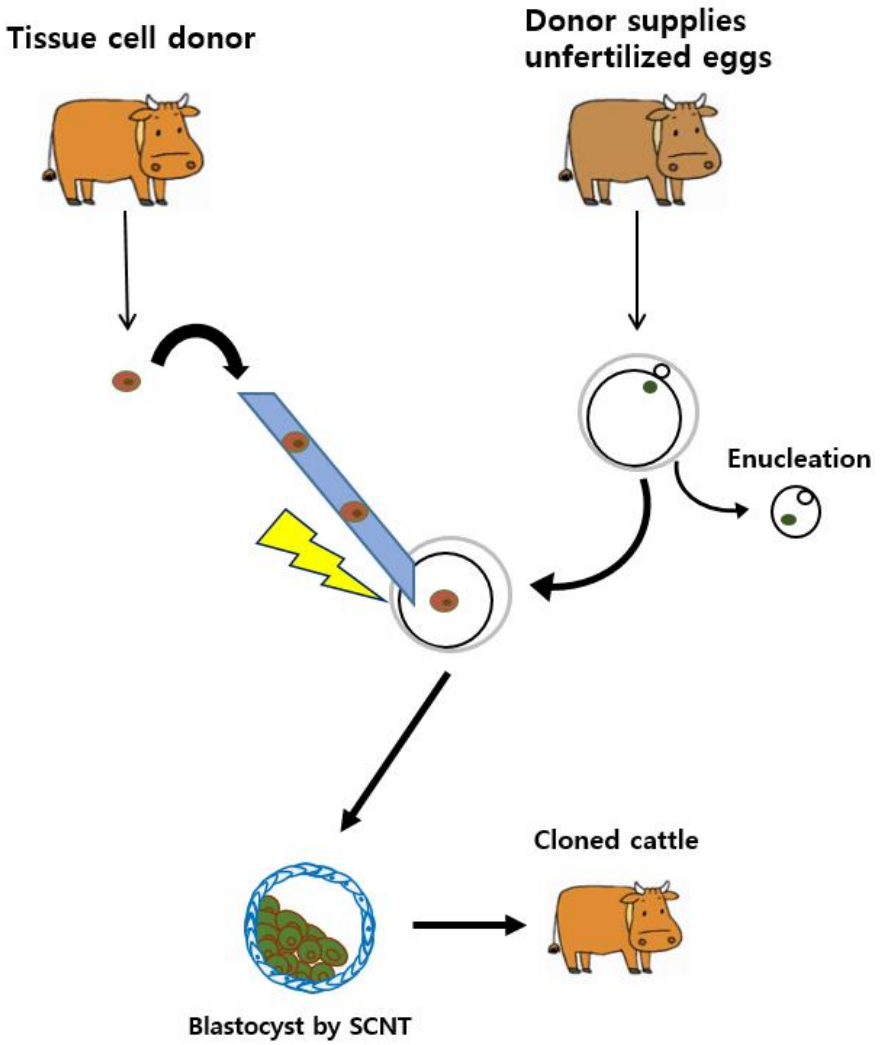


Figure 2. Representation of the somatic cell nuclear transfer procedure.

humans because of the similarities in surface markers and cellular morphologies between bovine ESCs (or ES-like cells) and human ESCs [5, 8, 9]. Another technique for generation PSCs is iPSCs formed by forced expression of four transcription factors in somatic cells were introduced in 2006 by Dr. Yamanaka and colleagues [4]. However, immunogenicity of iPSCs is still questionable [25], and abnormalities of iPSCs, which seem to occur during the reprogramming event, compromise genomic integrity by introducing *de novo* mutations and copy number variations [4]. Moreover, because the reprogramming processes by external factors in iPSCs are not understood completely.

Microarray

Microarray technology has revolutionized molecular biology, because of large-scale studies of gene expression. The ability of these arrays to simultaneously interrogate tens of thousands of transcripts has led to important advances in tackling a wide range of biological problems, including the identification of genes that are differentially expressed between diseased and healthy tissues, new insights into developmental processes, drug discovery, toxicology studies, small molecule screening. Nonetheless, array technology has several limitations. For example, background hybridization limits the accuracy of expression measurements, particularly for transcripts present in low abundance. Furthermore, probes differ considerably in their hybridization properties, and arrays are limited to interrogating only those genes for which probes are designed. Although there are several limitations, microarrays still remain the

most popular approach for transcript profiling and can be readily afforded by many laboratories.

In stem cell research, microarray platforms were also used in various aspects such as exploring the microenvironment, profiling stem cell markers and tracking cell fate decisions. Until now, lots of transcriptome analyses of humans and mice have been profiled not only in embryo level but also in PSCs. However, there are some reports of microarray data in cattle at the pre-implantation embryo level and no microarray data using bovine ESCs (bESCs) has been published.

Different from mice and humans, studies of ESCs in cattle have been remained largely obscure. Moreover, most culture conditions using hESCs or mESCs were failed to support bovine ESCs, suggesting that the mechanism of pluripotency in cattle may be considerably different from hESCs or mESCs. Because of this situation, High-throughput screening assays are essential to establish genuine ESCs in cattle.

INTRODUCTION

There have been attempts to generate ESCs in domestic animals [8, 9]. From an agricultural perspective, the establishment of ESCs in domestic ungulates can provide a more efficient way to produce genetically-modified animals. Although the generation of ESCs in bovine has been attempted by many labs over the world [6, 28, 29], most methods have failed to support their pluripotency as stem cells including self-renewal and differentiation. Moreover, no studies have focused on the causes of these failures. Therefore, in the present study, I suggest a series of experiments for the following three topics to create a new approach to overcome these failures.

1. The generation of bovine eSLCs by somatic cell nuclear transfer

This topic has been largely reproduced from an article published, entitled "*In vitro* culture of stem-like cells derived from somatic cell nuclear transfer bovine embryos of the Korean beef cattle species, HanWoo" [30].

ESC technology is a useful tool in large animals for the conservation of endangered animal species and efficient production of transgenic animals [26, 27]. Studies of SCNT-ESCs in large animal species like cattle can serve as a pre-clinical model for patient-specific stem cell therapies in humans because of the similarities in surface markers and cellular morphologies [5, 8, 9]. One of the most difficult challenges faced for SCNT-ESCs is the high incidence of chromosomal abnormalities that occur in SCNT embryos [31]. In the case of

cattle, most putative ESCs have been reported from *in vitro* fertilized or parthenogenetic embryos [5, 15, 26], while only one was from an SCNT embryo [9]. Although there have been many attempts to establish true ESC lines from bovine species under culture conditions modified either from mouse or human ESC culture protocols [5-9], only one study reported true ESC lines from *in vitro*-produced (IVP) bovine embryos [32]. Evidently, the ESC culture systems for mice and humans are suboptimal for the proliferation and maintenance of the pluripotency of bovine embryonic cells.

Recently, several reports have been applied inhibitors of differentiation for maintaining bovine pluripotency [15, 33]. The double inhibition of MEK and GSK3 signaling enhances the expression of pluripotency-related genes without significant changes in the expression of trophoblast-related genes during embryonic development in bovine species [33]. In addition, ESCs from parthenogenetic bovine embryos were successfully generated under the culture system using MEK, GSK3 and FGF signaling inhibitors [15]. After several passages and cryopreservations, these parthenogenetic bovine cells expressed genes of pluripotency and were also capable of differentiation into three germ layers *in vitro* [15].

In the present topic, I demonstrate the generation of eSLCs originated from SCNT bovine blastocysts using the three inhibitors. These cells are genetically identical to the donors and have the capacity for self-renewal and pluripotency after at least 50 passages.

2. Enhancing pluripotency of bovine eSLCs

This topic has been largely reproduced from an article published, entitled "Thiazovivin, a Rho kinase inhibitor, improves stemness maintenance of embryo-derived stem-like cells under chemically defined culture conditions in cattle" [34].

Recent studies have shown that small molecules, Y-27632, a Rho-associated kinase (ROCK) inhibitor, have a role in the maintenance of ESC properties, formation of iPSCs and the viability of stem cells after freezing and thawing. ROCK, a downstream target of Rho plays a role in cellular function through extracellular signaling. Inhibition of ROCK enhances the generation of human ESC from fair- or poor-quality (Grade III and IV) cleavage stage embryos [35] and also induces the increase and the stabilization of the E-cadherin protein at the plasma membrane of dissociated human ESC [36].

Recently, thiazovivin is demonstrated to be a more potent ROCKi than Y-27632 in human ESC. Thiazovivin enhances the survival of dissociated human ESC on matrigel by regulating E-cadherin-mediated cell-cell interaction [37]. Furthermore, thiazovivin is more effective than Y-27632 in achieving a greater colony-forming efficiency and obtaining larger colony size in intestine stem cells [38].

E-cadherin is a transmembrane glycoprotein that mediates Ca^{2+}-dependent, homophilic cell-cell adhesion in the epithelial tissues including ESC [39]. Because it is co-expressed with other ESC makers in undifferentiated human ESC, it can be used as an undifferentiated cell marker to identify human ESC [40]. Overexpression of the E-cadherin gene enhances reprogramming

efficiency, while knockdown of endogenous E-cadherin gene decreases reprogramming efficiency when generating iPSCs with Yamanaka's four factors [41]. In addition, expression of the E-cadherin can compensate for by enhanced expression of the Oct4 gene, a critical factor for maintenance of pluripotency, during somatic cell reprogramming [42].

In this topic, the purpose is to examine whether the treatment of thiazovivin can enhance the attachment and pluripotency to help the establishment of bovine eSLCs.

3. Microarray analysis of bovine eSLCs

This topic has been largely reproduced from an article published, entitled "Microarray analysis of embryo-derived bovine pluripotent cells: The vulnerable state of bovine embryonic stem cells" [43].

Cows are one of the most common and important domestic ungulates and are acknowledged as livestock for food and bioreactors [44]. There have been many attempts to establish ESCs in bovine species using general culture conditions for mESCs and hESCs [6, 28, 29]. However, those conventional methods are inappropriate for the survival of bESCs *in vitro*, as they lose their stem cell properties when involved in proliferation, pluripotency, or differentiation. These early efforts to generate bESCs were gradually discontinued due to less than promising results which showed that the majority of the cultured cell population lacked evidence of pluripotency or ability to sustain long-term growth.

There have been several attempts to generate authentic bESCs using

small molecules. Recently, eSLCs from bovine embryos were successfully generated using three inhibitors [30, 34]. Moreover, *Cdx2*, the trophoblast-specific gene, is still expressed in the eSLCs. Recently, it has been reported that *Cdx2*-knockdown embryo-derived stem cells are generated and have similar characteristics to genuine PSCs [45] although only one cell line was successfully established from 59 embryos. So far, many studies have failed to isolate true ESCs. However, there is limited information about the key aspects that fail during the establishment of bESCs, and little is known about their transcriptomes and biological functions. Although the eSLCs in this experiment are not complete bESCs, analyses of these cells can contribute to our understanding of the characteristics of embryo-derived PSCs in cattle.

To date, many genome-wide gene expression analyses of ESCs in humans and mice have been reported [46]. The early results of those microarrays were analyzed to verify the differences among various embryo resources, including IVP, parthenogenesis (PA), and nuclear transfer (NT) [47]. This technique has also been applied to compare somatic cells (SCs) with diverse stem cells from IVP, PA, and NT [48, 49]. Although two reports presented microarray data in cattle at the pre-implantation embryo level [50, 51], there are no reports of microarray data using bESCs.

In this topic, I investigated the global gene expression patterns of bovine eSLCs from three different origins, IVP-, NT- and PA-embryos, to validate their distinct characteristics including shared signaling pathways related to pluripotency. In addition, oncogenes and tumor suppressor genes were analyzed to understand the failure of teratoma formation in bovine ESCs.

MATERIALS AND METHODS

Chemicals

Most inorganic and organic compounds were purchased from Sigma-Aldrich Korea (Yong-in, Korea) and all liquid medium and supplements were from Life Technologies (Grand Island, NY, USA) unless indicated in the text.

Oocyte recovery and in vitro maturation (IVM)

Bovine ovaries were collected from the Korean native beef cattle, HanWoo, at a local slaughterhouse (Livestock products market, Naju, Korea) and transported to the laboratory within 2-3 h of collection in saline at 25-35°C. Cumulus-oocyte complexes (COCs) were recovered by aspiration of 3 to 8 mm follicles. COCs that were enclosed by more than three layers of compact cumulus cells and an evenly granulated ooplasm were selected and incubated in IVM medium under warmed and gas-equilibrated mineral oil for 20-22 h at 38.5°C under 5% CO_2. The IVM medium for oocytes is composed of tissue culture medium 199 with Earle's salts and L-glutamine (TCM199) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific Korea, Seoul, Korea), 10 μg/ml FSH-P (Folltropin-VTM, Vetrepharm, Belleville, ON, Canada), 0.2 mM sodium pyruvate, 1 μg/ml estradiol-17β, and 10 ng/ml epidermal growth factor.

In vitro production (IVP) of bovine embryos

The thawed HanWoo semen (purchased from HanWoo improvement center,

Seosan, Korea) was deposited on the top of a discontinuous Percoll gradient prepared by depositing 2 ml of 90% Percoll under 2 ml of 45% Percoll in a 15 ml centrifuge tube, and the sample was then centrifuged for 20 min at 252 x *g*. The pellet was removed and re-suspended in 300 μl of hTALP and centrifuged at 201 x *g* for 10 min. The active semen from the pellet was inseminated with a matured oocyte for 24 h (1 x 10^6 sperm cells/ml). After insemination, the cumulus cells were removed by repeated aspiration into a pipette and denuded fertilized oocytes were transferred to *in vitro* culture medium consisting of CR2 with 0.3% ff-BSA and 1% ITS for 3 days. Oocytes were then transferred to CR2 medium with 0.15% ff-BSA, 1% ITS, and 0.15% FBS for 5 days at 38.5°C in a humidified gas environment of 5% CO_2, 5% O_2, and 90% N_2.

Parthenogenesis and in vitro culture

Parthenogenetic activation was performed after IVM of the oocytes. The oocytes were activated in 5 μM Ca-ionophore A23187 for 5 min, followed by 2 mM 6-dimethylaminopurine (6-DMAP) for 3 h. After treatment, the activated oocytes were transferred and cultured *in vitro* as described above.

Somatic cell nuclear transfer

Matured oocytes were enucleated in HEPES-buffered TCM199 (hTCM199) supplemented with 20% FBS. The zona pellucida (ZP) was partially dissected with a fine glass needle to create a slit near the first polar body. The first polar body and the adjacent cytoplasm, presumably containing the metaphase II

chromosomes, were extruded by squeezing with the needle. The enucleated oocytes were placed and incubated in hTCM199 with 10% FBS before NT. A single donor cell isolated from ear skin tissue of the Korean native cattle, HanWoo, was injected into the perivitelline space of the enucleated oocyte through the slit made during enucleation. Then, karyoplast-cytoplast complexes were transferred into a cell fusion chamber with Zimmerman's cell fusion medium and sandwiched between fine electrical rods. Cell fusion was accomplished with a single DC pulse of 25 V/mm for 10 μs. After 30 min of electric stimulation, fusion was confirmed under a stereomicroscope. The fused couplets were activated in 5 μM Ca-ionophore A23187 for 5 min, followed by 2 mM 6-DMAP for 3 h. After treatment, the activated oocytes were transferred and cultured *in vitro* as described above.

Generation of eSLCs

ZP-free blastocysts were placed onto a mitomycin-C inactivated murine STO feeder cell layer and cultured at 38.5°C in a humidified gas atmosphere of 5% CO_2 in 3i medium, which consists of equal volumes of DMEM/F12-Glutamax™ and neurobasal media with 1% (v/v) N2 and 2% (v/v) B27 supplements plus the three inhibitors (3i): 0.8 μM PD184352 (Selleck Chemicals, Breda, Netherlands), 2 μM SU5402 (Tocris Bioscience, Ellisville, MO, USA), and 3 μM CHIR99021 (Tocris Bioscience). This was designated as passage zero (P0), and the medium was replaced every other day. After 10 days of culture, the initial outgrowths from the embryos were mechanically

dissociated into 2-4 pieces from each clump, and each piece was re-plated onto fresh STO feeder layers and designated as P1. The colonies were passaged mechanically every 4 to 5 days. Only multilayered cells in the central region of the colony (CMt) were picked up for passaging eSLCs (Fig. 3). The peripheral monolayer part (PMn) were either discarded or separately cultured for comparative analysis. Each colony from IVP-, NT- and PA-embryos was labeled I_x-P_y, N_x-P_y, and P_x-P_y respectively along with its specific number x, P_y the passage number.

Treatment of thiazovivin

To investigate the effect of thiazovivin, the 3iT medium is designed containing 3i medium plus 2 μM thiazovivin. In a previous study it was demonstrated that ROCK inhibition with 2 μM thiazovivin is effective and comparable to 10 μM Y-27632 in human ESC [37].

Karyotyping

Bovine eSLCs actively growing in log phase were treated with 0.1 mg/ml of colcemid for the induction of mitotic arrest. Cells were subsequently harvested by the standard cytogenetic methods of trypsin dispersal and hypotonic shock, and fixed using 3:1 methanol:acetic acid mixture. Mitotic cell slide preparations were analyzed by the G-banding method and interpreted by an investigator blinded to study condition.

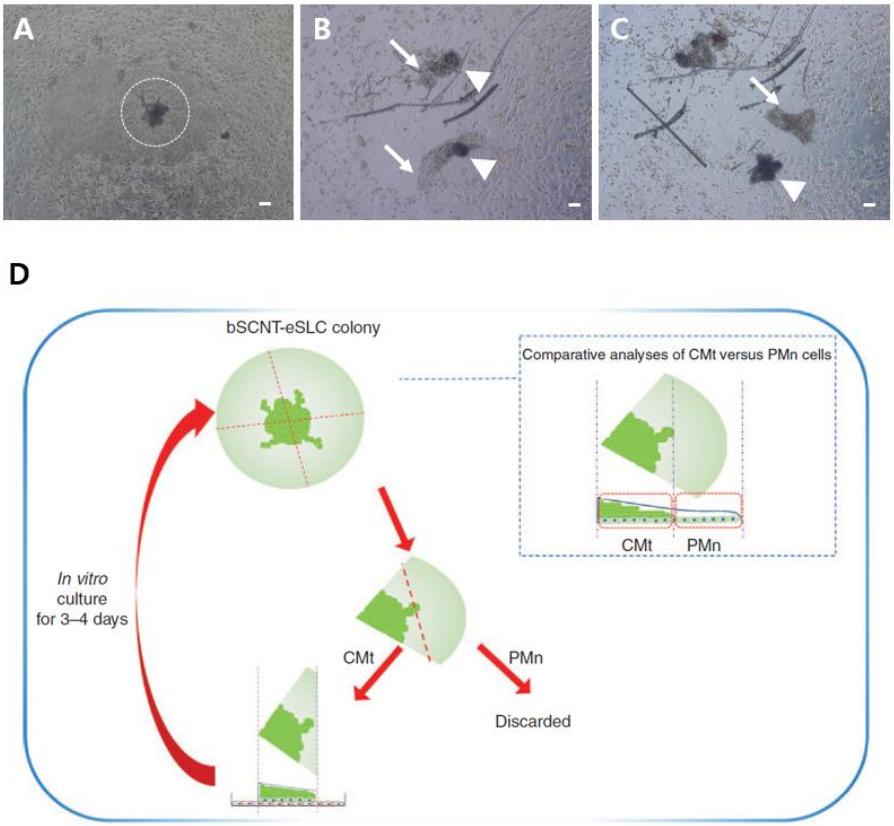


Figure 3. The scheme of *in vitro* culture cycle for passaging bovine somatic cell nuclear transfer embryo-derived stem-like cell (bSCNT-eSLC) colony. The methods for passaging the central multilayer (CMt) cell clump in bSCNT-eSLC colony. (A) Whole SCNT-eSLC colony. The dotted circle displays CMt part. (B) Split colony into two clumps which include both CMt (arrowhead) and peripheral monolayer (PMn, arrow) regions. (C) CMt was manually separated from PMn in the clump using a 26 gauge needle. (D) A bSCNT-eSLC colony was divided into two layers, central multilayer (CMt) and peripheral monolayer (PMn). For passaging, the colony was cut into four to six pieces, and only CMt part of the slice was transferred to new feeder cell layer. In general, PMn part was discarded, except for comparative analyses of PMn versus CMt part cell propagation and molecular characterization. Scale bar = 100 μm

Short tandem repeat (STR) loci analysis

Donor somatic cells and bSCNT-eSLCs were used for independent microsatellite analyses. Genomic DNA was extracted from samples using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Samples were amplified in DNAmplify PE9700 following the technical manual of Biotrace™ bovine identification kit (Gendocs, Daejeon, Korea). Gene scan analysis on the amplified fragments was performed on an ABI 3130XL genetic analysis for the following microsatellite markers: *Tgla227*, *Bm2113*, *Tgla53*, *Eth10*, *Sps115*, *Tgla126*, *Tgla122*, *Inra23*, *Eth3*, *Eth225* and *Bm1824* (Applied Biosystems, Inc., Foster City, CA, USA). Each amplification reaction contained 1 μl DNA, 2 μl primer mix, 4 μl PCR premix, 0.25 μl of Amplitaq Gold DNA polymerase and 2.75 μl sterile H_2O. Final reaction volumes were 10 μl each. The cycling program started with an initial 11-min denaturation step at 95°C, followed by 28 cycles of 1 min at 94°C, 1 min at 59°C, 1 min at 72°C, and the final extension step of 60 min at 60°C [52].

Proliferation Assays

To determine the effect of 3i culture medium on cellular proliferation, 10 μM of 5-bromo-2'-deoxyuridine (BrdU) was added to the culture medium, and incubation was continued for 24 h. Before colony analysis, the colonies newly passaged and cultured in 3i system for 48 h to prevent contamination of BrdU-positive bSCNT-eSLCs from the BrdU-positive feeder cells. Cells were fixed with 4 % paraformaldehyde in PBS (pH 7.4) at 37°C for 2 h, acid-treated with

2 N HCl in PBS for 30 min at 45°C, equilibrated with 0.1 M borate buffer (pH 8.5), and finally incubated with blocking buffer (20% Calf serum; 0.1% Triton X-100; 1% DMSO in PBS) for 2 h. Fixed cells were immunostained with antibodies against anti-BrdU mouse monoclonal antibody IgG (sc-32323, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) followed by incubation with the secondary antibodies FITC conjugated goat anti-mouse IgG (A11001, Life Technologies). The treated cells were covered with slow-fade anti-fade with DAPI (SlowFadeGold^{TM} with DAPI, Life Technologies) for nuclear staining and covered with a glass coverslip. Images were captured with the fluorescence microscope (FV-300, Olympus). To examine cell doubling time, bSCNT-eSLCs were plated in 96-well flat-bottom plates at a density of $3x10^3/cm^2$ and cultured in 3i system with the BrdU CHEMICON^{TM} Cell Proliferation Assay kit (2750, Millipore, Bedford, MA, USA) according to the manufacturer's instruction. Absorbance was measured at 450 nm with a microplate reader (Model 550, Bio-Rad, Hercules, CA, USA) from 4 to 40 h.

Alkaline phosphatase (AP) and immunofluorescence staining

The AP staining was performed using a commercial AP detection kit from Sigma. Immunofluorescence staining was performed according to standard protocols. Briefly, presumptive eSLCs from bovine SCNT blastocysts were fixed in 4% paraformaldehyde, permeabilized with 0.25% Triton X-100 and blocked with 1% goat serum in PBS. The fixed cells were immunostained with antibodies against octamer-binding transcription factor 4 (OCT4) (ab18976, Abcam, Cambridge, MA, USA), homeobox gene that encodes the NANOG

protein (NANOG) (ab21603, Abcam), sex determining region Y-box 2 (SOX2) (MAB4343, Millipore, Bedford, MA, USA), stage-specific embryonic antigen-4 (SSEA-4) (MAB4304, Millipore), tumor rejection antigen 1-60 (TRA-1-60) (MAB4360, Millipore), tumor rejection antigen 1-81 (TRA-1-81) (MAB4381, Millipore), caudal type homeobox 2 (Cdx2) (AM392, BioGenex, Fremont, CA, USA) and E-cadherin (1:100; ab11512; Abcam) followed by an incubation with FITC conjugated goat anti-mouse IgG (10 μg/ml; A11001, Life Technologies) or FITC conjugated anti-rabbit IgG (10 μg/ml; 65-6111; Life Technologies) or Cy3 conjugated anti-rat IgG (1 μg/ml; 112-1656-167; Jackson Immuno Research Lab Inc., West Grove, PA, USA) secondary antibodies. The treated cells were covered with slow-fade anti-fade with 4',6-diamidino-2-phenylindole (DAPI) (SlowFadeGold™ with DAPI, Life Technologies) for nuclear staining and covered with a glass coverslip. Images were captured with the fluorescence microscope (FV-300, Olympus, Tokyo, Japan).

Formation of embryoid bodies (EBs)

For EBs formation, bSCNT-eSLCs were harvested by treating with trypsin and transferred to 100 mm Petri dishes in DMEM containing 2 mmol/ml Glutamax™, 0.1% (v/v) β-mercaptoethanol, 1% (v/v) nonessential amino acid (NEAA), 1% (v/v) ITS, 1% (v/v) penicillin–streptomycin and 20% FBS at 38.5°C in a humidified gas environment of 5% CO_2 in air [15]. Culture medium was changed every 2 days. After 15 days of floating culture, non-attached EBs were collected to analyze gene expression.

Reverse transcription PCR and Real-time PCR

Total RNA from eSLCs, TE, ICM, whole blastocysts or EBs was extracted using the RNeasy mini kit (Qiagen), and M-MLV Reverse Transcriptase was used to synthesize cDNA according to the manufacturer's instructions. The reaction (total volume 50 μl) for PCR contained 50 ng cDNA, 20 pmol each of specific primers, 1 μl Dimethyl sulfoxide (DMSO) if required and 25 μl Sapphire Amp fast PCR Master Mix (Takara, Otsu, Japan). Each thermal cycle for amplification included incubation at 98°C for 5 s, at 56°C for 5 s, and at 72°C for 10 s; this cycle was repeated 35 times. PCR products were examined by 1% agarose gel electrophoresis. Nuclease-free water was also used as negative control. Real-time PCR was performed by 7500HT system™ (Applied Biosystems) using SYBR Premix Ex Taq (Takara). PCR volume was 20 μl, containing 1 μl reverse transcript product. Cycling conditions were 1 cycle of 95°C for 30 s and 40 cycles of 95°C for 5 s and 60°C for 30 s. The ΔΔCt method was used to determine relative quantitation of mRNA expression in samples, and fold change was determined as $2^{-\Delta\Delta Ct}$. The specific primer sequences representing pluripotency and three-germ layer differentiation marker genes are from previous reports [15, 53-55] and listed in Table 1.

Teratoma formation

Female nude mice of 6–8 weeks old were housed under pathogen-free conditions in a temperature controlled room on 12/12 h light/dark schedule with food and water *ad libitum*. All animal experiments including animal

Table 1. Primer sequences for reverse transcription and real time polymerase chain reaction

Gene name	Sequence	Accession No.
	Pluripotency	
Oct4	F: GCCTGGATTTTCCTAGCATCTAC R:GGCCAAGCAGGCTTTG	NM_174580.3
Cldn6	F: ACAAGCCTTTCCTTGCTGGTCA R:AGTAACAACTGGTGAGTGTGGGC	NM_001205697.1
Cnot1	F: TTG GAC AGA TGC ACC AGC AA R:AGT CGG ACA AAG GCA TCC AG	NM_001206039.1
Dppa3	F: AAAGCGGACGAGTATCGAGAAC R:CTGGGCGATGTGGCTAATTT	NM_001111108.2
Dppa4	F: TGCAAGTTGCCACTCAACTC R:TCTTACCCCTCTCCGCCTAT	NM_001206481.1
Fgf5	F: GCCGTGAAGACGGAGTCAGT R:GAACCCAGCCTTCTGTGTTAGC	NM_001078011.2
Klf2	F: GGCTTGAGGAGCGCAGTCCGGGCTCCCGCA R:CCGGGCTAGGAGGCGTCGACGGAAACGCGT	XM_015463970.1
Lefty2	F: GCGACTTCCTCTTCTTCCC R:GCAGATGGAAACCGATGC	NM_001206043.1
Nanog	F: CAGGGACTATGGAGCTCAGG R:CTGGATGCTGACAATCATGG	NM_001025344.1
Nrob1	F: CCTGCAGTGCGTGAAGTA R:AGGGTGTTGGCACTGATG	NM_001205857.1
Otx2	F: CAATGCAGTCACCAGCCATCTCA R:TGCCTCCTGGGACATTGATCA	NM_001193201.1
Pecam1	F: CTCACCAAGGTCTGGGAACAA R:TCTTCTCGGAATGTGGGCAT	NM_174571.3
Prdm14	F: ATTTTCGTTCCGCCGCCCCC R:TCAGCCCCGGCGCTATCGGT	NM_001191264.1
Rex-1	F: TGCCTGTCCTCACAACGGATGC R:AGTGTGGGTGCGCACGTGTG	NM_001192308.1

Sox2	F:GGCTGGATCGGCCAGAA R:AGGAAAATCAGGCGAAGAATAA	NM_001105463.2
T-brachyury	F:CCAGTACCCCAGCCTGTGGTCC R:TGATGCCAGAGGCATCTCC	NM_001192985.1
Zfp42	F:TGCCTGTCCTCACAACGGATGC R:AGTGTGGGTGCGCACGTGTG	XM_003587951.3
	Trophectoderm	
Gata3	F:ATGAAACCGAAACCCGATGG R:TTCACAGCACTAGAGAGACC	NM_001076804.1
IFN-τ	F:TGTTACCTGTCTGAGAACCACATGCT R:TCAAAGTGAGTTCAGATCTCCACC	NM_001245936.1
Tead4	F:AAGTTCTGGGCAGACCTCAA R:GTGCTTCAGCTTGTGGATGA	XM_010827947.2
	Ectoderm	
β-3-tubulin	F:CATCCAGAGCAAGAACAGCAG R:GATTCCTCCTCATCGTCTTCG	AY675081.1
Nestin	F:CACCTCAAGATGTCCCTCAGC R:TCTTCAGAAAGGTTGGCACAG	NM_001206591.1
Vimentin	F:GATGTTTCCAAGCCTGACCTC R:GGCGTTCCAGAGACTCGTTAG	NM_173969.3
	Endoderm	
A-fetoprotein	F:TGCAAGATGGCAGACCAGA R:CCTGTGAGGCTATGACGGAA	BC103123.1
Somatostatin	F:CCTGGAGCCTGAAGATTTGTC R:GTGAGAAGGGGTTTGGAGAAG	M31217.1
Transthyretin	F:GTCTCGCTGGACTGGTGTTT R:AATTCATGGAACGGGGAGAT	NM_173967.3
	Mesoderm	
Bmp4	F:TCGTTACCTCAAGGGAGTGG R:GGCTTTGGGGATACTGGAAT	NM_001045877.1
Connexin40	F:TGCGAGAACGTCTGCTATGAC R:GGCAATCCTTCCATTCACTTC	NM_001078022.1
	Imprinting	
Phlda2	F:GCTCCAGGTGTGGAAGAAGA	NM_001076521.2

	R:GACGCGTTCCAGTAGCTCTC	
H19	F: CTTGGAACACGGACTTCTTCAAG R:TCTACTTCAGCCGACCATCCA	NR_003958.2
Meg3	F: GTTTGGGACTGGGACGCTTA R:GGAGACGACGGACAGAGTTC	NR_037684.1
Igf2r	F: CCGGGAGATGGTAATGAGCA R:TCTCGTTCTCGTCGGCCT	NM_174352.2
Igf2	F: TCTACTTCAGCCGACCATCCA R:GTAAGTCTCCAGCAGGGCCA	NM_174087.3
Nap1l5	F: TCC AAC TGT GTG TCC CCA TC R:ACGAAGGCACAG CTAACACA	NM_001077878.2
Peg3	F: CGCCAAAGTCAGGGAGAG R:CTTAACTGCCAGGACACC	NM_001002887.2
Plagl1	F: TCTCTAAGGTTCTTCCCTGCCT R:TAAGCTTGGGGTCCAGAGGA	NM_001103289.1
	Oncogene	
Smo	F: GCTTCACCCGTCTACTACCC R:GCTCATGGAAATGCCAGTTC	NM_001192220.1
Bcl11a	F: GGGTATTTGTAA AGATGAGCCCAG R:GGG GTG TGT GAA GAA CCC G	NM_001076121.1
Ccnd1	F: AGCAGAAGTGCGAGGAAGAG R:GCCAGGTTCCACTTGAGTTT	NM_001046273.2
Maml2	F: CCCAACCCCTGCTCAAATCC R:GCAATTTTCTCCGCATCAGCC	NM_001098050.1
	Tumor suppressor	
Mlh1	F: TCCGGGAGATGCTGCATAA R:CAAGGCCCACTGAGGATTCA	NM_001075994.2
Msh2	F: AGACAGGTCGGAGTTGGGTA R:CCTCCTCTCTGAATAACCTGCC	NM_001034584.1
Socs1	F: CTCGTACCTCCTACCTCTTCATGTT R:ACAGCAGAAAAATAAAGCCAGAGA	XM_864316.5
Suz12	F: CATCCAAAAGGTGCTAGGATAGATG R:TGGGCCTGCACACAAGAATG	NM_001205587.2
Brca1	F: GGAGCCCTCATCATTCACCC	NM_178573.1

	R:CCCGATCACATGGAAGCCAG	
	Chromatin	
Hmga1	F: GCCTCCAAGCAGGAAAAGG R:TTGCTTCCCTTTGGTCGG	NM_001076523.1
Padi4	F: TGCATGCTGGGTCCAGATTT R:GCTCAGGAACTCGTCCACAT	NM_001192173.1
Chd1l	F: GTCTCTGATGCCCTGCCTAC R:GGGATGGGAACACTGCCTTTA	NM_001037820.1
Sycp3	F: GAGAAACAAGGGAAGAAAAGG R:TGCTGGAACAAAGTCAGAAAC	NM_001040588.2
Padi2	F: CTTCCCTCTGTCCGGTGGT R:GTAGTACAGGGGTTCCGCAC	NM_001105452.2
	Defensin	
Defb1	F: ATCCTCTAAGCTGCCGTCT R:AGCATTTTACTGAGGGCGT	NM_001324544.1
Defb3	F: AAGTGACTGCCCCTGCTTTG R:GTAAATCCTGACCCAGCAGACAG	NM_001282581.1
Defb7	F: CCTGTCTGCTGGGTCAGGATTTAC R:AGGTGCCAATCTGTCTCCTGTG	NM_001102362.2
	BMP signaling	
Bmp7	F: TGCCACTAGCTCTTCCTGGAA R:TGAGAGACCCAGGATCCAGAA	NM_001206015.1
Smad4	F: AACATTGGATGGAAGGCTTCA R:CCAGAGACGGGCATAGATCAC	NM_001076209.1
Smad5	F: GCAACGTTTCCTGATTCTTTCC R:GGCGGGTAGGGACTATTTGG	NM_001077107.3
Smad3	F: GGAGCCGAGTACAGGAGACA R:AAAGGTCCATTCAGGTGCAG;	NM_001205805.1
Id1	F: CTGGGATCTGGAGTTGGAGC R:GGAACACACGCCGCCTCT	NM_001097568.2
Bmpr1a	F: TCAGCGAACTATTGCCAAACAG R:CCCATCCACACTTCTCCGTATC	NM_001076800.1
	WNT signaling	
Wnt7a	F: GGT AGT CCT TCC TGC CCT TT	NM_001192788.1

	R:GTG TGT CCT GGC CTG ATT TT	
Wnt10a	F: ATGAGTGCCAGCACCAGTTC R:ATGCACTTTCTCGGAAACCTCT	NM_001099078.1
Ctnnbl1	F: AATCCGAGCTGGACCTCAATGACA R:TATCATGTCCAAGCAGCCCGAGAA	NM_174637.4
Fzd7	F: TTGTTTCTGGAACCTCCTTCCGCT R:GCATCATCCTGCAAGTCTTTGCCA	NM_001144091.1
Dkk3	F: GAGATGTTCCGCGAGGTTGA R:ATGCACTCGTGGCTCCTTTT	NM_001100306.2
Dkk1	F: GACTGGTGGAGGCGCTCGGA R:GCTGTGCCCAGAGCCGTCA	NM_001205544.1
Dvl1	F: CACTCAACATGGAGAGGCAC R:GAAGTTGACGTCGTTCACCT	NM_001206601.1
	LIF signaling	
Lif	F: CCTCTATTACACGGCCCAGG R:GTACAGCTCCACCAGCCG	NM_173931.1
Lifr	F: AGACATGCCCTTGGAGTGTG R: TCCCGCAAAAACAACCGTTC	NM_001192263.1
Stat3	F: CTCTCCCCACTTCTGCCAA R:GGGGTCACAACTGCTGCT	NM_001012671.2
Socs3	F: GCCACTCTCCAACATCTCTGT R:TCCAGGAACTCCCGAATGG	NM_174466.2
	House keeping	
Gapdh	F: AAAGCGGACGAGTATCGAGAAC R:CTGGGCGATGTGGCTAATTT	NM_001034034.2

management and surgical procedures were approved and performed under the guidelines of the Institutional Animal Care and Use Committee of Seoul National University (SNU-120321-7). To confirm pluripotency *in vivo*, bSCNT-eSLCs were delivered under the testis capsule of nude mice by injection (approximately 5×10^6 cells per site). Eight weeks after cell injection, mice were sacrificed, and the formed teratoma was extracted for histological examination. For immunohistochemistry, 4-μm-thick paraffin-embedded tumor tissue sections were stained with hematoxylin and eosin.

Microarray gene expression analysis

For microarrays, the synthesis of target cRNA probes and hybridization were performed using Agilent's Low RNA Input Linear Amplification kit (Agilent Technologies, Palo Alto, CA, USA) according to the manufacturer's instructions. The fragmented cRNA was resuspended with 2X hybridization buffer and directly pipetted onto the assembled Agilent's Bovine Oligo Microarray (44K). The arrays were hybridized at 65ºC for 17 h using the Agilent Hybridization oven and the hybridized microarrays were washed as described in the manufacturer's washing protocol (Agilent Technologies). The hybridized images were scanned using Agilent's DNA microarray scanner and quantified with Feature Extraction Software (Agilent Technologies). All data normalization and selection of fold-changed genes were performed using GeneSpringGX 7.3 (Agilent Technologies). The averages of normalized ratios were calculated by dividing the average of the normalized signal channel intensity by the average of the normalized control channel intensity.

Hierarchical clustering was performed with TIGR MeV Ver.4.9 software (Institute of Genomic Research, Rockville, MD, USA) [56]. Microarray data are available from Gene Expression Omnibus (GEO, http://www. ncbi. nlm.nih.gov/geo/) with the accession number GSE92672.

Gene Ontology (GO) annotation analysis

The functional annotation analysis of the co-up and downregulated gene lists was carried out using the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://www.david.abcc.ncifcrf.gov/) based on GO annotation [57], as well as GO terms of BP, MF and CC. The annotation with a false discovery rate (FDR) was adjusted. P <0.05 was considered significant.

Statistical analysis

All values are expressed as mean ± SD. To determine significance between two groups, comparisons were made using Student's *t*-test by Graphpad Prism V5.0 (Graphpad Software. San Diego, CA, USA). $P < 0.05$ was considered significant.

RESULTS

1. The generation of bovine eSLCs by somatic cell nuclear transfer

Optimization of culture condition for bovine eSLCs by various combination of inhibitors

To find proper culture condition for bovine eSLCs, IVF bovine blastocysts were seeded and cultured under several combinations of PD184352, SU5402 and CHIR99021. Among all combination groups tested, only 3i combination group was able to maintain bovine eSLC colonies over 20 passages (Table 2).

Embryo production and the generation of bSCNT-eSLC colonies

SCNT bovine embryos showed normal development up to the blastocyst stage *in vitro*. The ZP-free blastocysts were obtained mechanically by mouth-pipetting (Fig. 4A) and seeded onto the feeder layer with 3i culture medium. The rate of attachment and primary colony formation were evaluated. Initially, 2 or 3 blastocysts were seeded into each well, and 7 different eSLC colonies were generated from 21 ZP-free blastocysts in the 9 different wells (Table 3). Most ZP-free blastocysts were attached on the feeder layer within 3 days after seeding, and the primary colonies started to appear after that. In Group 1 where 2 embryos were seeded in each well, 7 out of the 12 blastocysts successfully attached (58.3%), and in Group 2 where 3 embryos were seeded in each well,

Table 2. Comparison of various bovine embryonic stem cell culture media

	No. of stem cell colonies at[*]				
	Passage 0[**]	*Passage 3*	*Passage 5*	*Passage 11*	*Passage 15*
PD184352	5	1	0	0	0
CHIR99021	5	1	1	0	0
SU5402 + CHIR99021	5	3	1	0	0
PD184352 + CHIR99021	5	3	2	2	0
3i[**]	22	22	21	20	20

[*]When passaging, only one piece of four slices of the colony, which had been selected randomly, was passaged and maintained for this experiment.

[**] Three blastocysts per well

[***]PD184352 + CHIR99021 + SU5402

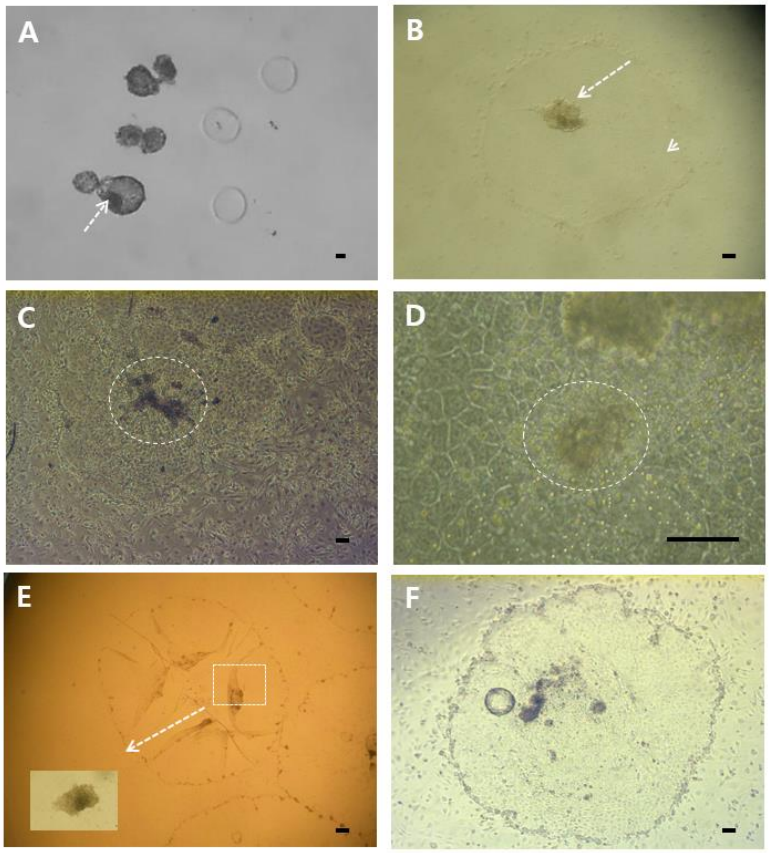


Figure 4. Generation of bovine somatic cell nuclear transfer embryo-derived stem-like cell (bSCNT-eSLC) colony. (A) Zona-pellucida-free blastocysts. The arrow indicates the intact ICM. (B) A primary bSCNT-eSLC colony. The arrow indicates central multilayer (CMt) part and the arrow head points to the boundary between the feeder cell layer and the peripheral monolayer (PMn) part of the eSLC colony. (C) The colony at passage 20 and (D) high magnification picture of the cell colony. The circle indicates the multilayer part of the bSCNT-eSLC colony. (E) Split cell slices for passaging. Only the central multilayer portion of the slice (dotted rectangle and small picture box at bottom-left) was collected for passaging. (F) Example of a colony with unexpected cystic cavities, which was not further passaged. Scale bar = 100 μm.

Table 3. Generation of bovine somatic cell nuclear transfer embryo-derived stem-like cells in multi-embryo seeding groups

	Group 1[a]	*Group 2*[b]	*Total*
No. of total embryos	12	9	21
No. of total wells	6	3	9
No. of attached blastocysts (%)	7 (58.3)	7 (77.7)	14 (66.6)
No. of outgrown primary colonies (%)	7 (58.3)	7 (77.7)	14 (66.6)
No. of stem cell lines over passage 50 (%)	4 (33.3)	3 (33.3)	7 (33.3)

[a]Two blastocysts per well

[b]Three blastocysts per well

7 out of 9 blastocysts attached (77.7%) on the feeder layer cells. In this experiment, every attached blastocyst grew well and formed a primary colony. This is comparable to the previous reports (13-67%) [29].

Although most previous studies also used a multi-embryo seeding method [6, 58-60], here, I attempted single-embryo seeding for the creation of single custom stem cell lines, because it is important for various future applications based on individual genetic traits. Hence, bSCNT-eSLC colonies were generated from the single-seeding group where only one ZP-free blastocyst was seeded in each well. In total, 8 out of 11 ZP-free blastocysts attached well on the feeder layer, and all of those grew successfully (Table 4). In this experimental group, the rate of primary colony formation was 72.7%, which was comparable to the multi-seeding groups (66.6%; Table 3). Moreover, all primary colonies developed into eSLC colonies, which went over P50. The efficacy of cell colony establishment was more than two times higher in the single seeding group than in the multi-seeding group (72.7% vs. 33.3%). In the same way, IVF and parthenogenetic single-embryo seeding were also able to generate eSLC colonies (Table 5).

Morphology of bSCNT-eSLC colonies

A primary bSCNT-eSLCs colony is spherical in shape with well recognized boundaries (Fig. 4B). The primary colony is quite large in its size and similar to a human ESC colony [2]. The colony can be divided into two morphological regions, CMt and PMn (Fig. 4B). Cells from CMt are smaller and more condensed in shape, while cells from PMn are large and flat like epithelial cells

Table 4. Generation of bovine somatic cell nuclear transfer embryo-derived stem-like cells in the single-embryo seeding group

Series	*No. of used embryos*	*No. of attached blastocysts (%)*	*No. of primary colonies (%)*	*No. of stem cell lines over passage 50 (%)*
1	6	4 (66.6)	4 (66.6)	4 (66.6)
2	5	4 (80.0)	4 (80.0)	4 (80.0)
Total	11	8 (72.7)	8 (72.7)	8 (72.7)

Table 5. Comparison of various bovine embryonic stem cell culture media

	*No. of stem cell colonies at**				
	Passage 0	*Passage 3*	*Passage 5*	*Passage 11*	*Passage 15*
PD184352	5	1	0	0	0
CHIR99021	5	1	1	0	0
SU5402 + CHIR99021	5	3	1	0	0
PD184352 + CHIR99021	5	3	2	2	0
3i**	22	22	21	20	20

*When passaging, only one piece of four slices of the colony, which had been selected randomly, was passaged and maintained for this experiment.

**PD184352 + CHIR99021 + SU5402

(Fig. 4C, D); these morphologies correspond with previous reports [58, 61]. During passaging, the CMt cells were separated and interposed randomly within the middle of the colony but can still be discriminated from the monolayer portion. Only the CMt cells were selected to generate and propagate progeny colonies (Fig. 3 and Fig. 4E). The progeny colonies maintained their PSC characteristics, including a CMt and a PMn with clear boundaries. If the colony showed unclear CMt, unexpected cystic cavities (Fig. 4F), or various differentiated cell types were found, the colony was discarded.

Cytogenetic and molecular characteristics of bSCNT-eSLCs

All established bSCNT-eSLC colonies were maintained in culture over 50 passages for more than 270 days with normal karyotypes consisting of 60 chromosomes at P50 (58XX, Fig. 5A). Microsatellite DNA analyses examining eleven loci to confirm that the bSCNT-eSLC colonies are genetically identical to the donor fetal fibroblasts used for SCNT (Fig. 5B).

The presence of BrdU-positive cells in the CMt region of the colony during passaging was observed, indicating that bSCNT-eSLCs are able to proliferate under the 3i culture system after repetitive subculture (Fig. 6A). ESC must meet several criteria; at a bare minimum is alkaline phosphate (AP) staining analysis. The bSCNT-eSLCs identified in this study are positive to AP activity regardless of their morphological differences, although cells from the CMt display much stronger AP activity (Fig. 6B). In addition, expression of pluripotency and TE specific markers were also confirmed by RT-PCR analysis (Fig. 6C).

With the exception of *FGF4*, the naïve state PSC markers (*Rex-1*, *Klf2, Klf4,*

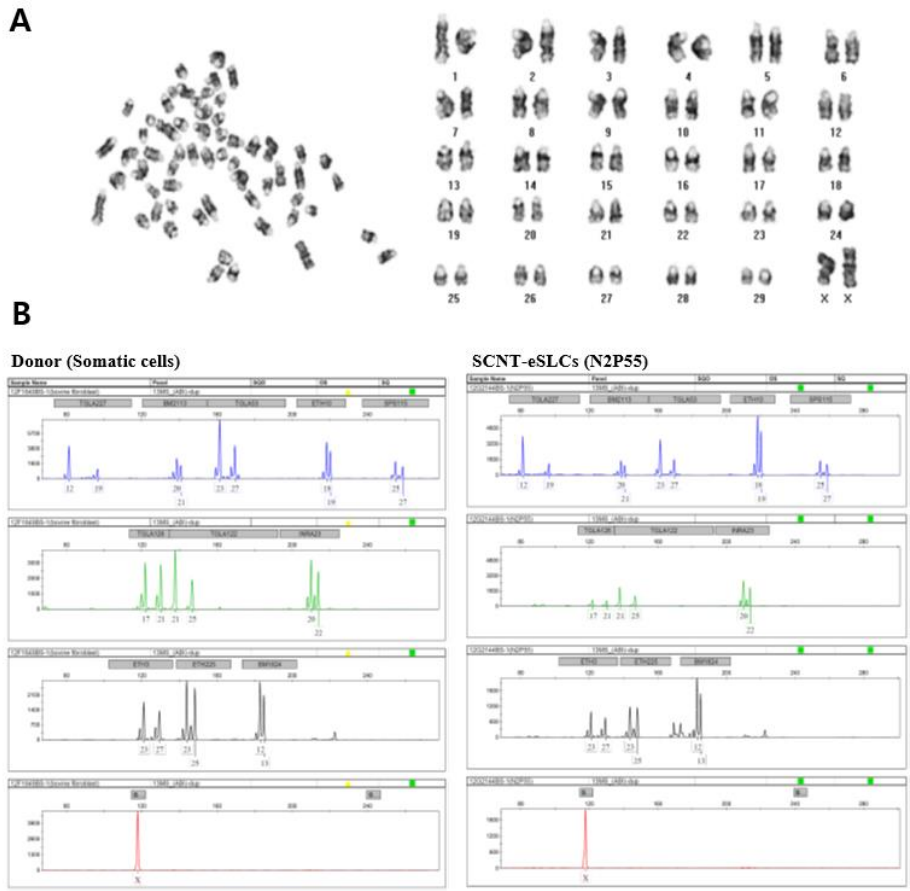
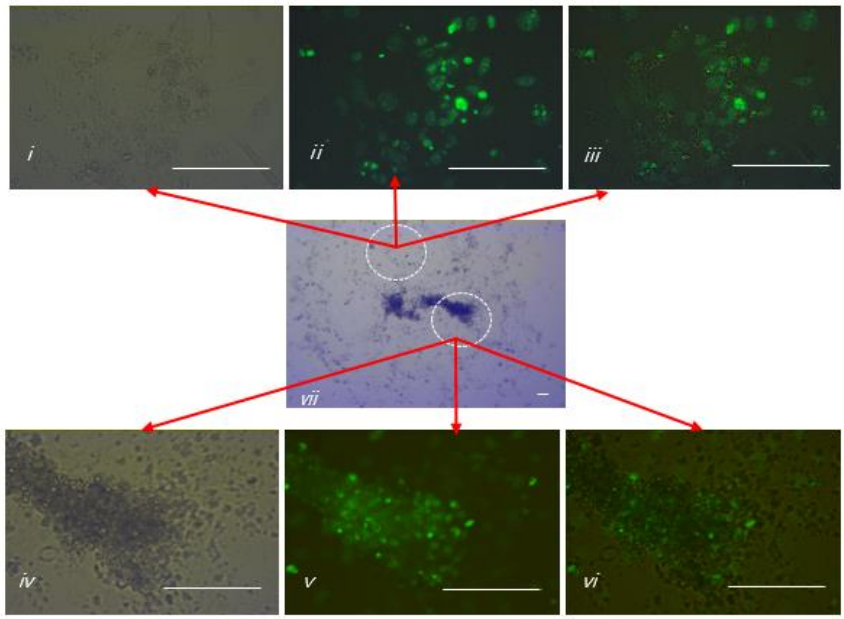
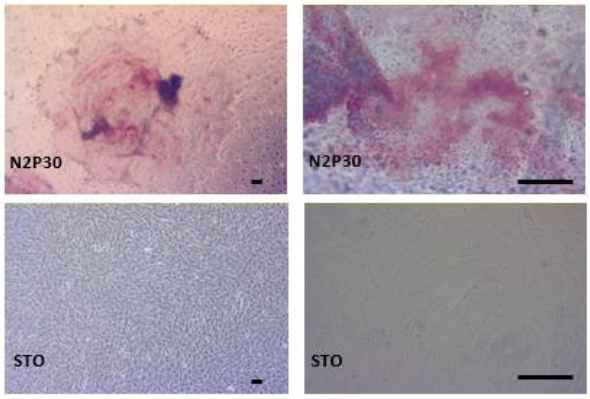


Figure 5. Cytogenetic characteristics of bovine somatic cell nuclear transfer embryo-derived stem-like cell (bSCNT-eSLC) colonies. (A) A normal karyotype consisting of 60 chromosomes (58XX) at P55 (N2P55; N2 line at passage 55). (B) Microsatellite DNA analyses examination of eleven loci of *Tgla227*, *Bm2113*, *Tgla53*, *Eth10*, *Sps115*, *Tgla126*, *Tgla122*, *Inra23*, *Eth3*, *Eth225* and *Bm1824* confirm that the bSCNT-eSLC colony is genetically identical to the donor fetal fibroblasts used for nuclear transfer.

A



B



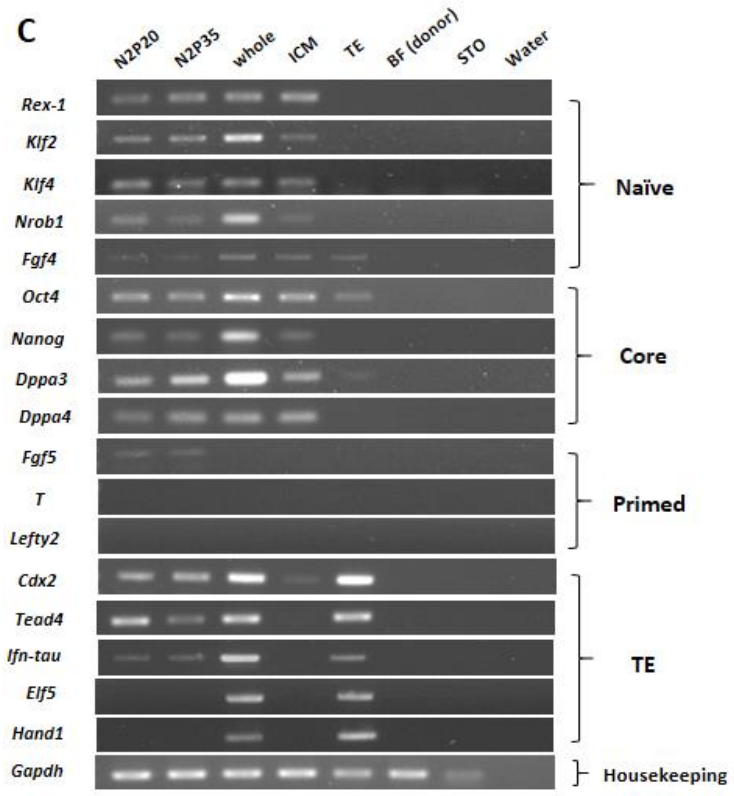


Figure 6. Molecular characteristics of bovine somatic cell nuclear transfer embryo-derived stem-like cell (bSCNT-eSLC) colonies. (A) 5-bromo-2'-deoxyuridine (BrdU) labeling of bSCNT-eSLC colony at P30 (N2P30). BrdU-positive cells are revealed in both peripheral monolayer (*i-iii*) and central multilayer (*iv-vi*) regions. All enlarged pictures (*i-vi*) were from the dotted circles in the colony (*vii*). (B) Alkaline phosphatase activity assay at P30 (N2P30). The bSCNT-eSLC colonies were positive for AP activity across the whole colony regardless of their morphological differences. The central multilayer portion displayed much stronger AP activity. STO cells were negative control. (C) Expression of the pluripotency and trophectoderm specific marker genes by RT-PCR analysis in bSCNT-eSLCs at P20 (N2P20) and P35 (N2P35), inner cell mass (ICM), trophoblast (trophectodermal cell; TE), whole embryo (whole), STO (feeder) and bovine fibroblast (BF as nuclear donor). The pluripotency markers were also classified naïve, core and primed in accordance with murine embryonic stem cells. Scale bar = 100 μm, T = *T-brachyury*.

Nrob1 and *Fgf4*) and core-pluripotency related factors (*Oct4, Nanog, Dppa3* and *Dppa4*) were highly expressed in the bSCNT-eSLCs. Among the primed state PSC markers, however, only *Fgf5* showed expression whereas *T-brachyury* and *Lefty2* were not expressed. These cells also expressed some typical TE specific markers such as *Cdx2, Tead4* and *Ifn-tau* whereas other TE markers, *Elf5* and *Hand1*, were not expressed.

All specific marker genes were not expressed in either the STO feeder cells or bovine adult fibroblasts used as nuclear donors. In TE from the blastocysts, only *Oct4*, *Dppa3*, *Fgf4* and TE specific markers were expressed. Immunofluorescence analysis indicates that the bSCNT-eSLCs were positive to the pluripotency markers such as OCT4, SOX2, NANOG, SSEA-4, TRA-1-60 and TRA-1-81 (Fig. 7). Positive expression of immunofluorescence markers was generally concentrated in and around the CMt.

In vitro and in vivo differentiation of bSCNT-eSLCs

To demonstrate *in vitro* differentiation potential of the eSLCs, EBs were formed from bSCNT-eSLCs and both cystic and solid types of EBs were obtained (Fig. 8A). Then, the generated EBs were analyzed by RT-PCR for specific markers of ectodermal, endodermal and mesodermal genes as well as for pluripotency genes (Fig. 8B). To confirm their *in vivo* differentiation potency, teratoma formation induction was attempted by introducing bSCNT-eSLCs under the testis capsule of nude mice. The transplanted cells formed exophytic tumorous mass (Fig. 9A, B). Even though after 8 weeks of cell introduction the tumors did not show complete differentiation of the three-germ layers, the tumors were

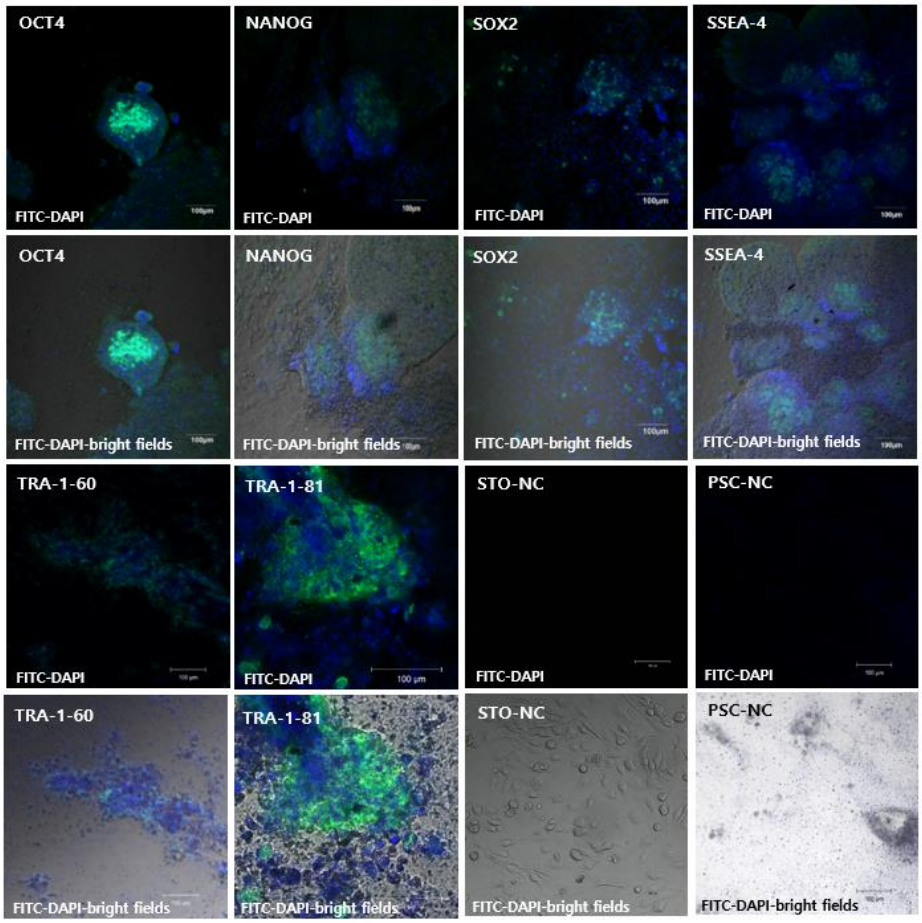


Figure 7. The images from immunofluorescence assay at P30. Bovine somatic cell nuclear transfer embryo-derived stem-like cells (bSCNT-eSLCs) are positive for the pluripotency markers OCT4, SOX2, NANOG, SSEA-4, TRA-1-60 and TRA-1-81. Fluorescence is generally highlighted in and around the central multilayer area. STO (feeder) and bSCNT-eSLCs without primary antibody were used as negative controls (STO-NC and eSLC-NC, respectively). Scale bar = 100 μm.

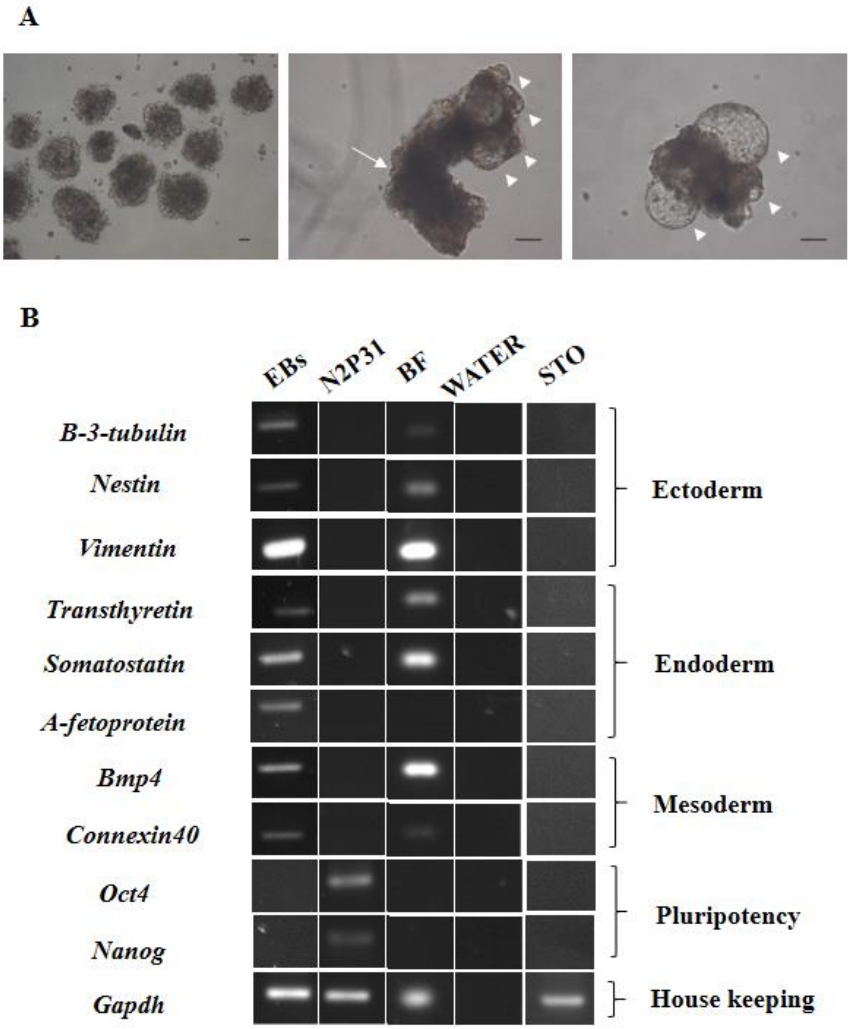


Figure 8. *In vitro* differentiation of bovine somatic cell nuclear transfer embryo-derived stem-like cells at P31 (N2P31). (A) Formed embryoid bodies (EBs) after 10 day of floating culture. The arrow and arrowheads indicate solid part and cystic part of EBs respectively. Scale bar = 100 μm. (B) Gene expression analyses of EBs by RT-PCR. The three germ layer specific markers of ectodermal (*β-3-tubulin, Nestin* and *Vimentin*), endodermal (*Somatostain, Tansthyretin* and *α-Fetoprotein*) and mesodermal (*Connexin40* and *Bmp4*) genes were expressed, while pluripotent marker genes of *Oct4* and *Nanog* were absent. BF: bovine fibroblast (nuclear donor).

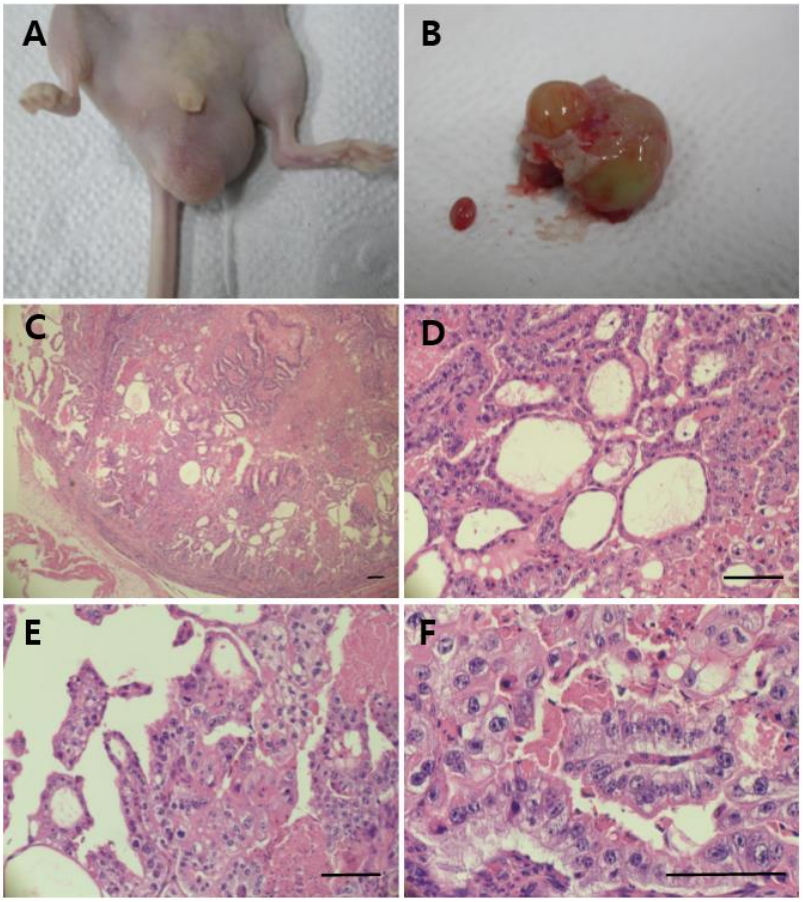


Figure 9. *In vivo* differentiation of bovine somatic cell nuclear transfer embryo-derived stem-like cells (bSCNT-eSLCs) at P31 (N2P31). (A and B) The embryonic carcinoma formed under the testis capsule of a nude mouse. (C) Representative histologic feature of the bSCNT-eSLC-derived embryonic carcinoma. The tumor consists of large primitive cells with areas of glandular (D) and papillary (E) formations. (F) The nuclei were large and overlapping, and prominent multiple nucleoli were present. Hematoxylin and eosin staining; Scale bar = 100 μm

typical embryonal carcinomas (Fig. 9C) formed by large primitive cells with areas of glandular (Fig. 9D) and papillary formations (Fig. 9E). The nuclei were large and overlapping, and nucleoli were multiple and prominent (Fig. 9F).

Comparative analysis of CMt and PMn

Since both pluripotency and TE specific marker genes are expressed in bSCNT-eSLCs, specific lineage markers of OCT4, NANOG and CDX2 were analyzed by immunofluorescence staining to localize the marker protein expression within the colony (Fig. 10). Although OCT4 and CDX2, or NANOG and CDX2 were largely co-localized throughout the whole colony (arrowheads, Fig. 10B, F), OCT4- or NANOG-only positive cell population without CDX2 expression also existed in CMt region of the colony (arrow, Fig. 10B, F). For the verification of more specific stem cell properties of the colony, cell population from CMt and PMn regions were separately passaged and analyzed. The colony derived from CMt presented small and condensed cells, whereas the colony from PMn revealed large and flat epithelial-like cells (Fig. 11A). The outgrowth rate and the number of AP-positive colonies were also remarkably different between two regions (Table 6 and Fig. 11B, C). Most colonies from CMt were able to outgrow while the large portion of colonies from PMn failed to maintain after passaging (80% vs. 25%) in this 3i culture condition. The AP staining results also showed that the numbers of AP-positive colonies from CMt are much higher than those from PMn (100% vs. 10%). The expression levels of pluripotency genes (*Oct4*, *Nanog* and *Sox2*) in CMt were significantly higher than those in PMn by Real-time PCR analysis, while the expression pattern of TE genes (*Cdx2*, *Tead4*, *Gata3* and *Ifn-tau*) was *vice versa* ($P<0.05$, Fig. 12).

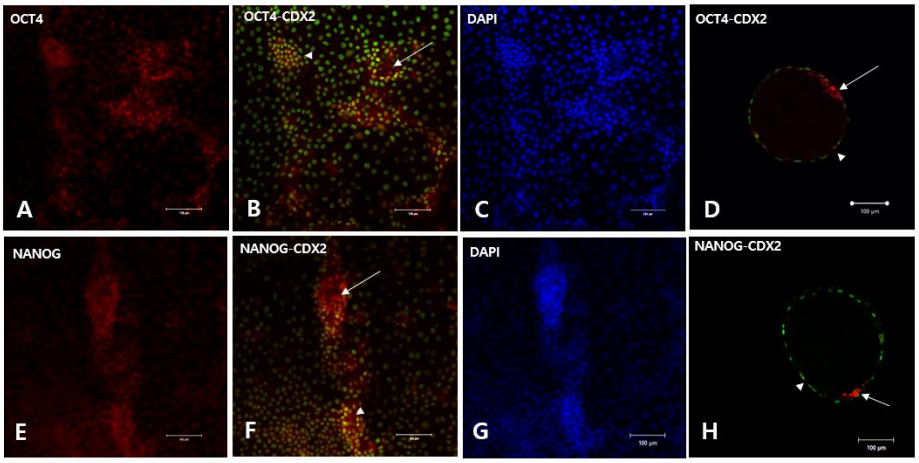


Figure 10. Immunofluorescence image of lineage specific marker expression in the central multilayer (CMt) part cells of the colony at P30 (N2P30). (A-C) Images from OCT4 (red) and CDX2 (green) stained cells. Nucleus was stained with DAPI (blue) (D) Distribution of OCT4 and CDX2 expressing cells in normal bovine blastocyst as positive control. (E-G) Images from NANOG (red) and CDX2 (green) stained cells. Nucleus was stained with DAPI (blue) (H) Distribution of NANOG and CDX2 expressing cells in normal bovine blastocyst as positive control. The arrowhead indicates the OCT4- or NANOG-positive cells with CDX2 expression. The arrow indicates OCT4- or NANOG-positive cells without CDX2 expression. Scale bar = 100 μm.

Table 6. Comparison of outgrowth rate and alkaline phosphatase activity between central multilayer cells and peripheral monolayer cells

	No. of passaged colonies			
	Total	*AP positive*		*Outgrowth (%)*
		Strong (%)	*Weak (%)*	
Central multilayer cells	20	15 (75)	1 (25)	16 (80)
Peripheral monolayer cells	20	0	2 (10)	5 (25)

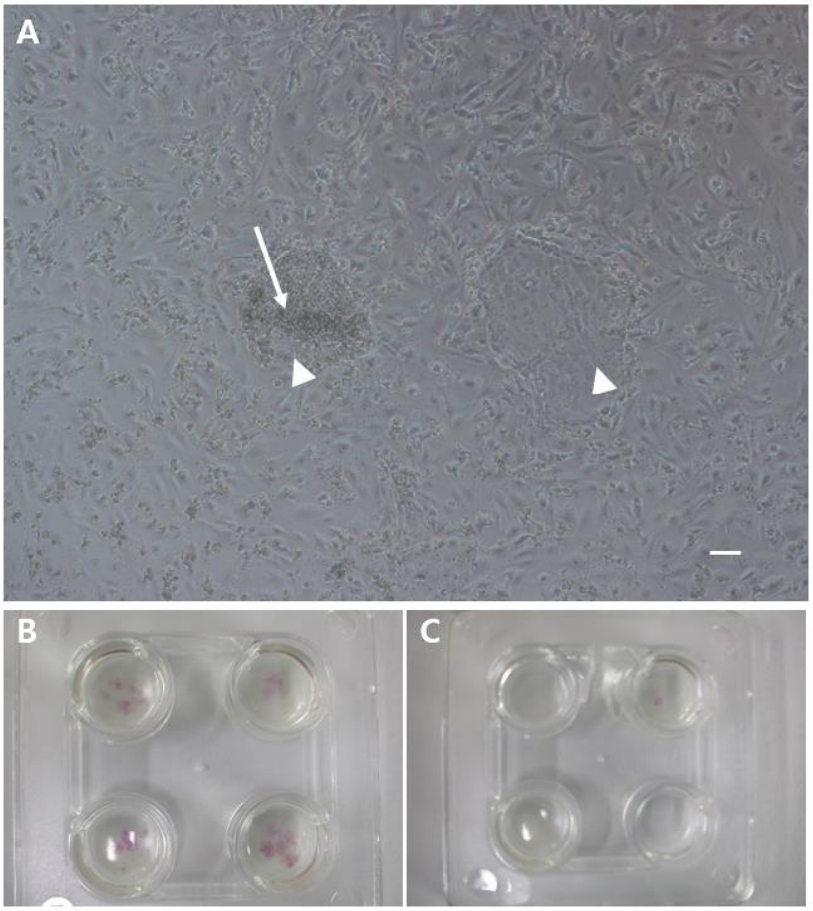


Figure 11. The comparison of central multilayer (CMt)-derived colonies and peripheral monolayer (PMn)-derived colonies after passaging. (A) The morphology of CMt-derived (Left) and PMn-derived passaged colony (Right). The arrow indicates small and condensed cells which are generally found in CMt. The arrowhead indicates large and flat cells generally found in PMn. (B and C) Alkaline phosphatase (AP) activity assay demonstrates that (B) most CMt-derived passaged colonies exhibit strong AP activity (C) while only few PMn-derived passaged colonies show weak AP activity. Scale bar = 100 μm.

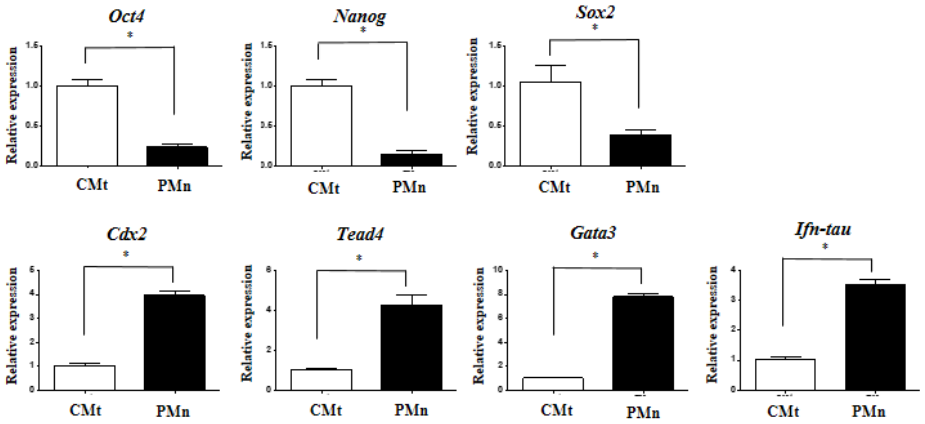


Figure 12. Quantitative gene expression analyses of central multilayer (CMt) and peripheral monolayer (PMn) parts of the colony at P31 (N2P31). The expression of pluripotency (*Oct4*, *Nanog* and *Sox2*) and trophectoderm (*Cdx2*, *Tead4*, *Gata3* and *Ifn-tau*) markers were analyzed by real-time PCR. The cells in CMt part have higher expression of pluripotency specific marker genes than those in PMn part, whilst TE specific marker expression shows the opposite results. Data were normalized to the expression level of *GAPDH* (house-keeping gene). **P*<0.05. N = 3.

2. Enhancing pluripotency of bovine eSLCs

The effect of thiazovivin on attachment

The ICM, TE or whole blastocyst at Days 7 to 9 was seeded and attached to the feeder layer, either in 3i or 3iT medium, and the rates of the attachment and the primary colony formation were evaluated. The attachment rates of the ICM, TE or whole blastocyst in 3iT medium (90.0%, 70.0% and 80.8%, respectively) were significantly greater ($P < 0.05$) than the rates in 3i medium (50.0, 40.0T and 46.2%, respectively). The formation rates of primary colonies from total seeded blastocysts in 3iT medium (50.0%, 35.0% and 76.9%, respectively) were also greater ($P < 0.05$) than the rates in 3i medium (25.0%, 25.0% and 38.5%, respectively). Neither the 3i nor 3iT culture conditions supported the proliferation of the TE-only part (data not shown), and among all experimental groups, the greatest yield of primary colonies was obtained from the 3iT medium group of the whole blastocyst seeding (Table 7). The results indicate that when generating eSLCs of cattle, cellular attachment and formation of primary colonies on the feeder layer can be increased by the whole blastocyst seeding and the thiazovivin treatment, using 3i medium condition.

The effect of thiazovivin on generation of eSLCs

The primary colonies achieved the outgrowth stage on the Day 7 of culture, when these colonies were passaged (Fig. 13). The colonies displayed a flat shape, were large in size and similar to a human ESC colony. The colony could be morphologically separated into two parts, the central multilayer and the

Table 7. Effect of thiazovivin on attachment of blastocyst and primary colony formation[†]

Group[*]	Part of embryo[**]	No. of total blastocysts	No. of attached/total blastocysts (%)	No. of primary colonies/total blastocysts (%)	Primary colonies/attached blastocysts (%)
3i	Whole	26	12 (46.2)[a]	10 (38.5)[a]	83.3 [a]
	ICM	20	10 (50.0)[a]	5 (25.0)[a]	50.0 [b]
	TE	20	8 (40.0) [a]	5 (25.0)[a]	62.5 [b]
3iT	Whole	26	21 (80.8)[b]	20 (76.9)[b]	95.2 [a]
	ICM	20	18 (90.0)[b]	10 (50.0)[c]	55.6 [b]
	TE	20	14 (70.0)[b]	7 (35.0)[a]	50.0 [b]

[†] Three Biological replicates

*3iT: 3i culture medium supplemented with thiazovivin

**Part of embryos when seeding on feeder layer. Whole: zona pellucida-free whole embryo; ICM: inner cell mass part; TE: trophectoderm part

Within a column, means without a common letter (a, b, c) differed ($P < 0.05$)

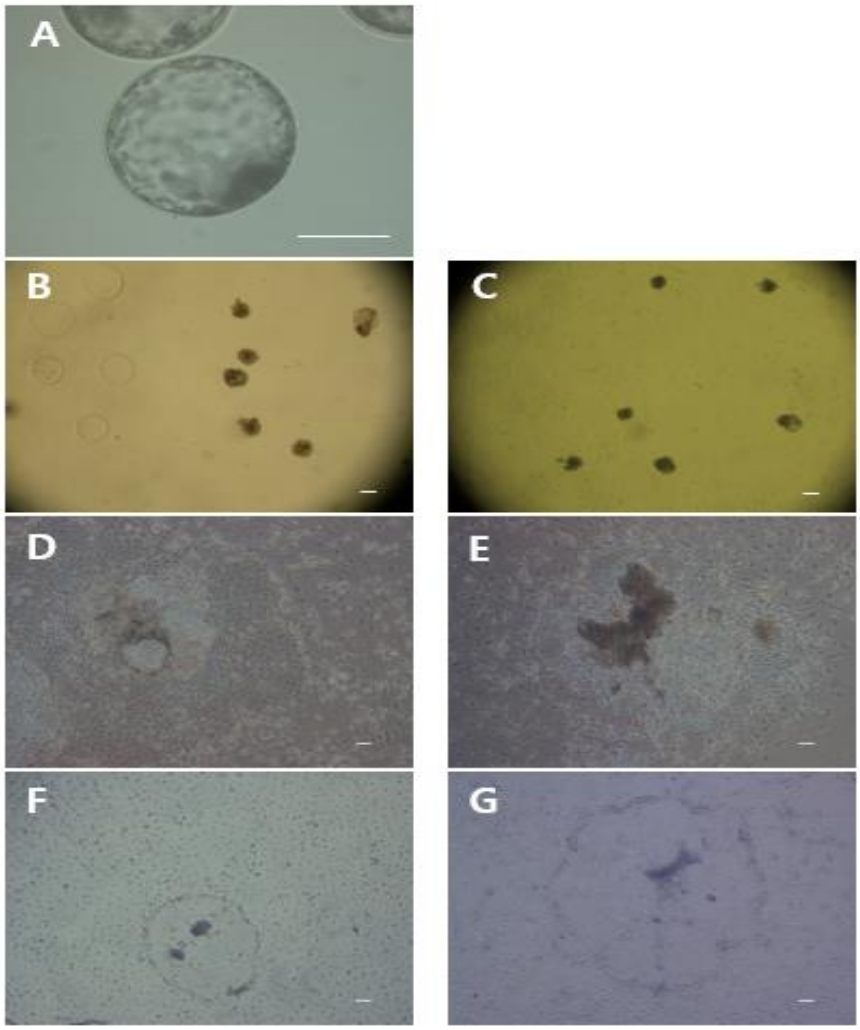


Figure 13. Morphology of embryo-derived stem-like cells (eSLCs) of cattle. (A) A good quality blastocyst after 7 days of *in vitro* fertilization (IVF). (B) Zona pellucida-free IVF blastocysts. (C) Blastocysts mounted on a STO feeder layer. Primary outgrown colony from the mounted blastocyst cultured in the medium containing either 3i (D) or 3i with thiazovivin (E). eSLC colony at passage 25 maintained in medium containing either 3i (F) or 3i with thiazovivin (G). Scale bar = 100 μm.

peripheral monolayer. The ES-like cell colonies at P15 were split by the mechanical method and cultured either in 3i or 3iT medium for passaging. The formation rate of outgrown colonies from the split putative colony that clumps in 3iT medium was greater than that in 3i medium after a subculture to P15. Therefore, thiazovivin treatment tends to reinforce putative colony outgrowth and supports the expansion of eSLC cultures during the subculture for passaging (Table 8).

Characterization of eSLCs

The putative eSLCs were passaged to P25 and the expression of pluripotency-related genes was analyzed by RT-PCR and qPCR. Under 3iT culture conditions, the expression of the pluripotency marker genes of *Oct4* and *Nanog* were greater than those under 3i control conditions (Fig. 14A, B). Naïve and primed pluripotency state-related markers were also analyzed. Naïve state markers were expressed in both groups, while *T* and *Lefty2*, the primed state markers, were not expressed (Fig. 14C). The eSLC colonies from both experimental groups were tested for the maintenance of an undifferentiated state by AP staining. The AP activity assays were positive in both groups (Fig. 15A, B). The ES cell-specific marker proteins such as OCT4, NANOG, SOX2 and TRA-1-60 were detected by immunofluorescence staining. The markers were all positive in the cultured colonies from both experimental groups (Fig. 15C-L). In the BrdU assay, the central part of eSLC colonies presented a BrdU-positive cells population (Fig. 16) and this confirmed the existence of actively growing cells in the central part.

Table 8. Effect of thiazovivin on outgrowth of primary colonies during *in vitro* culture[†]

Group	No. of putative colony clumps	No. (%) of outgrown colonies
3i	35	20 (57.1)[a]
3iT	35	31 (88.6)[b]

[†]Four Biological replicates

*3iT: 3i culture medium supplemented with thiazovivin

Within a column, means without a common letter (a, b) differed ($P < 0.05$)

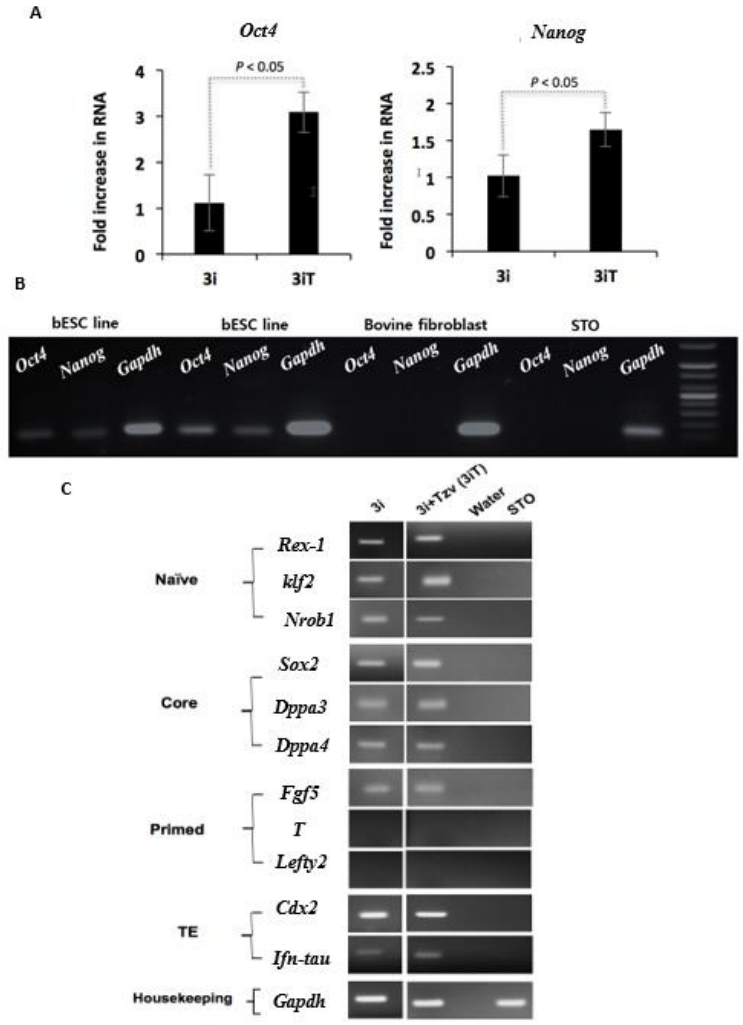


Figure 14. Gene expression analyses of pluripotency markers after thiazovivin treatment in culture. (A) Real-time quantitative PCR analysis of the pluripotency genes, *Oct4* and *Nanog*. All values are depicted as the level of expression relative to the 3i control group (values in 3i group = 1). Data represent means ± S.E.M from three experiments (n=3). The expression of the pluripotency marker genes Oct4 and Nanog was greater in the thiazovivin supplemented (3iT) group ($P < 0.05$). (B) The results of RT-PCR analysis also show that the 3iT group tends to express both pluripotency marker genes. (C) Naïve and primed pluripotency state marker genes were analyzed in 3i and 3iT groups by RT-PCR eSLCs at passage 25. Most marker genes are expressed in both groups except *T* (=*T-brachyury*) and *Lefty2*.

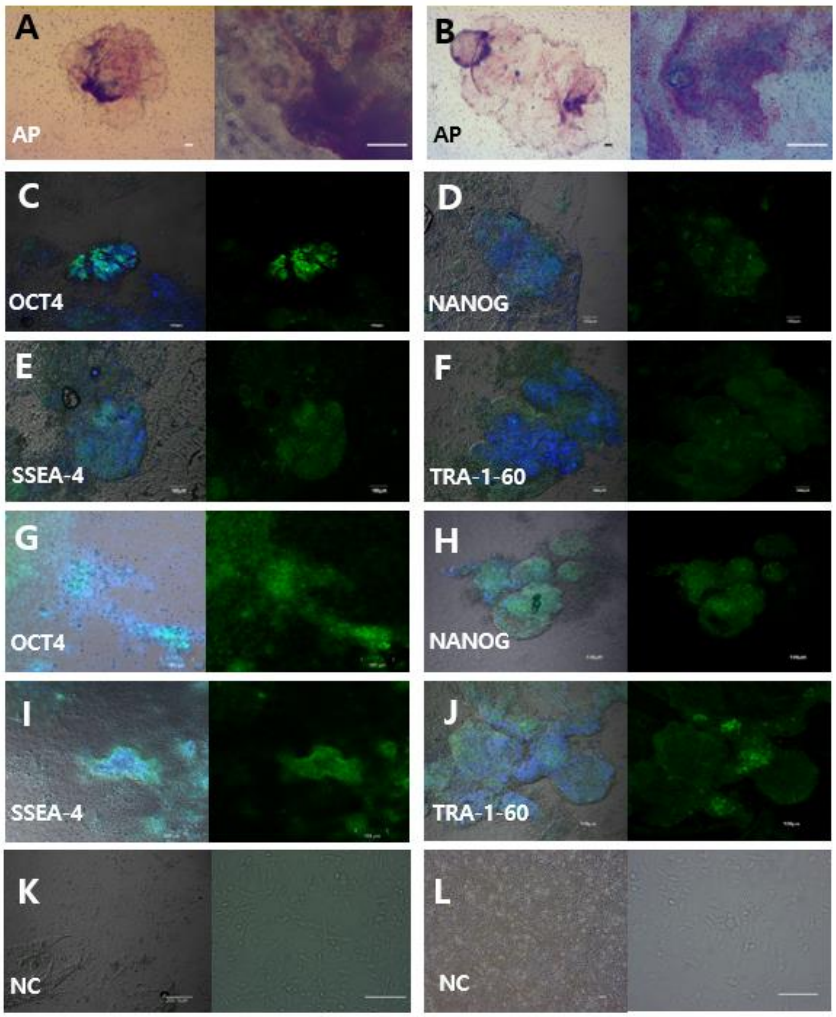


Figure 15. Immunofluorescence and alkaline phosphatase (AP) staining images of putative eSLCs of cattle cultured with or without thiazovivin. The cells cultured in the medium containing 3i only (A-D, K) or 3i with thiazovivin (E-H, L). (A-H) eSLC colonies were stained with OCT4, NANOG, SSEA-4 and TRA-1-60 antibodies as makers of embryonic stem cells (green) and with DAPI as a nuclear marker (blue). (I) Negative control without primary antibody. (J) STO cells as the feeder for eSLCs showing AP-negative. (K, L) The result of AP staining of cells (red); X40 (left) and X200 (right). All markers were positive in cultured colonies from both experimental groups. Scale bar = 100 μm (except left picture of I; Scale bar = 200 μm).

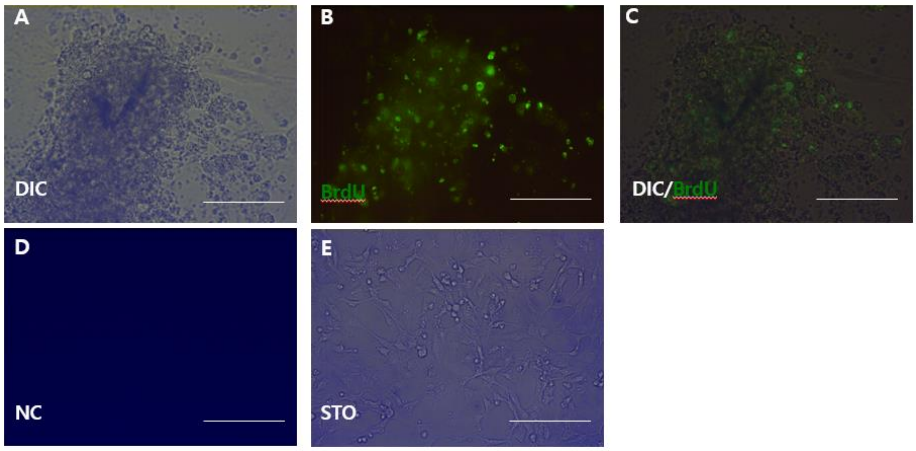


Figure 16. Proliferation of eSLCs of cattle. BrdU assay of eSLCs at passage 25. Central part of eSLC colony with dome-like shape (A) possessing BrdU-positive cell population (green; B, C). (D) Negative control without primary antibody. (E) Mitomycin C-treated STO cells showing BrdU-negative. Scale bar = 100 μm.

Detection of E-cadherin protein and gene in eSLCs

Immunofluorescence staining was performed to detect E-CADHERIN protein and qPCR analysis was conducted to analyze the expression of the *E-cadherin* gene in the eSLCs. The greater amount of E-CADHERIN protein was detected in the 3iT group compared with the 3i control group (Fig. 17A, B). The expression of the *E-cadherin* gene was also increased in the 3iT group when compared with the 3i control group (Fig. 17D).

To confirm the ability of eSLCs to differentiate into three developmental lineages *in vitro*, EBs were made from eSLCs via floating culture on low attachment plates for 15 days (Fig. 18A). Both cystic and solid types of EBs were formed from the culture (Fig. 18A) and solid type EBs were used for RT-PCR analysis. The pluripotency-related genes, *Oct4* and *Nanog*, were not detected whereas lineage-specific marker genes were expressed in EBs. The EBs derived from eSLCs treated with thiazovivin treatment tended to have greater expression of ectodermal lineage-specific genes than those derived from cells cultured without thiazovivin. In contrast, the result of endodermal lineage marker expression analyses was vice versa (Fig. 18B).

3. Microarray analysis of bovine eSLCs

Comparison of eSLCs and somatic cells (SCs)

To analyze the microarray data, I selected six different bovine eSLCs from three derivations of blastocysts: two IVP blastocysts, two NT blastocysts, and two

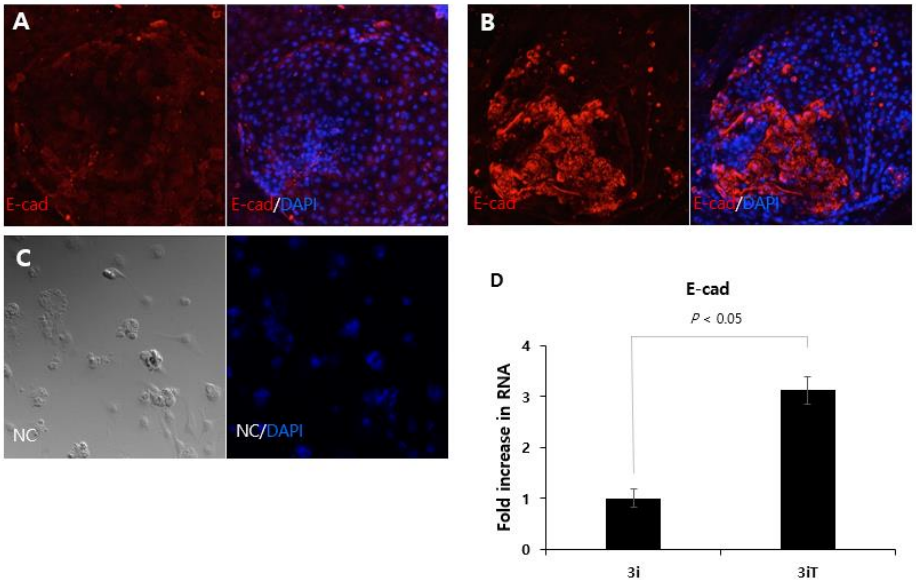


Figure 17. E-cadherin expression in putative eSLCs of cattle cultured with or without thiazovivin. Immunofluorescence staining images of E-CADHERIN (red) and DAPI (blue) in eSLCs. Cells were cultured in medium containing 3i only (A) or 3i with thiazovivin (3iT) (B) and negative control without primary antibody (C), X100. Higher levels of E-CADHERIN protein were detected in 3iT group compared with the 3i control group. (D) The expression of the *E-cadherin* gene was analyzed by real-time quantitative PCR (n=3). Values depicted by the rate to the expression in 3i control group (values in 3i group = 1). Data represent means ± S.E.M from three experiments. The expression of the *E-cadherin* gene was also greater in the 3iT group ($P<0.05$).

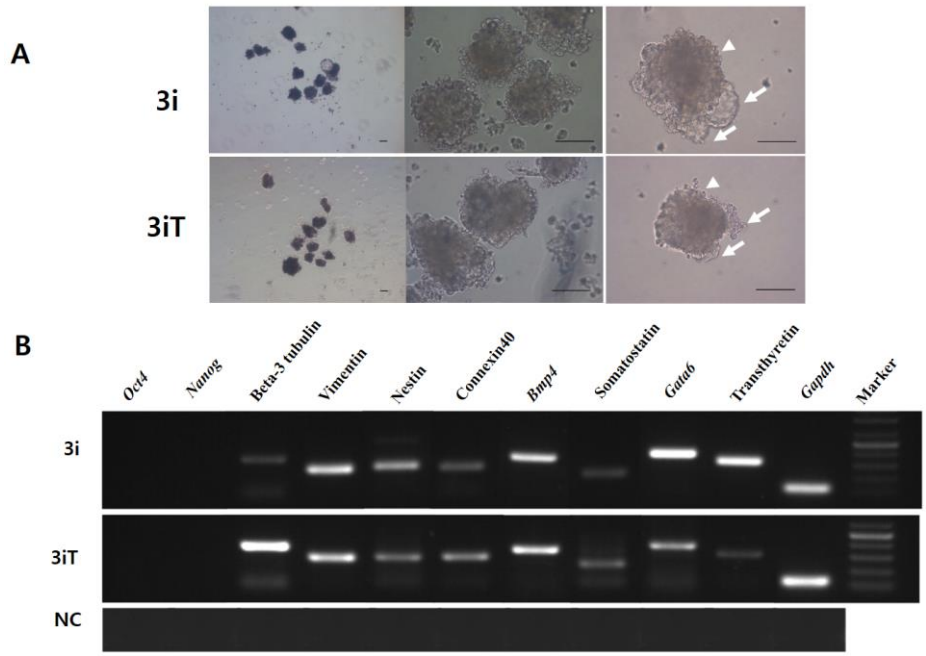


Figure 18. Formation and characterization of embryoid bodies (EBs) from putative eSLCs of cattle. (A) The EBs were formed by floating cultures of eSLCs maintained either in 3i or 3i with thiazovivin (3iT). The cell aggregates displayed a spherical morphology (solid and cystic type) in both experimental groups. Arrow represents the cystic part and Arrow head do the solid part. (B) Expression analysis of three germ layer marker genes in EBs of solid type by RT-PCR; Ectodermal (*Beta-3-tubulin*, *Vimentin* and *Nestin*), Mesodermal (*Connexin40* and *Bmp4*), Endodermal lineage-related genes (Somatostatin, *Gata*6 and Transthyretin) and pluripotency–related genes, as well as pluripotency markers *Oct4* and *Nanog*. The EBs formed in the 3iT group tended to have greater expression of ectodermal lineage specific genes whereas the result of endodermal lineage marker expression was vice versa. Negative control (NC) used water in place of sample cDNA. Scale bar = 100 μm.

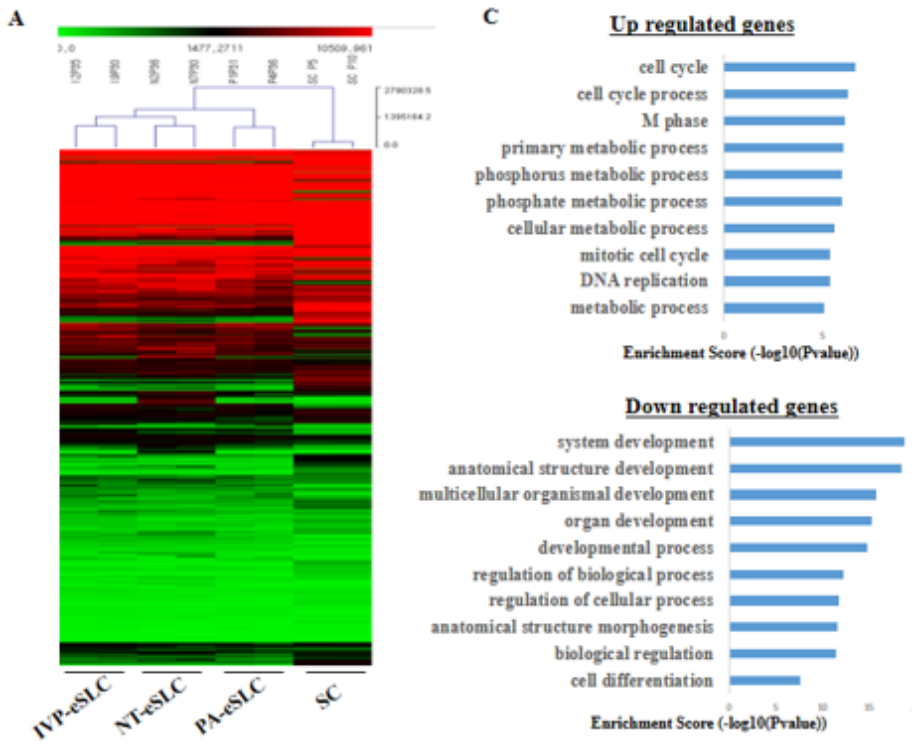
PA blastocysts. The lists of differentially expressed genes (DEGs), determined using an absent/present (A/P) classification and ≥ 2-fold difference as cut-offs, are available from GEO with the accession number GSE92672, and 10,203 genes were selected (Fig. 19A).

The selected 10,203 genes were used to compare groups. To improve the accuracy of gene expression alteration as DEGs, I compared the normalized single value of each sample and the average value of each sample. Finally, significant differences in gene expression were confirmed by real-time PCR.

To investigate characteristics of bovine eSLCs, they were compared with SCs. Hierarchical clustering with the 10,203 genes showed that there was little difference in gene expression among the six different eSLCs. Conversely, all eSLCs had significantly different gene expression from SCs (Fig. 19A).

Differences between eSLCs and SCs

To further investigate specific differences between eSLCs and SCs, eSLCs from IVP-blastocysts (IVP-eSLCs) were selected as typical eSLCs, because they originated from an IVP-blastocyst produced by a sperm and an oocyte, similar to normal fertilization *in vivo*. When I compared IVP-eSLCs and SCs, 3,414 genes were observed as DEGs: 1,552 of those genes were up-regulated and 1,862 genes were down-regulated (Fig. 19B). There were 289 GO terms in the BP group that were enriched by adjusting the FDR (P<0.05) for up-regulated genes. The 10 dominant GO terms were listed and the most of them (9 of 10 terms) were related to metabolic activity or cell cycle (Fig. 19C). There were also 419 GO terms in the BP group that were enriched by adjusting the FDR


A
IVP-eSLC
NT-eSLC
PA-eSLC
SC
C
Up regulated genes
cell cycle
cell cycle process
M phase
primary metabolic process
phosphorus metabolic process
phosphate metabolic process
cellular metabolic process
mitotic cell cycle
DNA replication
metabolic process
Enrichment Score (-log10(Pvalue))
Down regulated genes
system development
anatomical structure development
multicellular organismal development
organ development
developmental process
regulation of biological process
regulation of cellular process
anatomical structure morphogenesis
biological regulation
cell differentiation
Enrichment Score (-log10(Pvalue))


B
IVP-eSLCs vs. SCs
1862
1552
Up-regulated genes
Down-regulated genes
D
Pluripotency related DEG
12
39
Up-regulated genes
Down-regulated genes

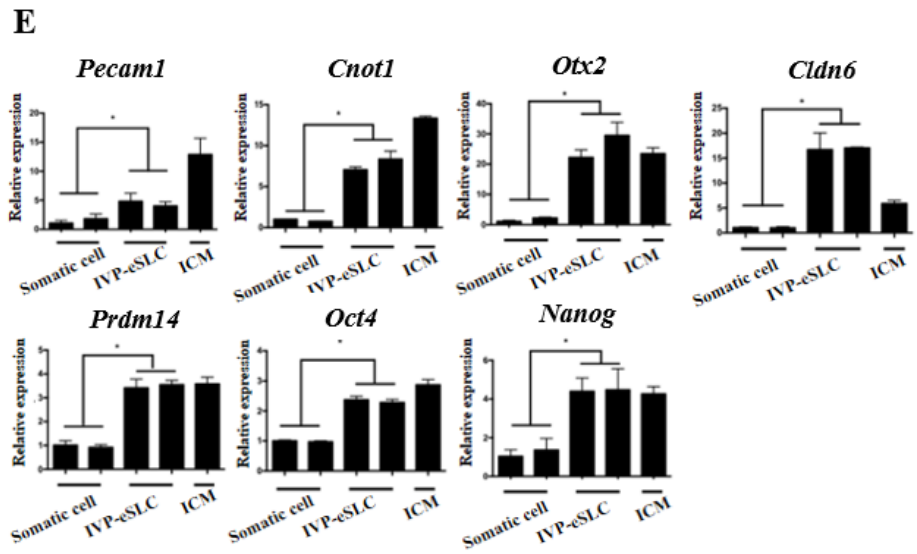


Figure 19. Comparison of gene expression between embryo-derived stem-like cells (eSLCs) and somatic cells (SCs) in cattle. (A) Hierarchical cluster of *in vitro* production (IVP)-, nuclear transfer (NT)-, parthenogenesis(PA)-eSLCs, and SCs. The gene expression pattern from three eSLCs are countlessly different from SCs. (B) Venn diagram of all differently expressed genes (DEGs) in IVP-eSLCs and SCs. (C) Top 10 biological processes associated with significantly up-regulated and down-regulated genes in IVP-eSLCs and SCs. (D) Venn diagram of DEGs related to pluripotency in IVP-eSLCs and SCs. (E) Gene expression profiles of representative genes related to pluripotency. These genes are highly expressed in three eSLCs, compared with the genes in SCs. ICM is also presented as a control. *P<0.05 (n=3)

(P<0.05) for down-regulated genes. The 10 dominant GO terms were listed and the many of them (6 of 10 terms) were related to development or cell differentiation (Fig. 19C).

To further investigate the properties of cultured IVP-eSLCs, I also analyzed pluripotency related genes. During the analysis, the microarray data were screened by GO terms (GO:0019827) related to stem cell maintenance. Among the 144 genes, 39 genes were up-regulated and 12 genes were down-regulated (Fig. 19D). Interestingly, these included core pluripotency markers including *Oct4* and *Nanog* as well as other markers that have not yet been identified in pluripotency, such as *Pecam1*, *Cnot1*, *Cldn6*, *Foxo1*, *Prdm14,* and *Otx2* (Fig. 19D). These genes were also confirmed by real-time PCR (Fig. 19E).

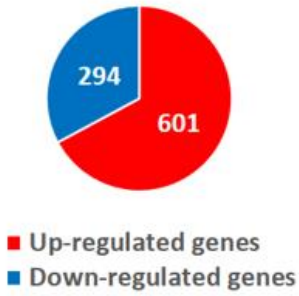
Gene expression profiles among eSLCs from three different origins

In order to further investigate characteristics of eSLCs, IVP-eSLCs were compared with PA- or NT-eSLCs. First, I examined the pattern of DEGs between NT- and IVP-eSLCs and identified 895 DEGs, with 601 up-regulated and 294 down-regulated genes (Fig. 20A). Although 77 chromatin remodeling related genes (GO:0006338) were not in the major group, they were also profiled between NT- and IVP-eSLCs. Only 5 genes, *Hmga1*, *Padi4*, *Chdil*, *Sycp3,* and *Padi2,* were revealed as DEGs in this study (Fig. 20B), and their expression patterns were confirmed by real-time PCR (Fig. 20C).

Next, the gene expression pattern in between PA- and IVP-eSLCs was analyzed. A total of 346 genes were differently expressed between PA- and IVP-eSLCs, with 78 up-regulated genes and 268 down-regulated genes (Fig. 20D).

A

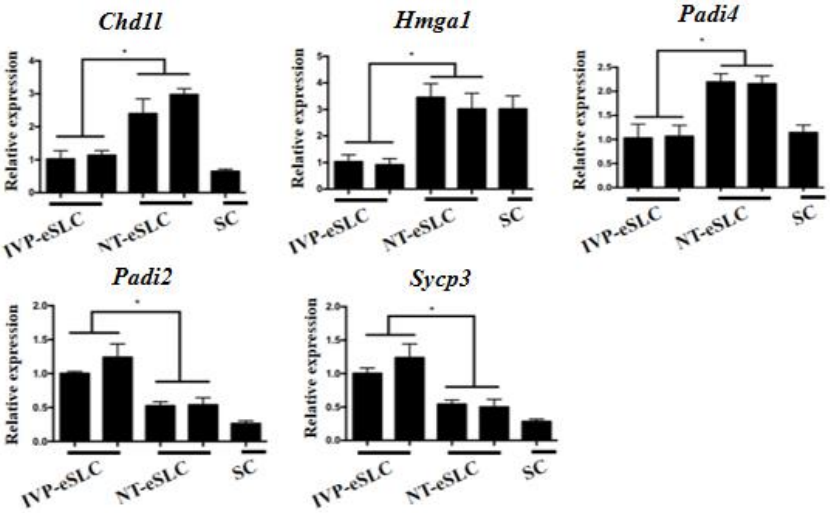
NT-eSLCs vs. IVP-eSLCs



B

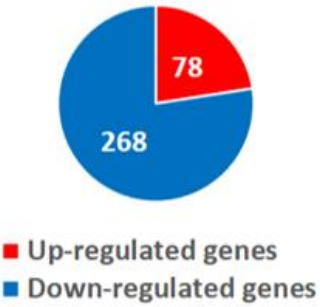
Chromatin remodeling gene

Gene	Fold change
Hmga1	4.727089
Padi4	2.1589981
Chd1l	2.0584542
Sycp3	0.4635187
Padi2	0.4597359



D

PA-eSLCs vs. IVP-eSLCs



E

Imprinting gene

Gene	Fold change
Phlda2	5.5497327
Ascl2	2.7512835
H19	2.6649958
Meg3	2.6293358
Tssc4	2.4949528
Igf2r	2.4294826
Igf2	0.450536
Snrpn	0.4065958
Nap1l5	0.3000457
Peg3	0.2890593
Plagl1	0.2464155

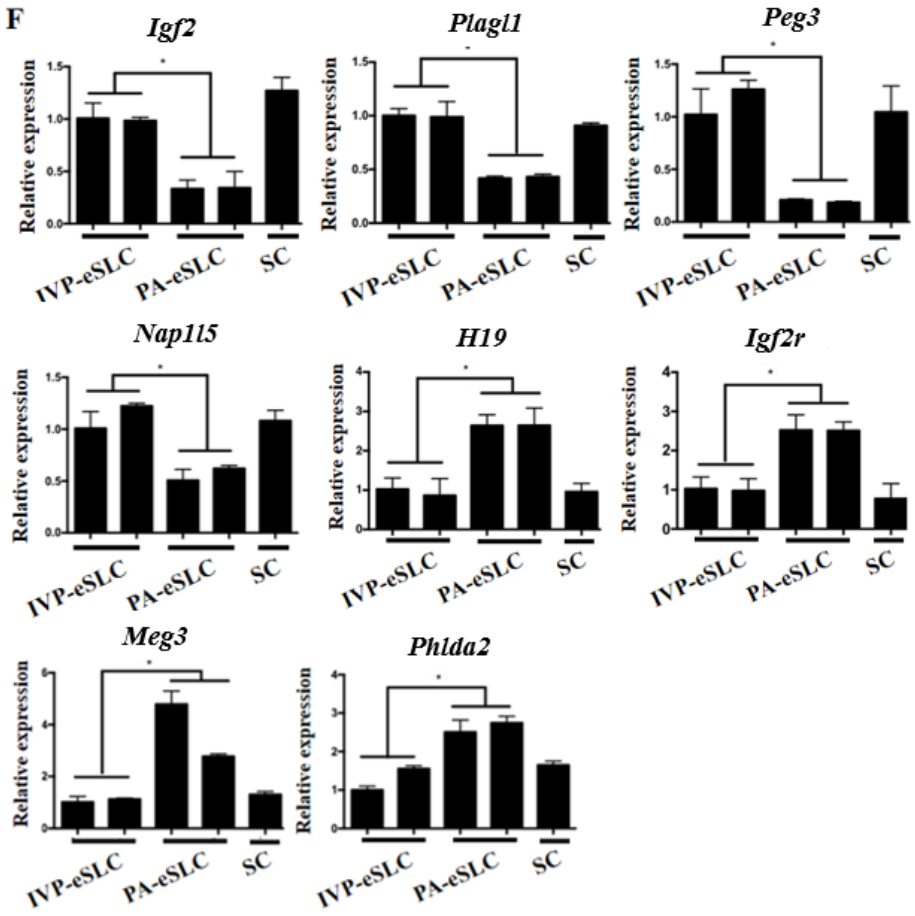


Figure 20. Comparison of differently expressed genes (DEGs) among embryo-derived stem-like cells (eSLCs) from three different origins. (A) Venn diagram of all DEGs in nuclear transfer-eSLCs (NT-eSLCs) and *in vitro* production-eSLCs (IVP-eSLCs). (B) Chromatin remodeling genes in NT-eSLCs and IVP-eSLCs. (C) Gene expression profiles of DEGs related to chromatin remodeling. (D) Venn diagram of all DEGs in parthenogenesis-eSLCs (PA-eSLCs) and IVP-eSLCs. (E) Imprinting genes in PA-eSLCs and IVP-eSLCs. The expression of paternally expressed imprinting genes is increased in PA-eSLCs compared with the gens in IVP-eSLCs, while maternally expressed imprinting genes are *vice versa*. (F) Gene expression profiles of DEGs related to imprinting. Somatic cells are also presented as a control. *P<0.05 (n=3)

Although these were not in the major group, 12 imprinting related genes were included in these DEGs (Fig. 20E). Surprisingly, among these genes, PA-eSLCs had higher expression of *Phlda2*, *Ascl2*, *H19*, *Meg3*, *Tssc4,* and *Igj2r* as imprinted maternally expressed genes than IVP-eSLCs (Fig. 20F). On the other hand, the expression of 5 imprinted paternally expressed genes, *Igf2*, *Snrpn*, *Nap1l5*, *Peg3,* and *Plagl1*, was down-regulated in PA-eSLCs compared to IVP-eSLCs (Fig. 20E). These genes were also confirmed by real-time PCR (Fig. 20F).

The expectation of signaling pathways for bovine pluripotency

Although there are many studies of stem cells, little is known about the signaling pathways related to pluripotency in bovines. Therefore, the co-expression pattern of whole genes in eSLCs may be a valuable tool for the discovery of important pathways related to pluripotency in bovines. To elucidate these pathways in more detail, I specifically searched for co-expressed genes that may be related to signaling pathways for pluripotency, and the biological pathways were analyzed by the Kyoto Encyclopedia of Genes and Genomes (KEGG) database in bovines [62]. In co-up-regulated genes among eSLCs, I identified 2,415 DEGs, with 1,014 co-up-regulated genes and 1,401 co-down-regulated genes (Fig. 21). By the KEGG database, there were 54 signaling pathways in DEGs, and some of them were related to the maintenance of pluripotency, including TGFβ, WNT, and LIF signaling (Fig. 21). In TGFβ signaling, the BMP family and SMAD family were contained in DEGs and several key genes were confirmed by real-time PCR (Fig. 22).

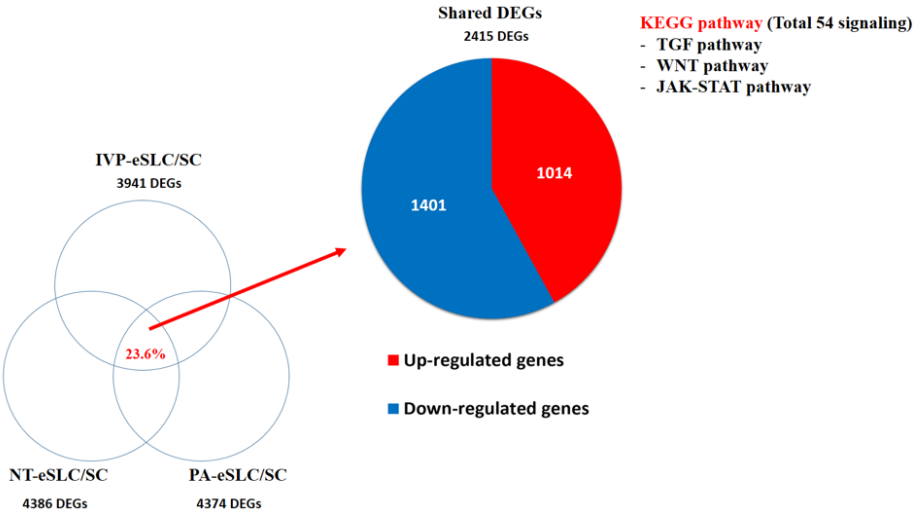
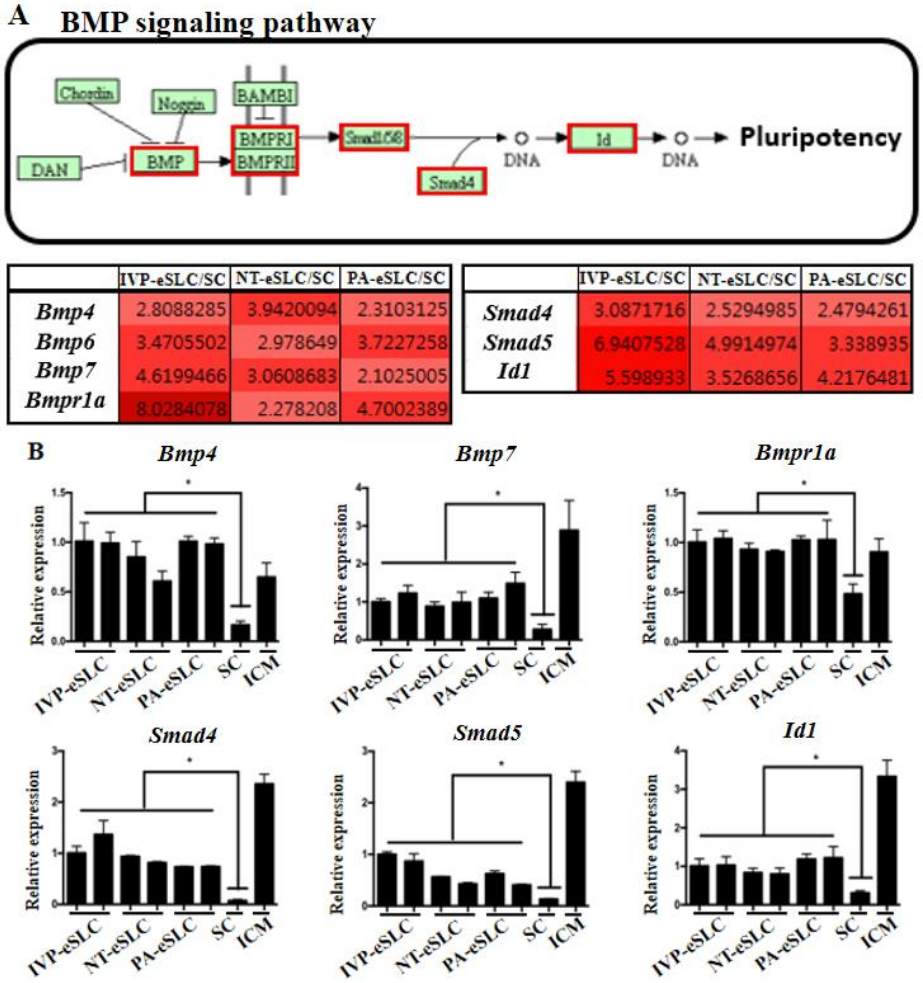


Figure 21. Differently expressed genes (DEGs) in embryo-derived stem-like cells (eSLCs) and the analysis of distinct pathways related to pluripotency. In total of 10203 genes, the DEG numbers of *in vitro* production (IVP)-, nuclear transfer (NT)-, parthenogenesis (PA)-eSLCs are 3941, 4386 and 4374, respectively. Among them, co-expressed DEGs are 2415 (23.6%). By KEGG analysis of the co-expressed DEGs, there are 54 signaling including TGF-β, WNT, and LIF pathways which are strongly related to pluripotency.



	IVP-eSLC/SC	NT-eSLC/SC	PA-eSLC/SC
Bmp4	2.8088285	3.9420094	2.3103125
Bmp6	3.4705502	2.978649	3.7227258
Bmp7	4.6199466	3.0608683	2.1025005
Bmpr1a	8.0284078	2.278208	4.7002389

	IVP-eSLC/SC	NT-eSLC/SC	PA-eSLC/SC
Smad4	3.0871716	2.5294985	2.4794261
Smad5	6.9407528	4.9914974	3.338935
Id1	5.598933	3.5268656	4.2176481

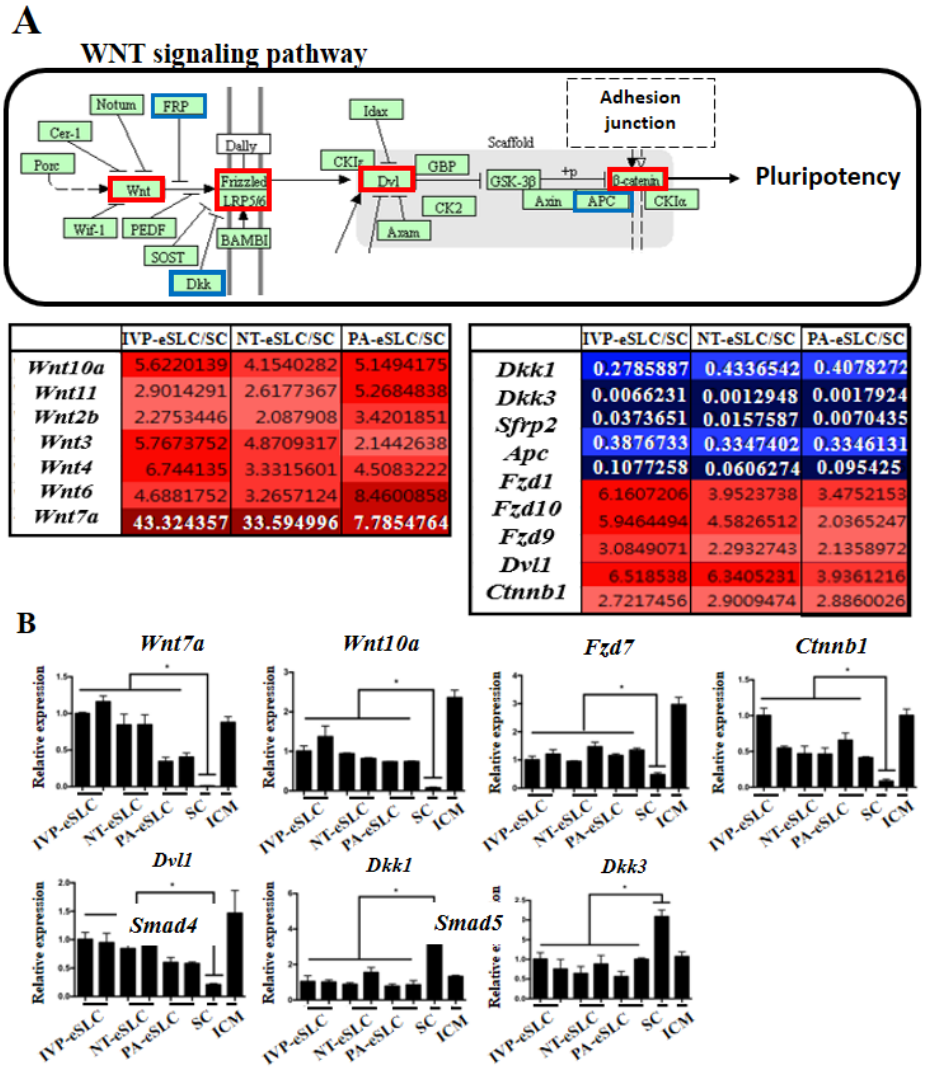
Figure 22. BMP signaling pathway in embryo-derived stem-like cells (eSLCs). (A) KEGG pathway map of BMP signaling related to core transcriptional network for pluripotency. Most differently expressed genes (DEGs) related to BMP signaling are up-regulated in eSLCs compared with the gens in somatic cells. The boxes outlined with red indicate relatively up-regulated DEGs. Fold change value is also provided with red in the table below (A). (B) Gene expression profiles of DEGs related to the BMP signaling pathway. ICM and somatic cell (SC) are also presented as a control. **P*<0.05 (n=3).

In WNT signaling, 19 genes such as *Wnt7a*, *Wnt10a*, *Fzd7*, *Dkk1,* and *Dvl1* were included in DEGs, and core genes were confirmed by real-time PCR (Fig. 23). In LIF signaling, *Lif*, *Stat3,* and *Socs3* were identified as DEGs and confirmed by real-time PCR (Fig. 24).

The expression pattern of tumor-related genes in eSLCs

To elucidate abnormal teratoma formation in bovine eSLCs, I attempted to examine 82 oncogenes and 63 tumor suppressor genes among eSLCs, as described on the Cancer Genes website and via literature searches [63]. Among the oncogenes, 7 co-up-regulated genes and 23 down-regulated DEGs were identified in eSLCs (Fig. 25A). The expression of key genes in down-regulated DEGs was confirmed by real-time PCR (Fig. 25B). Among tumor suppressors, 30 DEGs, with 21 co-up-regulated genes and 9 co-down-regulated genes were also identified (Fig. 25C). The expression of primary genes in up-regulated DEGs was confirmed by real-time PCR (Fig. 25D).

I also investigated *Defb1*, *Defb3*, *Defb7,* and *Smad3,* which may be related to teratoma formation. Interestingly, according to the data, *Smad3* expression was decreased, while the expression of *Defb1*, *Defb3,* and *Defb7* was increased in eSLCs compared to SCs (Fig. 25E). These genes were also confirmed by real-time PCR (Fig. 25F).



	IVP-eSLC/SC	NT-eSLC/SC	PA-eSLC/SC
Wnt10a	5.6220139	4.1540282	5.1494175
Wnt11	2.9014291	2.6177367	5.2684838
Wnt2b	2.2753446	2.087908	3.4201851
Wnt3	5.7673752	4.8709317	2.1442638
Wnt4	6.744135	3.3315601	4.5083222
Wnt6	4.6881752	3.2657124	8.4600858
Wnt7a	43.324357	33.594996	7.7854764

	IVP-eSLC/SC	NT-eSLC/SC	PA-eSLC/SC
Dkk1	0.2785887	0.4336542	0.4078272
Dkk3	0.0066231	0.0012948	0.0017924
Sfrp2	0.0373651	0.0157587	0.0070435
Apc	0.3876733	0.3347402	0.3346131
Fzd1	0.1077258	0.0606274	0.095425
Fzd10	6.1607206	3.9523738	3.4752153
Fzd9	5.9464494	4.5826512	2.0365247
Dvl1	3.0849071	2.2932743	2.1358972
Ctnnb1	6.518538	6.3405231	3.9361216
	2.7217456	2.9009474	2.8860026

Figure 23. The WNT signaling pathway in embryo-derived stem-like cells (eSLCs). (A) KEGG pathway map of WNT signaling related to core transcriptional network for pluripotency. Most differently expressed genes (DEGs) related to WNT signaling in eSLCs are up-regulated when compared with somatic cells (SCs), although some genes, such as *Fzd1* and *Apa*, are down-regulated. *Dkk1*, *Dkk3,* and *Sfrp2*, inhibitors of BMP signaling, are down-regulated in eSLCs. The boxes outlined with red indicate relatively up-regulated DEGs, while the ones outlined with blue point to relatively down-regulated DEGs. Fold change value is also provided with red (up-regulated genes) and blue (down-regulated genes) in the tables below (A). (B) Gene expression profiles of representative DEGs related to the BMP signaling pathway. ICM and somatic cell (SC) are also presented as a control. **P*<0.05 (n=3).

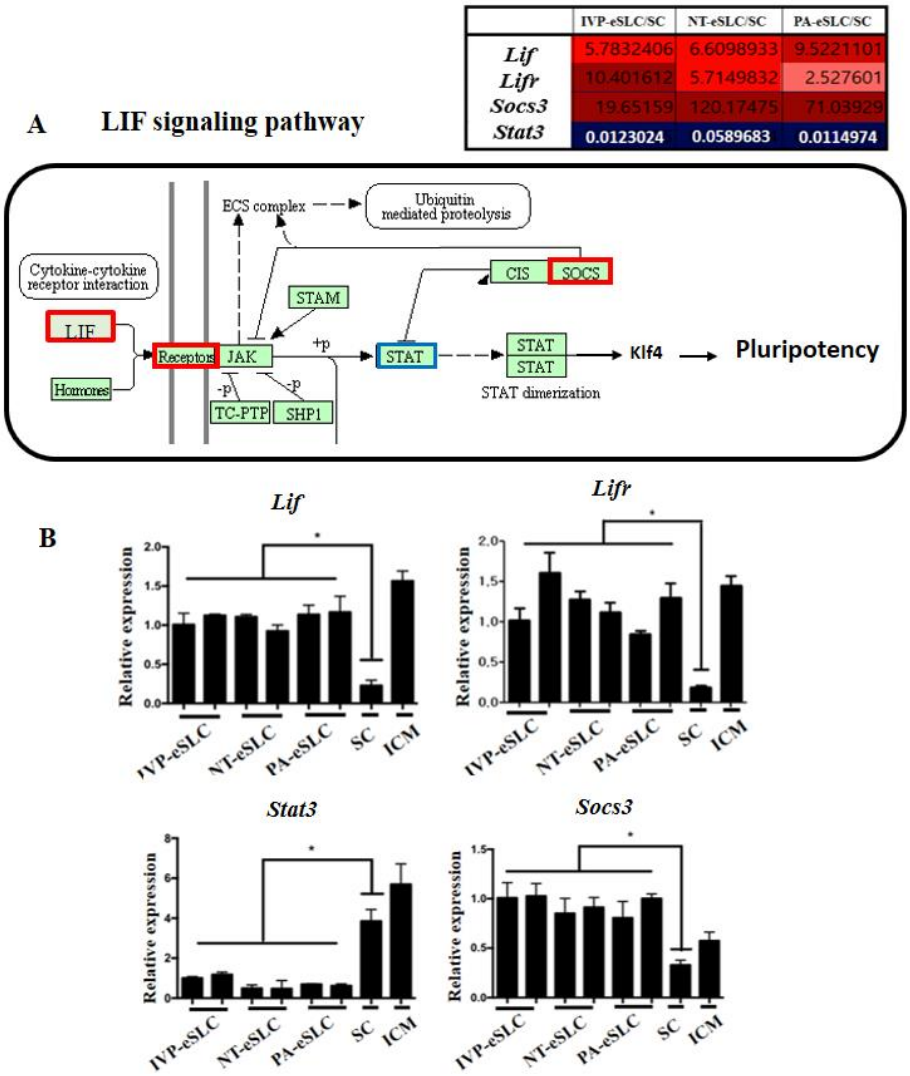
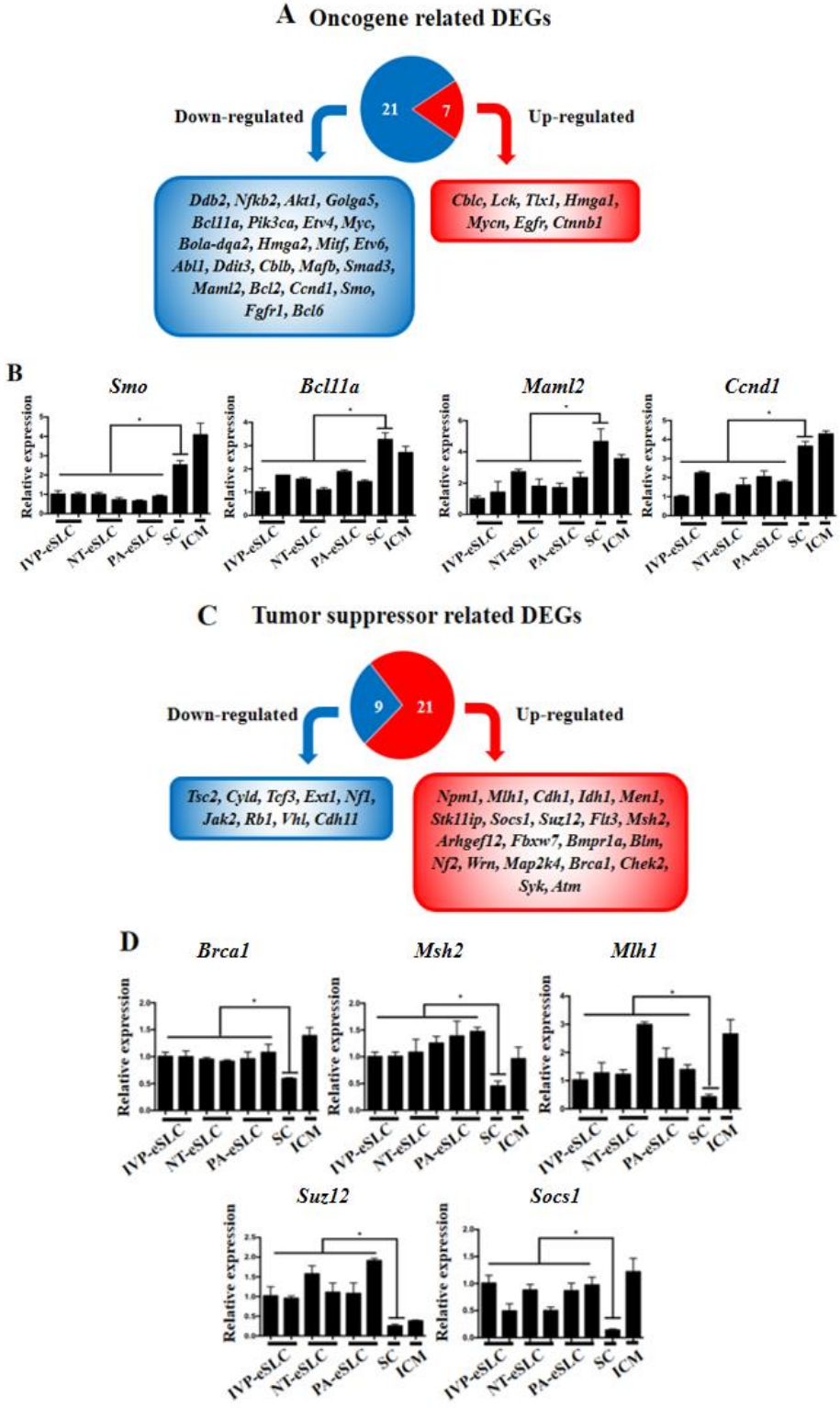


Figure 24. LIF signaling pathway in embryo-derived stem-like cells (eSLCs). (A) KEGG pathway map of LIF signaling related to core transcriptional network for pluripotency. *Lif*, *Lifr,* and *Socs3* genes are up-regulated in eSLCs when compared with somatic cells, while the *Stat3* gene is down-regulated. The boxes outlined with red indicate relatively up-regulated DEGs, while the ones outlined with blue mark relatively down-regulated DEGs. Fold change is also provided with red (up-regulated genes) and blue (down-regulated genes) in the table above (A). (B) Gene expression profiles of DEGs related to the LIF pathway. ICM and somatic cell (SC) are also presented as a control. *P<0.05 (n=3)


A Oncogene related DEGs
Down-regulated
21
7
Up-regulated
Ddb2, Nfkb2, Akt1, Golga5, Bcl11a, Pik3ca, Etv4, Myc, Bola-dqa2, Hmga2, Mitf, Etv6, Abl1, Ddit3, Cblb, Mafb, Smad3, Maml2, Bcl2, Ccnd1, Smo, Fgfr1, Bcl6
Cblc, Lck, Tlx1, Hmga1, Mycn, Egfr, Ctnnb1
B
Smo
Bcl11a
Maml2
Ccnd1
Relative expression
IVP-eSLC
NT-eSLC
PA-eSLC
SC
ICM
C Tumor suppressor related DEGs
Down-regulated
9
21
Up-regulated
Tsc2, Cyld, Tcf3, Ext1, Nf1, Jak2, Rb1, Vhl, Cdh11
Npm1, Mlh1, Cdh1, Idh1, Men1, Stk11ip, Socs1, Suz12, Flt3, Msh2, Arhgef12, Fbxw7, Bmpr1a, Blm, Nf2, Wrn, Map2k4, Brca1, Chek2, Syk, Atm
D
Brca1
Msh2
Mlh1
Suz12
Socs1

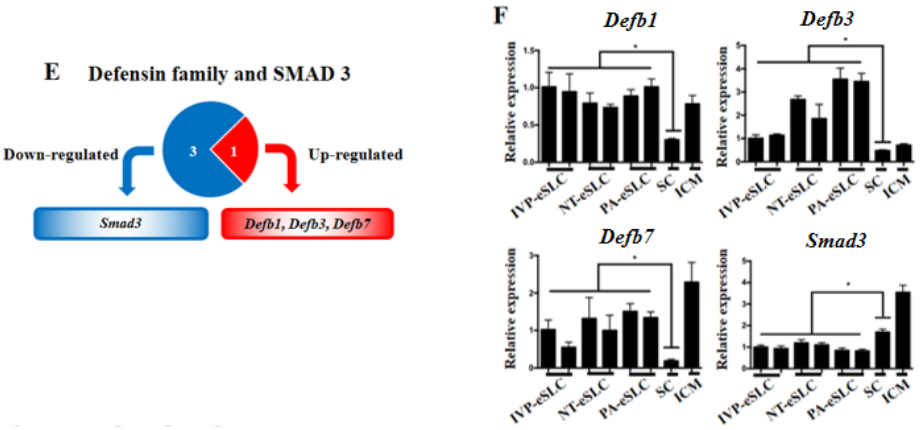


Figure 25. Oncogene- and tumor suppressor-related differently expressed genes (DEGs) in embryo-derived stem-like cells (eSLCs). (A) Venn diagram shows 7 up-regulated and 23 down-regulated DEGs related to oncogenes. (B) Gene expression profiles of representative differently expressed oncogenes. (C) Venn diagram shows 21 up-regulated and 9 down-regulated DEGs related to tumor suppressors. (D) Gene expression profiles of representative differently expressed tumor suppressors. (E) Venn diagram shows DEGs related to the Defensin family (tumor suppressor) and *Smad3* (oncogene). (F) Gene expression profiles of DEGs related to the Defensin family and *Smad3*. ICM and somatic cells (SCs) are also presented as a control. **P*<0.05 (n=3)

DISCUSSION

1. The generation of bovine eSLCs by somatic cell nuclear transfer

The objective of the present topic was to establish bovine SCNT-eSLCs and to demonstrate their stem cell characteristics such as self-renewal, pluripotency and differentiation potentials. Although most early reports on the cultivation of bovine ES-like cells based on the system for mouse ESCs, failed to generate true bovine ESCs [6, 58-60], recent reports have demonstrated that treatment of cells with various small molecule inhibitors of FGF, MEK, and GSK3, can establish ESCs even for non-permissive species such as outbred mice and rats [3, 11]. FGF, MEK and GSK3 signaling pathways are important for stem cell differentiation and apoptosis in mammals [11, 16-19]. In my preliminary studies, three different culture media based on human and mouse ESC media with slight modifications failed to maintain putative bovine ESCs beyond the 15^{th} passage [64]. In recent reports, small molecules combinations were shown to efficiently establish ESCs [3, 15]. In my study, various combinations of three small molecule inhibitors were tested to investigate optimal culture media for bovine PSCs (Table 2); only the 3i supplemented medium maintained PSC colonies over 20. Although other studies have shown effectiveness of different combinations of small molecules [53], my results are in agreement with findings from experiments on parthenogenetic ESCs [15]. Furthermore, the efficacy of cell colonies establishment was approximately twice higher in the single-seeded group (~72.2%) than that of the multi-seeded group (~33.3%),

implying that the 3i culture system is appropriate to maintain bSCNT-eSLCs for long-term propagation.

One of the fundamental properties of PSCs is continued proliferative self-renewal. Indeed, CMt of bSCNT-eSLC colony also actively proliferated during culture (Fig. 6A) as indicated by positively labeling with BrdU. Characterization of stem cell properties in livestock ESCs routinely analyze AP activity [7]. Although there are species differences, staining for AP [7] activity is still an important criterion for identifying ESCs, especially in bovine studies where there lacks consensus on the use of other pluripotency specific markers such as SSEA-1, -3 and -4 [8, 9, 58, 65]. The bSCNT-eSLC colonies of the present study exhibited mosaic pattern of AP activity (Fig. 6B), with stronger AP staining in the CMt portion than the PMn, which may indicate a higher pluripotency potential of the central portion. Alternatively, heavy staining may also have contribution from the multilayers of cells in the central region or both (Fig. 6B). Previous studies have also shown heterogenous staining of AP activity in bovine ES-like cells [7, 61, 66].

The RT-PCR results suggest that the colonies cultured under the 3i system may include cells possessing pluripotency because they were positive for core pluripotency markers and importantly, positive for the naïve-specific markers (Fig. 6C). In many species including cattle, *Rex-1* (*Zfp42*), *Klf2, Klf4, Nrob1* and *Fgf4* are used as naïve pluripotency-specific markers, whereas *Fgf5, T-brachyury* and *Lefty2* are considered as primed pluripotency-specific [11, 53, 67]. Unlike other naïve pluripotency markers, *Fgf4* showed weak expression in colonies owing to the FGF- or MEK-inhibitor in 3i system. Interestingly, *Fgf4*

is also well-known to induce trophoblast stem cells (TSCs) in mice [68] and the gene was expressed in bovine TE as well (Fig. 6C). The small molecule inhibitors of the 3i system may interrupt or suppress differentiation into trophoblastic lineage and may help eSLCs to maintain their PSC character.

It has been documented that *Rex-1* is important for maintenance of pluripotency and is one of the key factors used to distinguish naïve and primed state pluripotency [11, 67]. *Rex-1* was reported to be expressed in both ICM and TE of bovine blastocyst [55]. However, in the present study, *Rex-1* was only detected in ICM of the blastocyst and cultured bSCNT-eSLCs. Different from other primed state PSC markers such as *T-brachyury* and *Lefty2*, only *Fgf5* was expressed in bSCNT-eSLCs. This suggests that 3i culture system may still require additional component factor(s) to completely maintain naïve state pluripotency in cultured bovine embryonic cells. All primed state PSC markers were not detected in whole blastocysts, ICM and TE as these genes are normally expressed after implantation [69].

In the present study, it is necessary to use whole blastocyst stage embryo for the generation of bSCNT-eSLCs although the cells were from PMn part of the colony which included ICM-derived cells. Therefore, TE from bovine blastocyst was also analyzed with various specific markers for pluripotency and TE (Fig. 6C) to characterize the cultured cells. Different from bSCNT-eSLCs, only *Oct4*, *Dppa3*, *Fgf4* and TE specific markers were expressed in bovine TE in this study. *Oct4* was expressed in both bSCNT-eSLCs and TE whereas the expression of *Nanog* was only observed in bSCNT-eSLCs. According to some previous studies, *Oct4* and *Nanog* were expressed in bovine TE, and *Oct4*

expression did not differ between ICM and TE while *Nanog* expression was significantly higher in ICM than TE [28, 70]. It is interesting to note that *Dppa3*, unlike other pluripotency specific markers, is expressed in murine ICM [71], and was also expressed in bovine TE (Fig. 6C). This finding may be related to the expression of *Oct4* [72] in bovine TE cells in this study.

In the mouse, TE specific markers are not expressed in embryo-derived PSCs including ICM and ESCs [73, 74]. However, bSCNT-eSLCs generated and maintained in the 3i system expressed some of the TE specific genes such as *Cdx2, Tead4* and *Inf-tau*. This may suggest that the regulatory genes and mechanism related to pluripotency maintenance in human and mouse does not completely match with the ones in cattle. In the present study, *Cdx2* which regulates TE cell fate and is expressed in TE of murine blastocyst, was also expressed in ICM (Fig. 6C) as previously reported [55, 70, 75], although the expression level was lower than in TE. These results provide a clue as to why the establishment and maintenance of genuine embryonic PSCs are difficult in ruminant animals such as cattle [5, 6, 9, 15, 26]. Various genes participate in differentiation of embryonic cells into TE lineage [54, 69, 76]. According to the reports, both *Elf5* and *Hand1* are involved in TE differentiation both in murine species and in bovine species. Different from TE cells, *Elf5* and *Hand1* were not expressed in bSCNT-eSLCs in this study, providing support for the notion that bSCNT-eSLCs in 3i system may not be analogous to TSCs or TE cells [69, 76], although some TE specific genes were expressed.

Immunofluorescence analysis using specific antibody probes to OCT4, SOX2, NANOG, SSEA-4, TRA-1-60 and TRA-1-81 were all consistent with

characteristics of pluripotency in bSCNT-eSLC colonies. Unlike the AP expression, these markers were not uniformly expressed throughout the colony (Fig. 7). Most markers were localized only in the CMt portion of the colony, suggesting that the colony might consist of cell type(s) in addition to the pure PSCs. Nevertheless, the positive expression of the battery of pluripotency markers demonstrates that the ESC culture method used in this study, the 3i system, is competent to maintain the stem-ness of bSCNT-eSLCs by inhibiting differentiation and supporting pluripotency, even though not all areas of the stem cell colony displayed positive expression of the markers. However, the non-uniform marker expression may also be characteristic of bSCNT-eSLCs like that of human ESCs [77].

This study investigated the differentiation potential of bSCNT-eSLC colonies using both in *in vitro* and *in vivo* assays because there have been a few attempts to verify the *in vitro* differentiation potentials [9] and no reports of the *in vivo* differentiation potentials. As has been demonstrated for other ESCs, EBs could be formed from bSCNT-eSLCs *in vitro* and categorized into two types by their shape, cystic and solid (Fig. 8A). Both types of EBs were able to grow for more than 10 days while retaining their morphology Furthermore, these EBs expressed three germ layer markers similar to human and mouse EBs (Fig. 8B) [1, 2], which is different from simple clusters of differented cells or trophoblastic vesicles [78]. In the present study, I also studied the *in vivo* differentiation potential of bSCNT-eSLCs after transplantation in nude mice. However, I did not recover true teratomas demonstrating differentiation into all three germ layers as determined by the expression of germ layer marker genes

(Fig. 9). In addition, the formation rate of the tumors was also very low (Table 9), perhaps due to insufficient number of stem cells in whole colony [53, 65]. Although the tumors did not represent authentic teratomas, they displayed typical embryonic carcinomas (ECs) with large primitive cells with areas of glandular and papillary formations (Fig. 9A-F). To the best of my knowledge, the present study is the first report demonstrating the differentiation potency of PSCs *in vivo* as well as *in vitro* using SCNT-eSLCs in cattle.

Unlike human and mouse, previous bovine PSC studies have been limited to description of the pluripotency of colonies [5, 8, 9, 15, 26]. Hence the present study was undertaken to explore further properties of bovine eSLCs. First, the colony was divided into two parts as CMt and PMn. The region of CMt shows smaller and concentrated shape while the region of PMn is analogous to trophoblastic or epithelial cells, revealing large and flat morphology (Fig. 4). Intriguingly, after passaging of CMt and PMn parts separately, they presented different morphologies (Fig. 11A). The CMt-derived colonies after passaging subculture contained not only small and condensed CMt part cells, but also large and flat PMn part cells. On the other hand, the PMn-derived passaged colony only possessed PMn part cell population. The outgrowth rate and the number of AP-positive colonies in CMt-derived colonies were markedly higher than in PMn-derived colonies (Table 6). In addition, some PMn-derived colonies showed very weak AP activity, considering that the PMn-derived colonies may include very few numbers of eSLCs when compared with CMt-derived ones. Some reports showed that bovine trophectoderm is positive to AP staining [79] However, only few parts of PMn cells (which mostly contain

Table 9. Teratoma-like tumor formation after transplantation of bSCNT-eSLCs*

	No.	
	Injection into testis	*Teratoma-like tumor formation*
bSCNT-eSLCs	10	1 (embryonic carcinoma)
STO (feeder cells)	6	0

*Bovine somatic cell nuclear transfer-derived stem-like cells

trophectodermal cells) were positive for AP staining in the 3i conditions of this study, which is consistent with other reports [59, 80]. This suggests that 3i culture condition may be insufficient to retain AP activity in PMn, different from the activity in CMt. As a result, the CMt-derived colonies continue to proliferate while the number of PMn-derived colonies decreased and ultimately failed to sustain during the passaging. Quantitative PCR analyses revealed higher expression of pluripotency specific markers in the CMt region compared to those in the PMn region, whilst TE specific marker expression was opposite in direction (Fig. 12). Numerous studies of bovine eSLCs or ES-like cells reported that the cells show TSC-like morphology, in addition to their ESC morphology [9, 15, 32, 58, 59, 66] and it may be important to discover the differences between eSLCs and TSCs. Like those studies, the morphology of PMn in the present study is similar to TSCs or trophoblastic cells. *Oct4* is an essential transcription factor to maintain pluripotency and the gene sequence is highly conserved in mammals [81].

Oct4 is found in various PSCs and is used as the core reprogramming factor to generate somatic cell-derived induced PSCs. In mice, *Oct4* is expressed in early blastomere and only ICM-specific expression was observed at the blastocyst stage which is the first determination stage of cell fate between ICM and TE. This, suggest that *Oct4* plays an integral role in cell fate determination during early mammalian development. *Cdx2* is generally considered to be an arbiter of trophoblastic cells and required to restrict *Oct4* expression to ICM of the embryo [74, 82]. Indeed, *Cdx2* is expressed in TSCs [74, 82] arising from polar TE that differentiates into all trophoblastic lineages [68, 75]. It is

generally accepted that *Oct4* and *Cdx2* expressions are hallmarks to identify and characterize the status of embryo-derived cells. Due to failure to retain the colony under 3i system when TE and ICM were separated (Table 10), the whole blastocysts were used in this study and the expression of TE-specific markers were also determined. Formation of stem-like cell colony which can be generated only from whole blastocysts implies that CMt (mainly from ICM) and PMn (mostly from TE) may be interdepentant during the establishment and maintenance of eSLCs *in vitro* under 3i system.

Co-localization of OCT4 and CDX2 throughout the colony implies that bSCNT-eSLCs contain TSC population and/or the cells have dual properties. Interestingly, OCT4-positive cell without CDX2 expression were largely restricted to the CMt part of the colony (Fig. 10A-C). However, recent studies showed that OCT4 is also expressed in bovine trophoblastic cell line [76], implying that OCT4 may not be a good PSC marker in bovine species. Different from OCT4, NANOG is not detected in bovine trophoblastic cell line [76] and is also known as a naïve pluripotency marker [53], although a recent report showed the presence of mRNA in bovine TS-like cells. Here, I showed that the NANOG-positive cell population without CDX2 expression was limited to CMt part in the colony like OCT4-positive cells (Fig. 10B, F). The bSCNT-eSLC colonies in the present study expressed most pluripotency specific markers although they also expressed some marker genes specific for TE. In particular, the naïve pluripotency specific markers such as *Rex-1*, *Klf2, Klf4* and *Nrob1* were highly expressed in bSCNT-eSLCs which were not expressed in bovine TE cells (Fig. 6C). This is consistent with previous studies in mouse [73].

Table 10. The survival rate of CMt- and PMn-derived colonies[a)]

			No. of survived colonies at			
		Group[b)]	*Passage 1*	*Passage 2*	*Passage 3*	*Passage 4*
Passaging the initial stage of colonies[c)]	CMt	C1	1	3	10	29
		C2	1	2	6	18
		C3	1	2	4	8
		C4	1	2	6	16
		C5	1	2	5	15
		Total (%)	5 (100.0)	11 (55.0)	31 (70.4)	86 (69.3)
	PMn	P1	1	2	1	0
		P2	1	2	2	0
		P3	1	0	0	0
		P4	1	1	0	0
		P5	1	0	0	0
		Total (%)	5 (100.0)	5 (50)	3 (25)	0 (0)

			No. of survived colonies at		
		Group[b)]	*Passage 22*	*Passage 23*	*Passage 24*
Passaging the proliferating stage of colonies[d)]	CMt	C1	1	4	13
		C2	1	3	8
		C3	1	2	5
		C4	1	2	11
		C5	1	3	4
		Total (%)	5 (100)	14 (70)	41 (73.2)
	PMn	P1	1	0	0
		P2	1	0	0
		P3	1	1	0
		P4	1	0	0
		P5	1	1	0
		Total (%)	5 (100)	2 (20)	0 (0)

[a)]CMt: central multilayer; PMn: peripheral monolayer.
[b)]Only one colony slice were used for the next passage. The CMt colony was sliced to 4 pieces while the PMn colony was sliced to 2 pieces due to the inability of attachment and proliferation of the cells in PMn colony in 3i culture condition.
c)Outgrown seeded blastocysts = Passage 0
[d)]Until passage 21, the stem cell colony was maintained only with CMt-derived cells. Up to passage 21, PMn part of the colony was discarded.

The results above suggest that a part of the colony, mostly maintained and propagated in CMt area, is at pluripotency and the cells are different from trophoblastic cells. However, bSCNT-eSLC colony in 3i system includes sub-populations of more than one type of cells including TE specific marker expressing cells. Recently, TS-like cells were generated by the treatment of 2 inhibitors, which had used previously characterized PSCs from many species including cattle [3, 11, 53, 69]. Although there are some similar features (eg. Morphology), the cell colonies under 3i condition not only express core or primed pluripotency markers, but also many naïve state markers. Moreover, the cells were able to differentiate into three germ layers *in vitro*. It was also reported that some trophoblastic markers are expressed in ICM-derived cells [83]. Considering these results, the eSLC colonies under 3i condition are different from previous PSCs cultured with 2 inhibitors including recent TS-like cells.

2. Enhancing pluripotency of bovine eSLCs

The characteristics that define ESC such as morphology, expression of specific markers and activation of signaling pathways for maintenance of pluripotency and self-renewal, are well known in mice and humans [84]. However, in cattle, methods for the establishment of genuine ESC and their specific characteristics have not yet been defined [80].

The objectives of the present study were to establish PSCs from blastocysts of cattle and demonstrate the effect of thiazovivin on the generation and propagation of these eSLCs. Previous studies revealed that thiazovivin

treatment improves the generation of iPSCs and ESC of mice and humans [37], because ROCK inhibitors are known to improve the survival of various kinds of stem cells, including ES, iPSCs and adult stem cells [36, 37, 85]. thiazovivin, a ROCK inhibitor, was utilized in the present study to establish eSLCs. The effects of thiazovivin on the plating efficiency of seeded blastocysts and the survivability and the proliferation of putative SLC were subsequently investigated.

Small molecules can play an important role in both the propagation of ESC and the generation of iPSCs by enhancing their pluripotency, self-renewal or cellular reprogramming [35]. One of these small molecules, thiazovivin, improves survival and proliferation of ESC via the stabilization of the E-cadherin protein through direct inhibition of ROCK [37]. E-cadherin-related cell-cell contact controls survival and self-renewal of human ESC [37, 38]. Fate and function of various stem cells are influenced by the extra-cellular matrix (ECM) both *in vitro* and *in vivo*. Cell-ECM interactions are important for the attachment, proliferation, differentiation and survival of stem cells.

In the generation of ESC lines, the initial stages including outgrowth of cell clumps and formation of colonies from the zona pellucida-free blastocyst are important for the subsequent steps in long-term culture and propagation of ESC [86]. In humans, subculture of ESC is performed by mechanically splitting the cell clump. However, subsequent attachment of some of these clumps to the feeder layer is poor, and this step is very important to form an ESC colony [87]. Because previous studies utilized buffalo and cattle ES-like cells also used a mechanical split method similar to that used for human ESC cultures [64, 85],

it is likely that the initial attachment of the embryonic cell clump may also be a critical step to generate ESC from cattle. Recent data show that existence of cell populations expressing the *Cdx2* and *Ifn-tau* genes (TE-specific markers) supports the maintenance of pluripotency and propagation of eSLCs of cattle over 50 passages, although there are some eSLC populations expressing only pluripotency markers without TE-specific marker expression [30]. In the present study, the culture peripheral trophoblastic part of eSLC colonies under the 3i system was attempted. However, unlike eSLCs of cattle, the cells could not grow and/or be maintained in 3i medium although few attached clumps could only form primary colonies (Table 7) [30]. The results also suggest that eSLCs of cattle in the 3i system may not be analogous to TE or trophoblast stem cells, although some TE-specific genes are expressed [30]. The eSLCs of cattle display a flat-shaped morphology similar to human ESC. Interestingly, eSLCs of cattle express core, naïve state pluripotency and primed state pluripotency gene markers together, although the cells are similar to murine naïve state pluripotent cells (Fig. 14C) [30].

At this stage, in ESC generation, cell-cell contact and/or attachment to feeder layer cells are critical. Therefore, in the present study, thiazovivin, a ROCK inhibitor, is utilized while culturing the ICM or whole blastocysts seeded on the feeder cells, in an effort to improve embryo-derived cell attachment. Regardless of the cell's type, thiazovivin improved the attachment rate to the feeder cells. Although isolated ICM tended to demonstrate higher attachment efficiency, the primary colony forming efficiency was significantly higher in the whole blastocyst-seeding group. These results imply that

thiazovivin treatment increases the attachment rate of zona-free blastocysts and consequently, the outgrowth rate of primary colonies. Both of them are critical initial steps for the generation of eSLCs of cattle.

After the primary colony formation, putative cattle eSLC colonies were subcultured over 25 passages, and the cells showed a normal karyotype. The *Oct4* and *Nanog* pluripotency markers were analyzed by RT-PCR and qPCR. Expression of the *Oct4* gene and *Nanog* mRNA tended to be increased by thiazovivin treatment. Previous studies demonstrated that the addition of a ROCK inhibitor improved survival and proliferation of human ESC and mesenchymal stem cells [36]. The inhibitor increased the expression of the *Oct4* gene in human ESC and the expression of the *Nanog* gene in buffalo ES-like cells [85]. In the present study, *Oct4* gene expression was increased by thiazovivin treatment of eSLCs of cattle, implying that thiazovivin may enhance the potential for pluripotency in the eSLCs. However, AP activity and immunofluorescence staining results were not different from those in the 3i control group. Partial staining of AP activity in SLCs is in agreement with other studies with cattle [66]. In addition, thiazovivin treatment resulted in larger eSLC colonies in cattle (Fig. 15). The ability of eSLCs of cattle to undergo cellular differentiation was not changed by thiazovivin treatment. This is in agreement with a previous study that examined another ROCK inhibitor, which demonstrated that the differentiation potential was not significantly improved by Y-27632 treatment in buffalo ES-like cells, although the primary colony formation rate was increased by the treatment [85].

ROCK is a direct target of thiazovivin and inhibition of the ROCK pathway

by thiazovivin increased the survival and adhesion of human ESC by stabilizing E-cadherin in the cell membrane [37]. Hence, the changes in E-cadherin gene expression in the present study were induced by thiazovivin treatment as evidenced by immunofluorescence staining and real time PCR analysis. The greater fluorescence intensity of E-CADHERIN and the elevated *E-cadherin* gene expression in thiazovivin-treated cells suggests that the enhancement of *E-cadherin* by ROCK inhibition may support the survival and adhesion of putative eSLCs of cattle, as in the case with human ESC. Additionally, this may result in the improvement of the maintenance of stemness of eSLCs of cattle.

3. Microarray analysis of bovine eSLCs

The microarray technology has revealed a powerful tool for profiling the global gene expression and DEGs are suggested specific or universal characteristics. To understand their characteristics, PSCs in many species including humans and mice have been analyzed by this technology. However, it is still not well known yet about gene expression profiles of embryo-derived PSCs in cattle. In the present study, I analyzed the gene expression pattern of bovine eSLCs from three different origins: IVP-, NT-, and PA-blastocysts. These were compared with each other to understand their shared and distinct properties. In addition, these were compared with SCs to understand shared pathways for pluripotency and failure of teratoma formation by profiling tumor-related genes and tumor suppressor genes.

To understand characteristics of eSLCs and SCs, I analyzed their gene expression. The hierarchical clustering results show little difference in gene

expression among six different eSLCs, while all the eSLCs have immensely different gene expression from SCs (Fig. 19A), suggesting that properties of eSLCs may be significantly different from those of SCs.

To further verify differences between eSLCs and SCs in detail, IVP-eSLCs were compared with SCs. Among up-regulated DEGs, most GO terms in the BP group were related to metabolic activity and the cell cycle (Fig. 19C). Generally, the cell cycle of ESCs is shorter than that of SCs, because the durations of G1 and G2 are remarkably decreased [88]. This means that there is a rapid onset of stem cell proliferation and an enormous demand for energy, such as ATP. Because of this, the metabolic system in ESCs is also changed [89]. Consequentially, metabolism-related genes in ESCs were up-regulated compared to SCs. The results suggest that the metabolic system of IVP-eSLCs may be similar to ESCs and that they may have a short cell cycle, consistent with my previous report [30].

Expression of pluripotent genes and inhibition of differentiation genes are both necessary for maintenance of a pluripotent state. Recently, it has been documented that mESCs are able to maintain their unique properties, including self-renewal and potential of differentiation, by using inhibitors which suppress differentiation signaling pathways [11]. More recently, bovine eSLCs were also derived with these inhibitors [30]. According to the results in down-regulated DEGs, most GO terms in the BP group were related to differentiation and development (Fig. 19C). These results suggest that the expression of differentiation-associated genes in bovine eSLCs is decreased when compared with SCs, suggesting that the 3i system may repress the tendency to

differentiate in bovine eSLCs. These results may help to retain pluripotency in eSLCs.

One of the biggest differences between ESCs and SCs is the expression of pluripotent genes [90]. Comparing with SCs, eSLCs expressed 39 pluripotent DEGs including the core pluripotency markers, *Oct4* and *Nanog*. It has been documented that *Oct4* and *Nanog* expressions are essential not only to decide first cell fate, trophoblast and ICM, but also to maintain pluripotency of stem cells in mouse, as well as human [91]. In bovine, it has been also revealed that *Oct4* and *Nanog* are also expressed in embryos and embryo-derived cells [30]. Moreover, the overexpression of two genes was essential to generate bovine iPSCs [20]. These previous reports and the results suggest that *Oct4* and *Nanog* expressions may be indispensable to support bovine ESCs. Among these DEGs, some pluripotency related genes are well known in mESCs and hESCs, but have not yet been reported in bovine embryo-derived cells; these include *Pecam1*, *Cnot1*, *Otx2*, *Prdm14*, and *Cldn6* (Fig. 19D). These genes have been well-known as pluripotency markers in mESCs [92-96]. In this study, these up-regulated DEGs were also confirmed by real-time PCR and the results revealed that these genes were significantly increased in IVP-eSLCs compared to SCs (Fig. 19E). Surprisingly, their expression was similar in IVP-eSLCs and ICM, implying that these genes may act as pluripotency markers and can aid in distinguishing the population of true PSCs in bovines.

Recent evidence suggests that ESCs from NT- and PA-embryos contribute epigenetic modifications such as chromatin remodeling and imprinting [97, 98]. This suggests that the analysis of bovine eSLCs from NT- and PA-embryos

might be useful for predicting epigenetic deficiencies that induce unsuccessful development.

Although NT-embryos are produced by oocyte-derived reprogramming factors like IVP-embryos, the efficiency was extremely low and transcriptional abnormalities were revealed [99]. The major cause of these developmental failures may be due to epigenetic modifications such as chromatin remodeling [100]. Profiling of chromatin remodeling-related genes revealed 5 genes that were differentially expressed between IVP- and NT-eSLCs (Fig. 20B and C). The expression of *Hmga1, PadI4,* and *Chd1l* was significantly increased in NT-eSLCs compared to IVP-eSLCs (Fig. 20B and C). Interestingly, these genes were not only related to chromatin remodeling, but were also associated with pluripotency. [101, 102]. They may be sufficient to trigger a cascade of epigenetic problems, leading to low efficiency of differentiation and development of the NT-embryo, despite the small number of DEGs.

Comparisons between PA- and IVP-eSLCs revealed differences in imprinting gene expression, which is consistent with a previous study [49]. Although the expression of some genes did not increase exactly two fold, an increasing trend for imprinted maternally expressed genes and a decreasing trend for imprinted paternally expressed genes in PA-eSLCs by real-time PCR were observed when compared to IVP-eSLCs (Fig. 20E and F). PA-eSLCs maintained an abnormal expression pattern of imprinting-related genes, like the PA embryo, and may be useful for preventing the waste of embryos in imprinting studies.

Since establishing mESCs, many studies have generated ESCs in bovines

[26, 58, 65]. However, there have been no reports that identify signaling pathways that maintain PSCs. In order to verify the appropriate pathways associated with bovine pluripotency, I investigated and analyzed co-regulated genes among eSLCs with the KEGG database. According to the results, co-regulated genes associated with pluripotency are strongly related to the TGFβ, WNT, and LIF signaling pathways (Fig. 21).

Although BMP signaling, which belongs to the TGFβ superfamily, promotes non-neural differentiation, BMPs also maintain pluripotency by activation of inhibitor of differentiation (Id) genes [10]. In addition, in mice, BMPs are able to support pluripotency in the absence of both serum and feeder cells [13]. Moreover, recent evidence has revealed that BMP4 plays an indispensable role in establishing bovine iPSCs [103]. Although some genes were down-regulated in this study, core BMP signaling genes appeared in co-up-regulated DEGs. In particular, *Bmp4*, *Bmp7*, *Bmpr1a*, *Smad4*, *Smad5,* and *Id1* were up-regulated (Fig. 22A and B). Interestingly, the expression pattern of these genes in all eSLCs was similar to that of ICM, suggesting that BMP signaling may be activated and may support pluripotency of bovine eSLCs, similar to the role of ICM in embryos.

The WNT pathway is also important for the enhancement of proliferation and maintenance of pluripotency in ESCs, as it stabilizes cytoplasmic b-catenin by suppressing GSK3β [12]. According to this results, WNT signaling genes such as *Wnt7a*, *Wnt10a*, and *Fzd7* were involved in co-up-regulated DEGs (Fig. 23A and B). Moreover, *Dvl1* and *β-catenin*, which are downstream of WNT signaling, are also expressed highly in bovine eSLCs. On the other hand, *Dkk1*

and *Dkk3*, which are suppressors of WNT signaling, were down-regulated in bovine eSLCs (Fig. 23A and B). Interestingly, this gene expression pattern was similar to that revealed in ICM (Fig. 23B). These results suggest that the WNT pathway may be activated as one of the strongest regulators to support pluripotency in bovine eSLCs.

Recently, it has been documented that LIF signaling is essential in mESCs and naïve hESCs [104]. In this results, LIF signaling also appeared in DEGs. The expression of *Lif* and *Lifr* genes was up-regulated, while *Stat3* expression was down-regulated in eSLCs (Fig. 24A). Surprisingly, the expression of *Stat3* in eSLCs was in reverse of its expression in ICM, suggesting that the signal transmission between LIF and STAT3 may be disconnected. It has been reported that many culture systems in previous studies, even those including LIF, fail to generate true bESCs [6, 28, 29]. According to this results, the failure may be related with the disconnection between LIF and STAT3. For the maintenance of pluripotency in bESCs, the LIF signaling pathway may be activated by STAT3 signaling and/or downstream effectors which do not supplement or stimulate LIF itself.

SOCS3 inhibits JAK signaling by a binding mechanism, resulting in the inhibition of the LIF pathway for pluripotency [105]. According to the microarray and real-time PCR results, *Socs3* expression was significantly up-regulated in eSLCs compared to SCs (Fig. 24). Assuming that the increased expression of *Socs3* may inhibit JAK signaling, this may be a critical factor involved in destruction of the LIF pathway. This study therefore suggests that the reactivation of *Stat3* may be compulsory for establishment of true ESCs in

bovines, and *Socs3* inhibition may generate authentic bESCs.

Generating eSLCs in a 3i culture system with long-term proliferation and expression of pluripotent markers has been previously successful. However, the efficiency of *in vivo* differentiation is extremely low and teratoma formation was induced abnormally [30]. Many previous studies in the literature have also revealed similar problems [26, 53, 59], even in bovine iPSCs [20].

It was hypothesized that tumor-related genes may be changed in eSLCs, so I profiled oncogenes and tumor suppressor genes. Interestingly, among DEGs, most oncogenes (23 genes) were down-regulated, including *Smo*, *Bcl11a*, *Maml2,* and *Ccnd1* which are related to tumors and metastasis [106-109] (Fig. 25A). These results suggest that decreased oncogenes may reduce the frequency of teratoma formation and immature teratomas. In contrast, most tumor suppressor genes (21 of 30 genes) were highly up-regulated in eSLCs including *Brca1*, *Mlh1*, *Msh2*, *Suz12,* and *Socs1*, which are related to an increased risk of cancer [110-114]. These results indicate that up-regulation of these tumor suppressor genes may be associated with suppression of teratoma formation in bovine eSLCs.

Some genes that affect teratoma formation are not tumor-related. The defensin family is a well-known immune system-connected factor [115] that can suppress tumor formation [116]. I observed increased expression of defensin family genes including *Defb1*, *Defb3,* and *Defb7* (Fig. 25E and F). These results show that defensin family genes may also be candidates for teratoma formation in bovines. It has also been documented that *Smad3* is the mediator of signals from the TGFβ superfamily, which controls cell

proliferation, pluripotency, and differentiation [117]. Recently, it has been reported that *Smad3* is closely connected with teratoma formation from ESCs [118]. *Smad3* expression was down-regulated (Fig. 25E and F). It is speculated that decreased *Smad3* gene expression may be one of the reasons why teratomas are induced abnormally.

CONCLUSION

In conclusion, the purpose of this study was to generate PSCs from bovine embryos by suppressing differentiation and to analyze them to discover the characteristics of bovine specific PSCs. To investigate this issue, three experiments were conducted.

In the first experiment, several small molecules were tested as suppressors of differentiation, and as a result, finally, three inhibitors (3i) were selected. The cells in 3i were able to proliferate and were maintained in culture for over 50 passages with the normal karyotype and pluripotency markers. Although the cells were still defined as eSLCs because of their incomplete capacity for in vivo differentiation, the cells had OCT4-positive or NANOG-positive cell populations without CDX2 expression. It suggests that there are some populations which have true pluripotency in the 3i system. Although the existence of pluripotent stem cells in 3i was confirmed, there are still unsolved problems to achieve authentic pluripotency. To investigate these issues, I focused on finding a small molecule to improve pluripotency and ultimately selected thiazovivin, a ROCK inhibitor. Thiazovivin treatment enhanced the attachment and outgrowth of the blastocyst and primary eSLC colony propagation after subculturing on the feeder layer cells. This effect may result from an increase in E-cadherin caused by the thiazovivin induced ROCK inhibition. However, the treatment of thiazovivin was not enough to transform eSLCs into true ESCs because the effect of thiazovivin was not able to improve

the efficiency of teratoma formation. To elucidate the limitations of the eSLCs, microarray analysis was used to investigate their transcriptome. From this analysis, it was shown that various gene expressions for pluripotency were mostly upregulated in the eSLCs, and bovine specific pathways for pluripotency were also shown by the microarray analysis. It was also suggested that the low efficiency of teratoma in the eSLCs may be related to the gene expression patterns of oncogenes and tumor suppressors. Moreover, embryo specific gene expression patterns such as imprinting or chromatin remodeling were confirmed to be maintained in eSLCs from the embryos, suggesting that eSLCs in 3i could be used in the functional research of genes like mESCs or hESCs.

Taken all together, the results of this research have increased our understanding of the state in bovine PSCs, and further investigation of the signaling pathway for pluripotency in eSLCs may be useful for the generation of genuine bovine ESCs. Furthermore, this study provides new insights into the derivation and long-term maintenance of PSCs in domestic animals.

REFERENCES

[1] M.J. Evans, M.H. Kaufman, Establishment in culture of pluripotential cells from mouse embryos, Nature 292(5819) (1981) 154-156.

[2] J.A. Thomson, J. Itskovitz-Eldor, S.S. Shapiro, M.A. Waknitz, J.J. Swiergiel, V.S. Marshall, J.M. Jones, Embryonic stem cell lines derived from human blastocysts, Science 282(5391) (1998) 1145-1147.

[3] M. Buehr, S. Meek, K. Blair, J. Yang, J. Ure, J. Silva, R. McLay, J. Hall, Q.L. Ying, A. Smith, Capture of authentic embryonic stem cells from rat blastocysts, Cell 135(7) (2008) 1287-1298.

[4] K. Takahashi, S. Yamanaka, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, Cell 126(4) (2006) 663-676.

[5] T. Anand, D. Kumar, M.K. Singh, R.A. Shah, M.S. Chauhan, R.S. Manik, S.K. Singla, P. Palta, Buffalo (Bubalus bubalis) embryonic stem cell-like cells and preimplantation embryos exhibit comparable expression of pluripotency-related antigens, Reprod. Domest. Anim. 46(1) (2011) 50-58.

[6] S. Cao, F. Wang, Z. Chen, Z. Liu, C. Mei, H. Wu, J. Huang, C. Li, L. Zhou, L. Liu, Isolation and culture of primary bovine embryonic stem cell colonies by a novel method, J. Exp. Zool. 11(5) (2009) 368-376.

[7] P.S. Yadav, W.A. Kues, D. Herrmann, J.W. Carnwath, H. Niemann, Bovine ICM derived cells express the Oct4 ortholog, Mol. Reprod. Dev. 72(2) (2005) 182-190.

[8] M. Munoz, C. Diez, A. Jouneau, I. Hue, E. Gomez, Embryonic stem cells in cattle, Reprod. Domest. Anim. 43 Suppl 4 (2008) 32-37.

[9] L. Wang, E. Duan, L.Y. Sung, B.S. Jeong, X. Yang, X.C. Tian, Generation and characterization of pluripotent stem cells from cloned bovine embryos, Biol. Reprod. 73(1) (2005) 149-155.

[10] X. Qi, T.G. Li, J. Hao, J. Hu, J. Wang, H. Simmons, S. Miura, Y. Mishina, G.Q. Zhao, BMP4 supports self-renewal of embryonic stem cells by inhibiting mitogen-activated protein kinase pathways, Proc. Natl. Acad. Sci. U.S.A. 101(16) (2004) 6027-6032.

[11] Q.L. Ying, J. Wray, J. Nichols, L. Batlle-Morera, B. Doble, J. Woodgett, P. Cohen, A. Smith, The ground state of embryonic stem cell self-renewal, Nature 453(7194) (2008) 519-523.

[12] N. Sato, L. Meijer, L. Skaltsounis, P. Greengard, A.H. Brivanlou, Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor, Nat. Med. 10(1) (2004) 55-63.

[13] Q.L. Ying, J. Nichols, I. Chambers, A. Smith, BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3, Cell 115(3) (2003) 281-292.

[14] J.M. Sperger, X. Chen, J.S. Draper, J.E. Antosiewicz, C.H. Chon, S.B. Jones, J.D. Brooks, P.W. Andrews, P.O. Brown, J.A. Thomson, Gene expression patterns in human embryonic stem cells and human pluripotent germ cell tumors, Proc. Natl. Acad. Sci. U.S.A. 100(23) (2003) 13350-13355.

[15] M. Pashaiasl, K. Khodadadi, M.K. Holland, P.J. Verma, The efficient

generation of cell lines from bovine parthenotes, Cell. Reprogram. 12(5) (2010) 571-579.

[16] T. Kunath, M.K. Saba-El-Leil, M. Almousailleakh, J. Wray, S. Meloche, A. Smith, FGF stimulation of the Erk1/2 signalling cascade triggers transition of pluripotent embryonic stem cells from self-renewal to lineage commitment, Development 134(16) (2007) 2895-2902.

[17] M.P. Stavridis, J.S. Lunn, B.J. Collins, K.G. Storey, A discrete period of FGF-induced Erk1/2 signalling is required for vertebrate neural specification, Development 134(16) (2007) 2889-2894.

[18] T. Burdon, C. Stracey, I. Chambers, J. Nichols, A. Smith, Suppression of SHP-2 and ERK signalling promotes self-renewal of mouse embryonic stem cells, Dev. Biol. 210(1) (1999) 30-43.

[19] G.S. Sineva, V.A. Pospelov, Inhibition of GSK3beta enhances both adhesive and signalling activities of beta-catenin in mouse embryonic stem cells, Biol. Cell. 102(10) (2010) 549-560.

[20] T. Kawaguchi, T. Tsukiyama, K. Kimura, S. Matsuyama, N. Minami, M. Yamada, H. Imai, Generation of Naive Bovine Induced Pluripotent Stem Cells Using PiggyBac Transposition of Doxycycline-Inducible Transcription Factors, PloS one 10(8) (2015) e0135403.

[21] H. Semb, Human embryonic stem cells: origin, properties and applications, Apmis. 113(11-12) (2005) 743-750.

[22] A. Baguisi, E. Behboodi, D.T. Melican, W.G. Gavin, E.W. Overstrom, Y. Echelard, Production of goats by somatic cell nuclear transfer, Nat. Biotechnol. 17(5) (1999) 456-461.

[23] I. Wilmut, A.E. Schnieke, J. McWhir, A.J. Kind, K.H. Campbell, Viable offspring derived from fetal and adult mammalian cells, Nature 385(6619) (1997) 810-813.

[24] M. Tachibana, P. Amato, M. Sparman, R.L. Stouffer, D. Wolf, S. Mitalipov, Human embryonic stem cells derived by somatic cell nuclear transfer, Cell 153(6) (2013) 1228-1238.

[25] T. Zhao, Z.N. Zhang, Z. Rong, Y. Xu, Immunogenicity of induced pluripotent stem cells, Nature 474(7350) (2011) 212-215.

[26] G. Gong, L. Jiang, X. Yang, X.C. Tian, Culture conditions and enzymatic passaging of bovine ESC-like cells, Cell. Reprogram. 12(2) (2010) 151-160.

[27] H. Niemann, W.A. Kues, Transgenic farm animals: an update, Reprod. Fertil. Dev. 19(6) (2007) 762-770.

[28] M. Munoz, A. Rodriguez, C. De Frutos, J.N. Caamano, C. Diez, N. Facal, E. Gomez, Conventional pluripotency markers are unspecific for bovine embryonic-derived cell-lines, Theriogenology 69(9) (2008) 1159-1164.

[29] N.C. Talbot, C.E. Rexroad, Jr., In vitro pluripotency of epiblasts derived from bovine blastocysts, Mol. Reprod. Dev. 42(1) (1995) 35-52.

[30] D. Kim, S. Park, Y.G. Jung, S. Roh, In vitro culture of stem-like cells derived from somatic cell nuclear transfer bovine embryos of the Korean beef cattle species, HanWoo, Reprod. Fertil. Dev. 28 (2015) 1762-1780.

[31] L.E. Young, K.D. Sinclair, I. Wilmut, Large offspring syndrome in cattle and sheep, Rev. Reprod. 3(3) (1998) 155-163.

[32] M.L. Lim, I. Vassiliev, N.M. Richings, A.B. Firsova, C. Zhang, P.J. Verma, A novel, efficient method to derive bovine and mouse embryonic stem cells

with in vivo differentiation potential by treatment with 5-azacytidine, Theriogenology 76(1) (2011) 133-142.

[33] Z. McLean, F. Meng, H. Henderson, P. Turner, B. Oback, Increased MAP kinase inhibition enhances epiblast-specific gene expression in bovine blastocysts, Biol. Reprod. 91(2) (2014) 49.

[34] S. Park, D. Kim, Y.G. Jung, S. Roh, Thiazovivin, a Rho kinase inhibitor, improves stemness maintenance of embryo-derived stem-like cells under chemically defined culture conditions in cattle, Anim. Reprod. Sci. 161 (2015) 47-57.

[35] A. Taei, S.N. Hassani, P. Eftekhari-Yazdi, M. Rezazadeh Valojerdi, M. Nokhbatolfoghahai, N.S. Masoudi, M. Pakzad, H. Gourabi, H. Baharvand, Enhanced generation of human embryonic stem cells from single blastomeres of fair and poor-quality cleavage embryos via inhibition of glycogen synthase kinase beta and Rho-associated kinase signaling, Hum. Reprod. 28(10) (2013) 2661-2671.

[36] K. Watanabe, M. Ueno, D. Kamiya, A. Nishiyama, M. Matsumura, T. Wataya, J.B. Takahashi, S. Nishikawa, S. Nishikawa, K. Muguruma, Y. Sasai, A ROCK inhibitor permits survival of dissociated human embryonic stem cells, Nat. Biotechnol. 25(6) (2007) 681-686.

[37] Y. Xu, X. Zhu, H.S. Hahm, W. Wei, E. Hao, A. Hayek, S. Ding, Revealing a core signaling regulatory mechanism for pluripotent stem cell survival and self-renewal by small molecules, Proc. Natl. Acad. Sci. U.S.A. 107(18) (2010) 8129-8134.

[38] F. Wang, D. Scoville, X.C. He, M.M. Mahe, A. Box, Wong, M.G. Martin,

M. Helmrath, L. Li, Isolation and characterization of intestinal stem cells based on surface marker combinations and colony-formation assay, Gastroenterology 145(2) (2013) 383-95 e1-21.

[39] H. Oda, M. Takeichi, Evolution Structural and functional diversity of cadherin at the adherens junction, J. Cell. Biol. 193(7) (2011) 1137-1146.

[40] Y. Sun, L.G. Villa-Diaz, R.H. Lam, W. Chen, P.H. Krebsbach, J. Fu, Mechanics regulates fate decisions of human embryonic stem cells, PloS one 7(5) (2012) e37178.

[41] T.T. Chen, D.T. Yuan, B. Wei, J. Jiang, J.H. Kang, K. Ling, Y.J. Gu, J.S. Li, L. Xiao, G. Pei, E-Cadherin-Mediated Cell-Cell Contact Is Critical for Induced Pluripotent Stem Cell Generation, Stem cells 28(8) (2010) 1315-1325.

[42] T. Redmer, S. Diecke, T. Grigoryan, A. Quiroga-Negreira, W. Birchmeier, D. Besser, E-cadherin is crucial for embryonic stem cell pluripotency and can replace OCT4 during somatic cell reprogramming, Embo. Rep. 12(7) (2011) 720-726.

[43] D. Kim, Y.G. Jung, S. Roh, Microarray analysis of embryo-derived bovine pluripotent cells: The vulnerable state of bovine embryonic stem cells, PloS one 12(3) (2017) e0173278.

[44] P. Yang, J. Wang, G. Gong, X. Sun, R. Zhang, Z. Du, Y. Liu, R. Li, F. Ding, B. Tang, Y. Dai, N. Li, Cattle mammary bioreactor generated by a novel procedure of transgenic cloning for large-scale production of functional human lactoferrin, PloS one 3(10) (2008) e3453.

[45] X. Wu, M. Song, X. Yang, X. Liu, K. Liu, C. Jiao, J. Wang, C. Bai, G. Su, X. Liu, G. Li, Establishment of bovine embryonic stem cells after knockdown

of CDX2, Sci. Rep. 6 (2016) 28343.

[46] S.N. Brimble, X. Zeng, D.A. Weiler, Y. Luo, Y. Liu, I.G. Lyons, W.J. Freed, A.J. Robins, M.S. Rao, T.C. Schulz, Karyotypic stability, genotyping, differentiation, feeder-free maintenance, and gene expression sampling in three human embryonic stem cell lines derived prior to August 9, 2001, Stem. Cells. Dev. 13(6) (2004) 585-597.

[47] A.A. Sharov, Y. Piao, M.S. Ko, Gene expression profiling of mouse embryos with microarrays, Methods. Enzymol. 477 (2010) 511-541.

[48] G. Chang, S. Liu, F. Wang, Y. Zhang, Z. Kou, D. Chen, S. Gao, Differential methylation status of imprinted genes in nuclear transfer derived ES (NT-ES) cells, Genomics 93(2) (2009) 112-119.

[49] Q. Mai, Y. Yu, T. Li, L. Wang, M.J. Chen, S.Z. Huang, C. Zhou, Q. Zhou, Derivation of human embryonic stem cell lines from parthenogenetic blastocysts, Cell. Res. 17(12) (2007) 1008-1019.

[50] K. Ushizawa, C.B. Herath, K. Kaneyama, S. Shiojima, A. Hirasawa, T. Takahashi, K. Imai, T. Tokunaga, Y. Tsunoda, G. Tsujimoto, K. Hashizume, cDNA microarray analysis of bovine embryo gene expression profiles during the pre-implantation period, Reprod. Biol. Endocrinol. 2 (2004) 77.

[51] C. Robert, J. Nieminen, I. Dufort, D. Gagne, J.R. Grant, G. Cagnone, D. Plourde, P. Rigault, N. Juge, M.A. Sirard, Combining resources to obtain a comprehensive survey of the bovine embryo transcriptome through deep sequencing and microarrays, Mol. Reprod. Dev. 78(9) (2011) 651-664.

[52] Z. Suadi, L.C. Siew, R. Tie, W.B. Hui, A. Asam, S.H. Thiew, L.K. Boon, STR data for the AmpFlSTR Identifiler loci from the three main ethnic

indigenous population groups (Iban, Bidayuh, and Melanau) in Sarawak, Malaysia, J. Forensic. Sci. 52(1) (2007) 231-234.

[53] V. Verma, B. Huang, P.K. Kallingappa, B. Oback, Dual kinase inhibition promotes pluripotency in finite bovine embryonic cell lines, Stem. Cells. Dev. 22(11) (2013) 1728-1742.

[54] T. Fujii, S. Moriyasu, H. Hirayama, T. Hashizume, K. Sawai, Aberrant expression patterns of genes involved in segregation of inner cell mass and trophectoderm lineages in bovine embryos derived from somatic cell nuclear transfer, Cell. Reprogram.. 12(5) (2010) 617-625.

[55] Z.E. Madeja, J. Sosnowski, K. Hryniewicz, E. Warzych, P. Pawlak, N. Rozwadowska, B. Plusa, D. Lechniak, Changes in sub-cellular localisation of trophoblast and inner cell mass specific transcription factors during bovine preimplantation development, BMC. Dev. Biol. 13 (2013) 32.

[56] A.I. Saeed, V. Sharov, J. White, J. Li, W. Liang, V. Trush, J. Quackenbush, TM4: a free, open-source system for microarray data management and analysis, Biotechniques 34(2) (2003) 374-378.

[57] G. Dennis, Jr., B.T. Sherman, D.A. Hosack, J. Yang, W. Gao, H.C. Lane, R.A. Lempicki, DAVID: Database for Annotation, Visualization, and Integrated Discovery, Genome. Biol. 4(5) (2003) P3.

[58] S. Saito, N. Strelchenko, H. Niemann, Bovine embryonic stem cell-like cell-lines cultured over several passages, Roux. Arch. Dev. Biol. 201(3) (1992) 134-141.

[59] M. Jin, A. Wu, S. Dorzhin, Q. Yue, Y. Ma, D. Liu, Culture conditions for bovine embryonic stem cell-like cells isolated from blastocysts after external

fertilization, Cytotechnology 64(4) (2012) 379-389.

[60] J.B. Cibelli, S.L. Stice, P.J. Golueke, J.J. Kane, J. Jerry, C. Blackwell, F.A.P. de Leon, J.M. Robl, Transgenic bovine chimeric offspring produced from somatic cell-derived stem-like cells, Nat. Biotechnol. 16(7) (1998) 642-646.

[61] A.E. Van Stekelenburg-Hamers, T.A. Van Achterberg, H.G. Rebel, J.E. Flechon, K.H. Campbell, S.M. Weima, C.L. Mummery, Isolation and characterization of permanent cell lines from inner cell mass cells of bovine blastocysts, Mol. Reprod. Dev. 40(4) (1995) 444-454.

[62] M. Kanehisa, S. Goto, S. Kawashima, Y. Okuno, M. Hattori, The KEGG resource for deciphering the genome, Nucleic. Acids. Res. 32(Database issue) (2004) 277-280.

[63] E.J. Walker, C. Zhang, P. Castelo-Branco, C. Hawkins, W. Wilson, S. Croul, D. Malkin, U. Tabori, Monoallelic expression determines oncogenic progression and outcome in benign and malignant brain tumors, Cancer. Res. 72(3) (2012) 636-644.

[64] S.P. D kim, S Roh, Comparison of Three Different Culture Systems for Establishment and Long-Term Culture of Embryonic Stem-like Cells from In Vitro-Produced Bovine Embryos, J. Emb. Trans. 27(3) (2012) 189-192.

[65] M. Mitalipova, Z. Beyhan, N.L. First, Pluripotency of bovine embryonic cell line derived from precompacting embryos, Cloning 3(2) (2001) 59-67.

[66] J.O. Gjorret, P. Maddox-Hyttel, Attempts towards derivation and establishment of bovine embryonic stem cell-like cultures, Reprod. Fertil. Dev. 17(1-2) (2005) 113-124.

[67] J. Nichols, A. Smith, Naive and primed pluripotent states, Cell stem cell

4(6) (2009) 487-492.

[68] S. Tanaka, T. Kunath, A.K. Hadjantonakis, A. Nagy, J. Rossant, Promotion of trophoblast stem cell proliferation by FGF4, Science 282(5396) (1998) 2072-2075.

[69] X. Huang, X. Han, B. Uyunbilig, M. Zhang, S. Duo, Y. Zuo, Y. Zhao, T. Yun, D. Tai, C. Wang, J. Li, X. Li, R. Li, Establishment of Bovine Trophoblast Stem-Like Cells from In Vitro-Produced Blastocyst-Stage Embryos Using Two Inhibitors, Stem. Cells. Dev. 23(13) (2014) 1501-1514.

[70] M. Ozawa, M. Sakatani, J. Yao, S. Shanker, F. Yu, R. Yamashita, S. Wakabayashi, K. Nakai, K.B. Dobbs, M.J. Sudano, W.G. Farmerie, P.J. Hansen, Global gene expression of the inner cell mass and trophectoderm of the bovine blastocyst, BMC Dev. Biol. 12 (2012) 33.

[71] K. Hayashi, S.M. Lopes, F. Tang, M.A. Surani, Dynamic equilibrium and heterogeneity of mouse pluripotent stem cells with distinct functional and epigenetic states, Cell stem cell 3(4) (2008) 391-401.

[72] M. Zuccotti, V. Merico, L. Sacchi, M. Bellone, T.C. Brink, M. Stefanelli, C.A. Redi, R. Bellazzi, J. Adjaye, S. Garagna, Oct-4 regulates the expression of Stella and Foxj2 at the Nanog locus: implications for the developmental competence of mouse oocytes, Hum. Reprod. 24(9) (2009) 2225-2237.

[73] P. Kuckenberg, M. Peitz, C. Kubaczka, A. Becker, A. Egert, E. Wardelmann, A. Zimmer, O. Brustle, H. Schorle, Lineage conversion of murine extraembryonic trophoblast stem cells to pluripotent stem cells, Mol. Cell. Biol. 31(8) (2011) 1748-1756.

[74] D. Strumpf, C.A. Mao, Y. Yamanaka, A. Ralston, K. Chawengsaksophak,

F. Beck, J. Rossant, Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst, Development 132(9) (2005) 2093-2102.

[75] R.M. Roberts, S.J. Fisher, Trophoblast stem cells, Biol. Reprod. 84(3) (2011) 412-421.

[76] A.T. Schiffmacher, C.L. Keefer, CDX2 regulates multiple trophoblast genes in bovine trophectoderm CT-1 cells, Mol. Reprod. Dev. 80(10) (2013) 826-839.

[77] M.H. Stewart, M. Bosse, K. Chadwick, P. Menendez, S.C. Bendall, M. Bhatia, Clonal isolation of hESCs reveals heterogeneity within the pluripotent stem cell compartment, Nat. Methods 3(10) (2006) 807-815.

[78] K. Tiruthani, P. Sarkar, B. Rao, Trophoblast differentiation of human embryonic stem cells, Biotechnol. J. 8(4) (2013) 421-433.

[79] J.L. Edwards, A.M. Powell, C.E. Rexroad, Alkaline phosphatase activity in bovine oocytes and preimplantation embryos as affected by removal of the zona pellucida and culture medium constituents, Reprod. Fertil. Dev. 15(5) (2003) 285-292.

[80] M. Roach, L. Wang, X. Yang, X.C. Tian, Bovine embryonic stem cells, Methods. Enzymol. 418 (2006) 21-37.

[81] V. Nordhoff, K. Hubner, A. Bauer, I. Orlova, A. Malapetsa, H.R. Scholer, Comparative analysis of human, bovine, and murine Oct-4 upstream promoter sequences, Mamm. Genome. 12(4) (2001) 309-317.

[82] S.L. Palmieri, W. Peter, H. Hess, H.R. Scholer, Oct-4 Transcription Factor Is Differentially Expressed in the Mouse Embryo during Establishment of the

First 2 Extraembryonic Cell Lineages Involved in Implantation, Dev. Biol. 166(1) (1994) 259-267.

[83] J.A. Desmarais, S.P. Demers, J. Suzuki, S. Laflamme, P. Vincent, S. Laverty, L.C. Smith, Trophoblast stem cell marker gene expression in inner cell mass-derived cells from parthenogenetic equine embryos, Reproduction 141(3) (2011) 321-332.

[84] A. Schnerch, C. Cerdan, M. Bhatia, Distinguishing Between Mouse and Human Pluripotent Stem Cell Regulation: The Best Laid Plans of Mice and Men, Stem cells 28(3) (2010) 419-430.

[85] R. Sharma, A. George, M.S. Chauhan, S. Singla, R.S. Manik, P. Palta, ROCK inhibitor Y-27632 enhances the survivability of dissociated buffalo (Bubalus bubalis) embryonic stem cell-like cells, Reprod. Fertil. Dev. 25(2) (2013) 446-455.

[86] A.E. Sutherland, P.G. Calarco, C.H. Damsky, Expression and function of cell surface extracellular matrix receptors in mouse blastocyst attachment and outgrowth, J. Cell. Biol. 106(4) (1988) 1331-1348.

[87] B.C. Heng, H. Liu, T. Cao, Late-adhering human embryonic stem cell clumps during serial passage can yield morphologically 'normal-looking' colonies, Ann. Clin. Lab. Sci. 35(4) (2005) 459-462.

[88] A. Ballabeni, I.H. Park, R. Zhao, W. Wang, P.H. Lerou, G.Q. Daley, M.W. Kirschner, Cell cycle adaptations of embryonic stem cells, Proc. Natl. Acad. Sci. U.S.A. 108(48) (2011) 19252-19257.

[89] K. Ito, T. Suda, Metabolic requirements for the maintenance of self-renewing stem cells, Nat Rev Mol. Cell. Biol. 15(4) (2014) 243-256.

[90] Y.H. Loh, Q. Wu, J.L. Chew, V.B. Vega, W. Zhang, X. Chen, G. Bourque, J. George, B. Leong, J. Liu, K.Y. Wong, K.W. Sung, C.W. Lee, X.D. Zhao, B. Lim, H.H. Ng, The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells, Nat. Genet. 38(4) (2006) 431-440.

[91] N. Ivanova, R. Dobrin, R. Lu, I. Kotenko, J. Levorse, C. DeCoste, X. Schafer, Y. Lun, I.R. Lemischka, Dissecting self-renewal in stem cells with RNA interference, Nature 442(7102) (2006) 533-538.

[92] T. Furusawa, K. Ohkoshi, C. Honda, S. Takahashi, T. Tokunaga, Embryonic stem cells expressing both platelet endothelial cell adhesion molecule-1 and stage-specific embryonic antigen-1 differentiate predominantly into epiblast cells in a chimeric embryo, Biol. Reprod. 70(5) (2004) 1452-1457.

[93] X. Zheng, R. Dumitru, B.L. Lackford, J.M. Freudenberg, A.P. Singh, T.K. Archer, R. Jothi, G. Hu, Cnot1, Cnot2, and Cnot3 maintain mouse and human ESC identity and inhibit extraembryonic differentiation, Stem cells 30(5) (2012) 910-922.

[94] S.H. Yang, T. Kalkan, C. Morissroe, H. Marks, H. Stunnenberg, A. Smith, A.D. Sharrocks, Otx2 and Oct4 drive early enhancer activation during embryonic stem cell transition from naive pluripotency, Cell. Rep. 7(6) (2014) 1968-1981.

[95] Z. Ma, T. Swigut, A. Valouev, A. Rada-Iglesias, J. Wysocka, Sequence-specific regulator Prdm14 safeguards mouse ESCs from entering extraembryonic endoderm fates, Nat. Struct. Mol. Biol. 18(2) (2011) 120-127.

[96] L. Wang, Y. Xue, Y. Shen, W. Li, Y. Cheng, X. Yan, W. Shi, J. Wang, Z. Gong, G. Yang, C. Guo, Y. Zhou, X. Wang, Q. Zhou, F. Zeng, Claudin 6: a novel

surface marker for characterizing mouse pluripotent stem cells, Cell. Res. 22(6) (2012) 1082-1085.

[97] S.M. Mitalipov, Genomic imprinting in primate embryos and embryonic stem cells, Reprod. Fertil. Dev. 18(8) (2006) 817-821.

[98] F. Farifteh, M. Salehi, M. Bandehpour, M. Nariman, M. Ghafari Novin, T. Hosseini, S. Nematollahi, M. Noroozian, S. Keshavarzi, A. Hosseini, Histone modification of embryonic stem cells produced by somatic cell nuclear transfer and fertilized blastocysts, Cell. J. 15(4) (2014) 316-323.

[99] M. Panarace, J.I. Aguero, M. Garrote, G. Jauregui, A. Segovia, S. Mower, M. Medina, How healthy are clones and their progeny: 5 years of field experience, Theriogenology 67(1) (2007) 142-151.

[100] Y.K. Kang, D.B. Koo, J.S. Park, Y.H. Choi, A.S. Chung, K.K. Lee, Y.M. Han, Aberrant methylation of donor genome in cloned bovine embryos, Nat. Genet. 28(2) (2001) 173-177.

[101] M.A. Christophorou, G. Castelo-Branco, R.P. Halley-Stott, M.L. Nielsen, J.B. Gurdon, T. Kouzarides, Citrullination regulates pluripotency and histone H1 binding to chromatin, Nature 507(7490) (2014) 104-108.

[102] S.H. Chiou, B.H. Jiang, Y.L. Yu, S.J. Chou, P.H. Tsai, W.C. Chang, L.K. Chen, L.H. Chen, Y. Chien, G.Y. Chiou, Poly(ADP-ribose) polymerase 1 regulates nuclear reprogramming and promotes iPSC generation without c-Myc, J. Exp. Med. 210(1) (2013) 85-98.

[103] S.W. Wang, S.S. Wang, D.C. Wu, Y.C. Lin, C.C. Ku, C.C. Wu, C.Y. Chai, S. Saito, K.K. Yokoyama, Androgen receptor-mediated apoptosis in bovine testicular induced pluripotent stem cells in response to phthalate esters, Cell.

Death. Dis. 4 (2013) e907.

[104] O. Gafni, L. Weinberger, A.A. Mansour, Y.S. Manor, E. Chomsky, N. Novershtern, J.H. Hanna, Derivation of novel human ground state naive pluripotent stem cells, Nature 504(7479) (2013) 282-286.

[105] Y. Li, J. McClintick, L. Zhong, H.J. Edenberg, M.C. Yoder, R.J. Chan, Murine embryonic stem cell differentiation is promoted by SOCS-3 and inhibited by the zinc finger transcription factor Klf4, Blood 105(2) (2005) 635-637.

[106] A.R. Hallahan, J.I. Pritchard, S. Hansen, M. Benson, J. Stoeck, B.A. Hatton, T.L. Russell, R.G. Ellenbogen, I.D. Bernstein, P.A. Beachy, J.M. Olson, The SmoA1 mouse model reveals that notch signaling is critical for the growth and survival of sonic hedgehog-induced medulloblastomas, Cancer. Res. 64(21) (2004) 7794-800.

[107] W.T. Khaled, S. Choon Lee, J. Stingl, X. Chen, H. Raza Ali, O.M. Rueda, F. Hadi, S.F. Chin, P. Liu, BCL11A is a triple-negative breast cancer gene with critical functions in stem and progenitor cells, Nat. Commun. 6 (2015) 5987.

[108] T. Komiya, Y. Park, S. Modi, A.B. Coxon, H. Oh, F.J. Kaye, Sustained expression of Mect1-Maml2 is essential for tumor cell growth in salivary gland cancers carrying the t(11;19) translocation, Oncogene 25(45) (2006) 6128-32.

[109] P. Jares, D. Colomer, E. Campo, Genetic and molecular pathogenesis of mantle cell lymphoma: perspectives for new targeted therapeutics, Nat. Rev. Cancer. 7(10) (2007) 750-762.

[110] D. Ford, D.F. Easton, D.T. Bishop, S.A. Narod, D.E. Goldgar, Risks of cancer in BRCA1-mutation carriers. Breast Cancer Linkage Consortium,

Lancet 343(8899) (1994) 692-695.

[111] R.R. Iyer, A. Pluciennik, V. Burdett, P.L. Modrich, DNA mismatch repair: functions and mechanisms, Chem. Rev. 106(2) (2006) 302-323.

[112] R. Fishel, M.K. Lescoe, M.R. Rao, J. Garber, M. Kane, R. Kolodner, The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer, Cell 77(1) (1994) 1 p following 166.

[113] M. Zhang, Y. Wang, S. Jones, M. Sausen, K. McMahon, R. Sharma, Q. Wang, A.J. Belzberg, K. Chaichana, K.W. Kinzler, N. Papadopoulos, B. Vogelstein, C. Bettegowda, Somatic mutations of SUZ12 in malignant peripheral nerve sheath tumors, Nat. Genet. 46(11) (2014) 1170-1172.

[114] S. Laner-Plamberger, F. Wolff, C. Hauser-Kronberger, A.M. Frischauf, T. Eichberger, Hedgehog/GLI signaling activates suppressor of cytokine signaling 1 (SOCS1) in epidermal and neural tumor cells, PloS one 8(9) (2013) e75317.

[115] T. Ganz, Defensins: antimicrobial peptides of innate immunity, Nature reviews. Immunology 3(9) (2003) 710-720.

[116] C.D. Donald, C.Q. Sun, S.D. Lim, J. Macoska, C. Cohen, M.B. Amin, A.N. Young, T.A. Ganz, F.F. Marshall, J.A. Petros, Cancer-specific loss of beta-defensin 1 in renal and prostatic carcinomas, Lab. Invest. 83(4) (2003) 501-505.

[117] A. Moustakas, S. Souchelnytskyi, C.H. Heldin, Smad regulation in TGF-beta signal transduction, J. Cell. Sci. 114(Pt 24) (2001) 4359-4369.

[118] P. Li, Y. Chen, X. Meng, K.Y. Kwok, X. Huang, K.W. Choy, C.C. Wang, H. Lan, P. Yuan, Suppression of malignancy by Smad3 in mouse embryonic stem cell formed teratoma, Stem. Cell. Rev. 9(5) (2013) 709-720.

국 문 초 록

소 배아유래 전분화능 줄기세포의 생산 및 분석

김 대 환

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배아줄기세포는 자가증식능력과 모든 세포로 분화할 수 있는 전분화능을 가지고 있다. 이러한 두 가지 특징 때문에 배아줄기세포는 동물 및 사람의 다양한 기초연구에 활용되고 있다. 오랫동안 소에서도 배아줄기세포를 생산하기 위한 노력이 있었으나, 진정한 의미의 배아줄기세포는 아직까지 보고된 바 없다. 본 연구에서는 소분자물질을 포함하는 배양액을 통해 소 배아줄기세포를 확립하고 다양한 분자생물학적 기법을 통해 그 성상을 분석하였다. 이러한 연구를 위해 먼저 배아줄기세포 생산에 적합한 소분자물질을 선별하고 증식력, 전능성 마커 및 분화능 검증을 포함하는 전분화능 줄기세포의 지표를 통해 배양된 세포의 성상을 분석하였다. 마이크로어레이를 통해 전사체 분석을 실시하였으며 관련된 차별발현유전자는 Real-time PCR기법을 통해 검증하였다. 또한 전분화능 관련 신호경로는 KEGG분석을 이용하였다. 생산된 배아줄기세포는 단층의 평평한 형태를 보였으며 alkaline phosphatase 염색에 양성을 보였다. 또한 배양된 세포는

정상적인 핵형 및 전분화능 유전자발현을 보이며 50계대 이상 증식이 유지되었다. 비록 불완전한 체내분화능 때문에 유사배아줄기세포로 정의하였지만, 생산된 세포내부에 Cdx2의 발현없이 Oct4 또는 Nanog 유전자 만 발현하는 전분화능 세포집단의 존재를 확인하였다. 전분화능을 향상시키기 위해 티아조비빈을 처리한 결과 배반포 부착 및 계대 후 증식력이 유의적인 차이를 보이며 증가하는 것을 확인하였다. 또한 전분화능 및 E-cadherin 유전자발현 역시 티아조비빈을 처리한 실험군에서 증가하였으나 여전히 체내분화능을 향상시키는 데는 실패하였다. 유사줄기세포의 한계를 분석하기 위해 마이크로어레이 분석을 진행하였다. 암과 관련된 유전자분석결과 발암유전자는 감소하고 항암유전자는 증가하였다. 이러한 결과는 배양된 줄기세포의 체내분화능이 약화된 원인과 관련이 있을 것으로 생각된다. 또한 TGFβ, WNT 그리고 LIF 신호경로가 소 전분화능 유지와 밀접한 관련이 있는 것으로 확인되었다. 다른 두 신호경로와 달리 LIF 신호경로는 비활성화 되어있어 LIF 신호경로의 재활성이 완전한 전분화능 획득에 중요한 역할을 할 것으로 생각된다. 결론적으로 본 연구에서는 세가지 억제제를 이용해 유사배아줄기세포를 생산하였다. 또한 세포의 성상을 검증하는 동안 소 전분화능 유지 한계의 원인을 규명하였다. 이러한 결과는, 소에서 특징적으로 나타나는 전분화능에 대한 이해를 넓히고, 나아가 대동물 유래 배아줄기세포 확립에 큰 도움을 줄 것으로 생각된다.

주요어: 소, 배아, 전분화능 줄기세포, 핵이식, 소분자물질, 마이크로어레이, 차별발현유전자

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