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Effects of Leukemia Inhibitory Factor, Stem Cell Factor, and Basic Fibroblast Growth Factor on the Proliferation of Bovine ICM Cells In Vitro

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Abstract: *Experiments were conducted to determine the effects of leukemia inhibitory factor (LIF), stem cell factor (SF), and basic fibroblast growth factor (bFGF) on the proliferation of undifferentiated primitive ectodermal cells isolated from bovine inner cell masses (ICM). ICMs of bovine hatched blastocysts produced at 10 days post-insemination (dpi) were immunosurgically isolated. Either one or three ICMs were cultured on mouse embryonic fibroblasts (MEF) in a 100 μl droplet of control medium with or without several combinations of murine recombinant LIF (1,000 units/ml), human recombinant SF (40 ng/ml), and human recombinant bFGF (40 ng/ml) as follows: 1) LIF, 2) SF, 3) bFGF, 4) LIF+SF, 5) LIF+bFGF, 6) SF+bFGF and 7) LIF+SF+bFGF at 37°C under 5% CO_2 in air. Attachment of ICMs to a feeder layer and proliferation of ectodermal cells were observed daily. After 6 to 7 days of culture, the primary ectodermal cells outgrown from ICM clumps were mechanically isolated with a 26G needle and mechanically disaggregated by pipetting through a fine pipette to make small clumps of cells. The primary ectodermal cell clumps were cultured and serially passaged every 6 to 7 days. The attachment of isolated ICMs started 1 day after culture in each treatment, and the rates of attachment ranged from 74 to 94%. The rates of ectodermal cells outgrowing from 1 ICM and 3 ICMs (37 and 56% respectively) were higher in the LIF+SF containing medium than those in the control medium and bFGF containing medium (16 and 17%; 22 and 22% respectively). When primary ectodermal cells that had been isolated from ICMs were cultured in the LIF+SF containing medium, the undifferentiated ectodermal cells were serially passaged (6 passages) during 2 months in culture. The results of this study demonstrate that 1) groups of 3 ICMs gave superior results to single ICM for the formation of ectodermal cell colonies from the ICM clumps and 2) LIF and SF are important factors in supporting the primary outgrowth and serial passage of ectodermal cells, as well as for maintaining an undifferentiated state as determined by a histochemical staining of alkaline phosphatase.*

Key words: *LIF, SF, bFGF, Bovine ICM cells, Proliferation in vitro*

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Since the establishment of embryonic stem (ES) cells in the mouse [1, 2], investigators worldwide have been attempting to develop stable techniques for establishing ES cells from large domestic animals, including pig [3–7], sheep [8–10], goat [9–11] and cattle [12–14]. There have been reports of live calf births through the nuclear transfer of ICM cells [15] and ICM cells cultured for a short period [16]. Nevertheless, although bovine ES-like cells at less than 10 passages had the ability to generate blastocysts by nuclear transfer, after embryo transfer, development of the nuclear transferred embryos was limited to only 55 days of pregnancy, due to a deficiency in placental development [13]; so that further improvements are necessary to establish a method for long term culture of bovine ES-like cells.

LIF plays an important role in early embryonic development [17], and it is essential for the maintenance of totipotent mouse ES cells [18]. Furthermore, LIF is able to enhance embryo development *in vitro* in sheep [19], and cattle [20, 21]. It is known that LIF and SF promote the proliferation of mouse primordial germ cells (PGCs) *in vitro* [22], and also support their survival *in vitro*, probably by greatly reducing the occurrence of apoptosis in mouse PGCs [23, 24]. Furthermore, LIF, SF and bFGF

are known to be required to support the proliferation of bovine PGCs [25]. The effects of LIF, SF and bFGF on the proliferation of bovine ICMs have not yet been investigated. We therefore examined the effects of LIF, SF and bFGF on the proliferation of undifferentiated ectodermal cells isolated from bovine ICMs.

Materials and Methods

Production of bovine embryos

The embryos used in this experiment were *in vitro*-produced from slaughterhouse Japanese black cow ovaries by methods described by Kim *et al.* [26]. Briefly, Cumulus-oocyte complexes (COC) were matured in TCM-199 supplemented with 10% bovine follicular fluid, 0.02 AU/ml FSH, 50 iu/ml hCG, 1 μg/ml E_2, 0.5 mM sodium pyruvate and 50 μg/ml gentamicin (maturation medium). A group of 20 COC were placed in a 100 μl droplet of maturation medium and cultured for 24 h. Frozen-thawed Japanese black bull spermatozoa were washed twice by centrifugation at 500 × g for 5 min with modified Tyrode's medum (TALP) supplemented with 5 mM caffeine. The final sperm pellet was resuspended at a density of 2–4 × 10^6 cells/ml in a 100 μl droplet of the same medium supplemented with 5 mM caffeine, 10 mg/ml BSA and 50 μg/ml heparin. The sperm suspension was incubated for 1 h. Then COC that had been matured for 24 h were washed 4 times with TALP supplemented with 5 mM caffeine, 10 mg/ml BSA and 50 μg/ml heparin, and 10 COC were placed into a 100 μl droplet of sperm suspension. At 6 h post-insemination (hpi), cumulus cells were partially removed from the oocytes by repeated passage through a fine pipette and washed 4 times with bovine embryo culture medium (BECM) [27] without glucose and supplemented with 10% fetal calf serum (FCS, FCS, JRH, Lenexa, Australia). Twenty oocytes were cultured in a 100 μl droplet of the same medium. At 48 hpi, embryos at the 8-cell stage were mechanically freed from cumulus cells with a small glass pipette and 20 embryos were co-cultured with bovine oviduct epithelial cells (BOEC) [28] in BECM supplemented with 10% FCS. At 120 hpi, embryos at the morula stage were selected and 10 to 12 morulae were co-cultured with BOEC in a 100 μl droplet of the same medium supplemented with 2.78 mM glucose. The *in vitro* embryo culture was terminated at 10 dpi. All culture systems were performed under paraffin oil in 60 mm culture dishes in a CO_2 incubator (5% CO_2 in air at 39°C).

Isolation and culture of ICM

ICMs were immunosurgically isolated [29] from hatched blastocysts at 10 dpi (Fig. 1A) as follows: Embryos were incubated at 39°C under 5% CO_2 in air for 40 min in anti-bovine splenocyte serum diluted (1:8) in alpha Minimum Essential Medium (MEM, Sigma Chemical Co., St. Louis, MO, USA, M-0644) supplemented with 0.4% ovalbumin (Sigma, A-5503), washed in alpha MEM+0.4% ovalbumin 3 times, and then incubated at 39°C under 5% CO_2 in air for 40 min in guinea pig complement (Gibco, Grand Island, NY, USA 19195-015) diluted (1:8) in alpha MEM+0.4% ovalbumin. Anti-bovine splenocyte serum was prepared in rabbits by intravenous injection of 2 × 10^8 bovine splenocytes. After washing with alpha MEM+0.4% ovalbumin 3 times, lysed trophoblastic cells were removed by mouth-operated pipetting and isolated ICMs (Fig. 1B) were washed 3 times in culture medium. The basal medium used was alpha MEM supplemented with 20% FCS, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, 3.5 mg/ml glucose, 100 units/ml penicillin and 100 μg/ml streptomycin. One or three ICMs were cultured on mitomycin C inactivated mouse embryonic fibroblasts (MEF) in a 100 μl droplet of basal medium with or without several combinations of 1,000 units/ml murine recombinant LIF (Esgro®, Gibco, 13275-011), 40 ng/ml human recombinant SF (Sigma, S-9790) and 40 ng/ml human recombinant bFGF (Gibco, 13256-029) as follows: 1) LIF, 2) SF, 3) bFGF, 4) LIF+SF, 5) LIF+bFGF, 6) SF+bFGF and 7) LIF+SF+bFGF at 37°C under 5% CO_2 in air. The medium was renewed every 2–3 days. Attachment of ICMs to a feeder layer and proliferation of ectodermal cells were observed under an inverted microscope.

Feeder cells

MEF were obtained from 13 to 15 day-old C57BL/6 mouse embryos and prepared as previously described [30]. MEF were routinely plated on gelatinized 25 cm^2 culture flasks (Iwaki, Tokyo, Japan) and then cultured in alpha MEM supplemented with 10% FCS, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C under 5% CO_2 in air. The cells were used until the fifth passage as feeder cells. When the cells reached confluence, they were mitotically inactivated by treatment with 10 μg/ml mitomycin C (Sigma, M-0503) for 3 h, trypsinized, and seeded in a 100 μl droplet of alpha MEM-10% FCS onto gelatinized 60 mm culture dishes (Nunc, Denmark), which were covered with paraffin oil and maintained at 37°C under 5% CO_2 in air.

Passaging ectodermal cell colonies

After 6 to 7 days of culture, the primary ectodermal cell colonies outgrown from ICM clumps were isolated

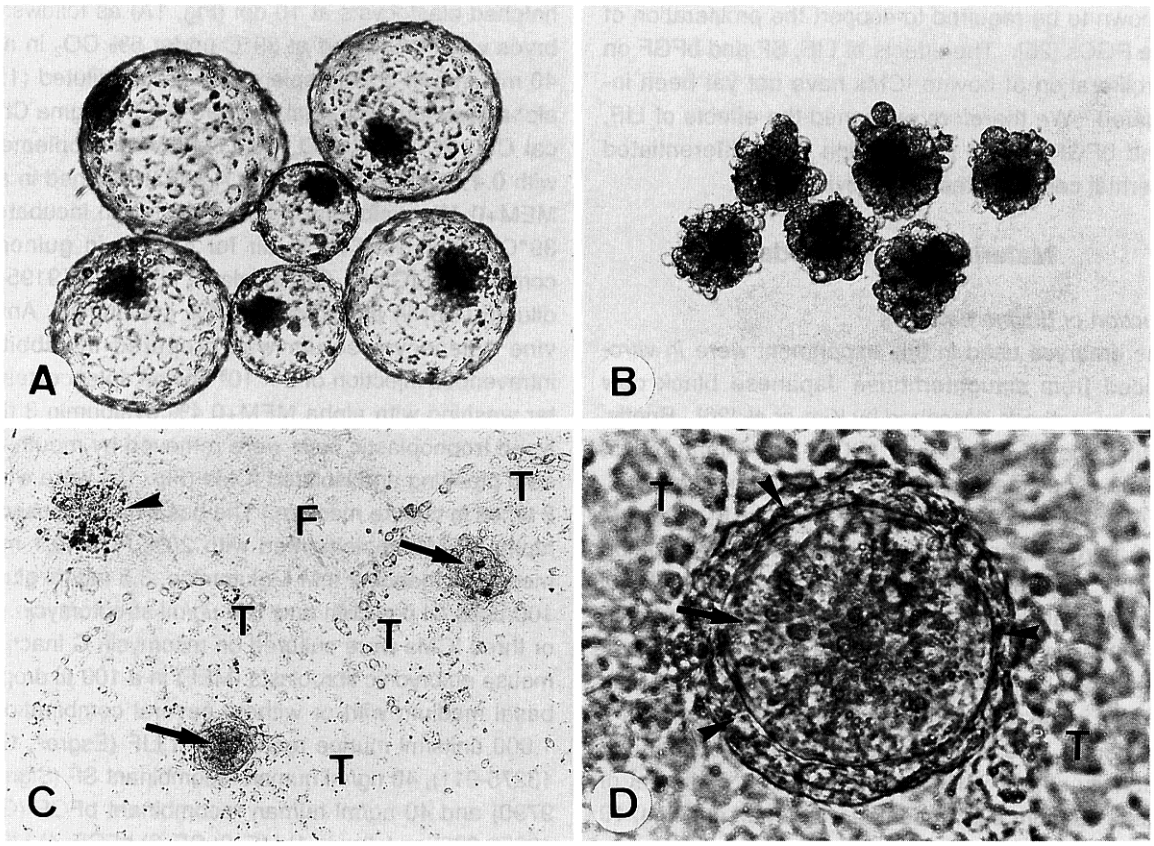

Fig. 1. (A) *In vitro*-produced hatched blastocysts, × 100. (B) ICM clumps immunosurgically isolated from hatched blastocysts shown in panel (A), × 200. (C) Three ICM clumps on feeder cells at 3 days of culture, primary ectodermal cell colonies (arrow), nongrowing ICM clump (arrowhead), T: trophoblastic cells, F: feeder cells, × 100. (D) Primary ectodermal cells outgrown from ICM clumps after 5 days of culture (arrow), primitive endodermal cells (arrowhead), T: trophoblastic cells, × 400.

from the feeder layer by cutting with a 26G needle. The primitive endoderm cells surrounding primary ectodermal cell colonies were cut off with a 26G needle. Primary ectodermal cell colonies were mechanically disaggregated by pipetting through a fine pipette to make small clumps of cells (10–20 cells/clump) in culture medium. The clumps of primary ectodermal cells disaggregated from each colony were then cultured on fresh feeder cells in the same culture medium which had been initially used, and then serially passaged every 6 to 7 days.

Results

When isolated ICM clumps were seeded onto feeder cells, attachment to feeder cells was observed one day after initiation of the culture. As shown in Table 1, when 1 ICM clump was cultured in a drop of basal medium, the lowest rate of attachment was observed (74%), whereas when 3 ICM clumps were cultured in a drop of LIF+SF containing medium, the highest frequency of attachment (94%) was observed. Approximately 1 to 2 days after ICMs attached to feeder cells, trophoblastic-like cells grew as a sheet on the feeder cells and surrounded ICM cells (Fig. 1C). Ectodermal cell colonies outgrown from ICM clumps were observed approximately 3 days after initiation of culture and showed clear-cut borders at 4 days of culture. The ectodermal cell colonies grew as a cluster on the top of trophoblastic-like cell sheets, and primitive endoderm cells also grew actively and then surrounded the ectodermal cell colonies (Fig. 1D).

As shown in Table 1, the proportion of the formation of ectodermal cell colonies from 1 ICM cultured in a drop of medium was lower than that of 3 ICMs cultured in a drop of medium in each different type of medium. The highest rate of formation of primary ectodermal cell colonies from 1 ICM and 3 ICMs per drop (37 and 57%

Table 1. Effects of leukemia inhibitory factor (LIF, 1,000 units/ml), stem cell factor (SF, 40 ng/ml) and basic fibroblast growth factor (bFGF, 40 ng/ml) on the proliferation of bovine ICM cells *in vitro*

Factors supplemented	No. of ICMs cultured/drop	No. of ICMs cultured	No.(%) of ICMs attached	No. (%) of ectodermal cell colonies forming	No. (%) of ectodermal cells surviving to passage					
					1	2	3	4	5	6
Control*	1	19	14 (74)	3 (16)	1 (5)	0	0	0	0	0
	3	18	15 (83)	4 (22)	2 (11)	0	0	0	0	0
LIF	1	18	15 (83)	5 (28)	3 (17)	2 (11)	1 (6)	0	0	0
	3	18	16 (89)	6 (33)	4 (22)	2 (11)	2 (11)	1 (6)	0	0
SF	1	18	15 (83)	6 (33)	4 (22)	2 (11)	1 (6)	0	0	0
	3	18	16 (89)	7 (39)	5 (28)	3 (17)	2 (11)	1 (6)	0	0
bFGF	1	18	14 (78)	3 (17)	1 (6)	0	0	0	0	0
	3	18	16 (89)	4 (22)	2 (11)	1 (6)	0	0	0	0
LIF + SF	1	19	16 (84)	7 (37)	6 (32)	4 (21)	2 (11)	1 (5)	0	0
	3	18	17 (94)	10 (56)	8 (44)	6 (33)	4 (22)	3 (17)	1 (6)	1 (6)
LIF + bFGF	1	17	14 (82)	5 (29)	3 (18)	2 (12)	1 (6)	0	0	0
	3	18	16 (89)	6 (33)	4 (22)	2 (11)	2 (11)	1 (6)	0	0
SF + bFGF	1	17	15 (88)	5 (29)	3 (18)	2 (12)	1 (6)	0	0	0
	3	18	15 (83)	7 (39)	5 (28)	4 (22)	2 (11)	1 (6)	0	0
LIF + SF + bFGF	1	18	16 (89)	6 (33)	5 (28)	3 (17)	2 (11)	1 (6)	0	0
	3	18	16 (89)	9 (50)	7 (39)	4 (22)	3 (17)	2 (11)	1 (6)	0

*alpha MEM supplemented with 20% FCS, 0.1 mM 2-mercaptoethanol, 2mM L-glutamine, 3.5 mg/ml glucose, 100 units/ml penicillin and 100 ug/ml streptomycin.

respectively) was obtained in the LIF+SF containing medium. In the control medium and bFGF containing medium, lower efficiencies in the formation of primary ectodermal cell colonies from 1 ICM (16 and 17% respectively) or 3 ICMs per drop (22 and 22% respectively) were obtained. The maximum number of passages (6 passages) of undifferentiated ectodermal cells was obtained from primary ectodermal cell colonies that had been disaggregated and cultured in the LIF+SF containing medium. Large cell clumps (10–20 cells/clump) and small cell clumps (<10 cells/clump) resulted at the time of disaggregation of ectodermal cell colonies. The large cell clumps were able to attach to feeder cells and proliferate after passage (Fig. 2A), whereas the small cell clumps barely survived due to failure to attach to feeder cells. In some cases, small cell clumps attaching to feeder cells were quickly differentiated into epithelial types of cells approximately 1 or 2 days after passage (Fig. 2B). Within 2 to 3 days after the first passage, ectodermal cells showed signs of spontaneous differentiation and were often characterized by a flattening of the cells (Fig. 2C). The differentiated cells forming a typical epithelial type of cell as a monolayer in a cobblestone-like sheet surrounded the ectodermal cell colony and formed vesicles (Fig. 2B, D) 5 to 6 days after passage. The number of ectodermal cells with undifferentiated morphology decreased from passage to passage and finally all of the cells were differentiated.

Discussion

As we previously reported [31], ICMs isolated from hatched blastocysts at 10 dpi exhibit the highest activities of growth when cultured on MEF. Talbot *et al.* [32] also reported that the formation of an ICM colony depended both on age and the source of blastocysts, and is essential for determining the optimal culture conditions for the establishment of bovine ES cells. The addition of LIF or recombinant LIF to the culture medium is necessary for the isolation of ES cells, including germ-line competent ES cells from mouse embryos [33–35]. The culture conditions for mouse ES cells are now generally applied for the isolation of livestock ES cells, but although BRL cell-conditioned medium or LIF has been added to culture medium, the isolation of ES cells from livestock has not yet been successful [5, 8, 12]. Anderson [36] suggested that culture conditions for mouse ES cells including murine LIF are not effective in preventing the initiation of differentiation in cultured ES cells of livestock species. LIF, SF and bFGF are known to be required to support the proliferation of bovine PGCs [25]. In the present experiments, it was shown that LIF and SF are required to support the formation of primary ectodermal cell colonies and to maintain an undifferentiated state for several passages. The proportions of attachment of ICM clumps to feeder cells and of the

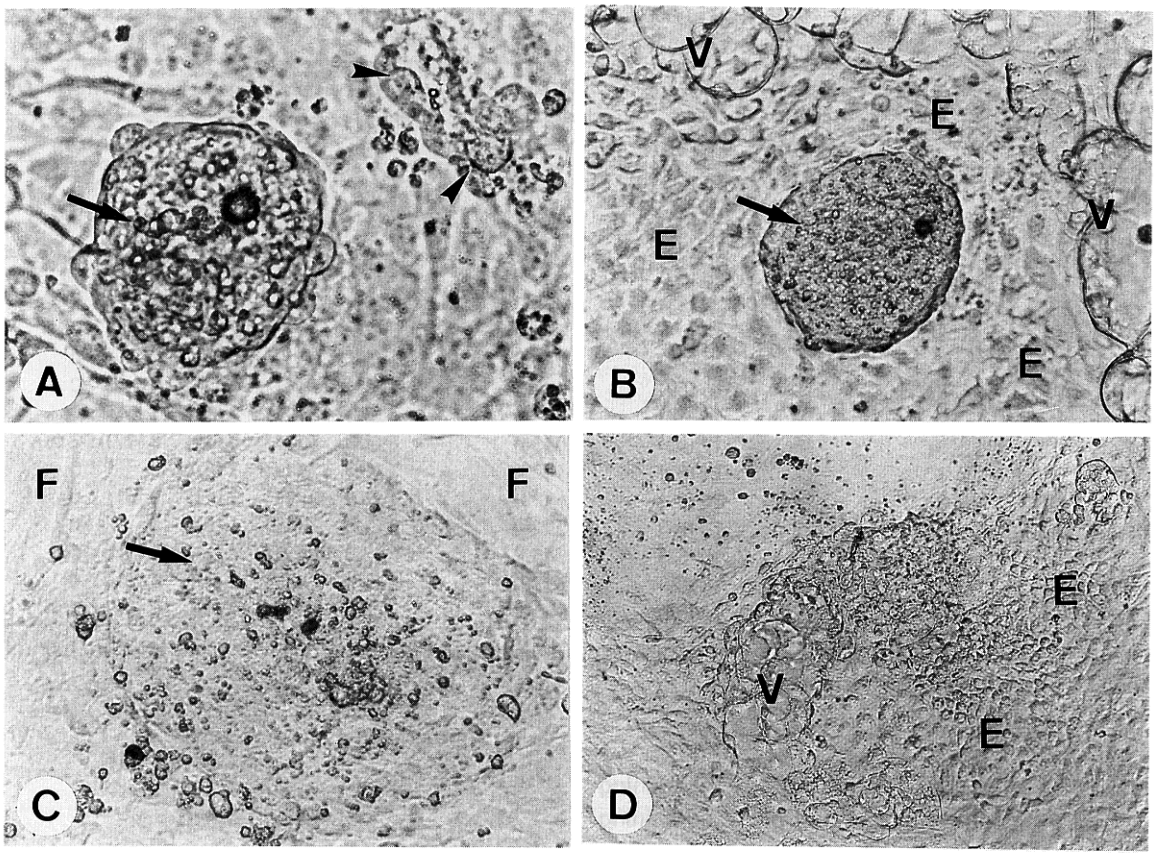

Fig. 2. (A) Ectodermal cell colony growing from a disaggregated large cell clump (arrow) 3 days after the first passage and a differentiated ectodermal cell colony growing from small cell clumps (arrowhead), × 400. (B) Ectodermal cell colony (arrow) 6 days after the fourth passage, E: epithelial-like cells monolayer, V: vesicles of epithelial-like cells, × 200. (C) Flattened ectodermal cell colony at 3 days of culture (arrow), F: feeder cells, × 200, (D) Flattened ectodermal cell colony shown in panel (C) was further cultured for 3 days, E: epithelial-like cells differentiated from ectodermal cells, V: vesicles of epithelial-like cells, × 100.

formation of primary ectodermal cell colonies from ICM clumps in control medium were similar to those of bFGF containing medium. Furthermore, even when bFGF was added to LIF+SF containing medium, the proportions of the attachment of ICM clumps and the formation of primary ectodermal cell colonies did not increase, compared to those of LIF+SF containing medium. These results suggest that bFGF does not support the proliferation of bovine ICMs. Contrary to our results, others have reported that the addition of bFGF to culture medium promoted the proliferation of isolated porcine ICMs [37].

The results of our experiments suggest that culturing groups of 3 ICMs rather than one in the same culture droplet gives an advantage in ICM attachment, with a higher rate of formation of primary ectodermal cell colonies. Campbell *et al.* [38] reported the successful establishment of a sheep embryonic cell line by culturing groups of 4-6 microdissected embryonic discs on feeder layers. It is known that the preimplantation mammalian embryo exhibits a stage-specific expression of receptors and their ligands during development [39], and that embryos do respond in a dose dependent manner to growth factors added to culture media [40–43]. Culturing embryos in groups and in a reduced volume of culture medium will allow any embryo-derived factor to increase in concentration over time, thereby facilitating its action either in an autocrine and/or in a paracrine manner [44].

It seems to be difficult to completely remove trophectoderm and primitive endoderm from immunosurgically isolated ICMs. The primary culture of isolated ICM clumps results in the proliferation of a mixed cell population with ectodermal cells, trophoblastic cells and primitive endoderm cells. However, it is possible that they were separated from the cultured ICMs [32]. When

the disaggregated ectodermal cells were serially cultured onto new feeder cells, they showed the growth characteristics of differentiated cells, mainly of formed epithelial-like cells. The trophectoderm and primitive endoderm cells derived from bovine blastocysts produced only polarized epithelial monolayers that formed domes and vesicles, respectively, being similar to those produced by pig and sheep trophectoderm and primitive endoderm cells [45]. Since it was found that the primitive endoderm promotes the differentiation of early epiblast cells to a stage when they can no longer form ES cells, contamination of primitive endoderm during passaging of the primary ectodermal cell populations could affect the successful isolation of ES cells [46].

The development of the passaging technique seems to be one of the important steps for successful isolation of bovine ES cells. Only large cell clumps which mechanically disaggregated from ectodermal cell colonies survived after passage, but we were unable to maintain undifferentiated ectodermal cells beyond 6 passages. We have already shown that dissociation of bovine ectodermal cell colonies into single cells by trypsin treatment resulted in unsuccessful serial passage [31].

In conclusion, data from our experiments demonstrate that culturing groups of 3 ICMs gave results superior to culturing single ICM for the formation of primary ectodermal cell colonies. LIF and SF are important factors for supporting outgrowth of the primary ectodermal cells and also for maintaining an undifferentiated state after serial passage of these cells.

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