ScienceAsia 26 (2000) : 81-86

Establishment and Long-Term Maintenance of Bovine Embryonic Stem Cell Lines Using Mouse and Bovine Mixed Feeder Cells and Their Survival after Cryopreservation

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Received 15 Jul 1999
Accepted 16 Mar 2000

ABSTRACT To investigate the efficiency of establishment and maintenance of bovine embryonic stem (ES) cell lines, the inner cell mass (ICM) of embryos derived from *in vitro* production was isolated for ES cell preparation by removal of trophoblasts with protease. Feeder cells from bovine, mouse and mixed fibroblasts were produced from mouse and bovine embryonic fibroblasts. The feeder cells were selected and purified through 30 passages. Stabilized feeder cells with their good contact and forming monolayer were used for long-term culture of bovine ES cell lines. The efficiency of bovine ES cell attachment on mouse or mixed feeder cell monolayer increased when compared to those using bovine feeder cells. Bovine ES cell lines were cultured and maintained in undifferentiated state longer on mixed fibroblasts (258±19 days) than bovine (225±6 days) or mouse (196±49 days) fibroblasts. The ES cell lines were identified as pluripotent by alkaline phosphatase (AP) staining and forming the embryoid body. Vitrification procedure for cryopreservation of ES cells showed a higher survival rate than the conventional freezing method. These data suggest that mixed fibroblast feeder cells were more efficient for long-term culture of bovine ES cell lines than using only mouse or bovine fibroblasts.

KEYWORDS: ES cells, bovine, mixed feeder cells, cryopreservation.

INTRODUCTION

Embryonic stem (ES) cells are totipotent cells deriving from inner cell mass (ICM) of preimplantation embryos,[1-2] embryonic germ (EG) cells and fetal germ cells.[3-4] They are capable of unlimited and undifferentiated proliferation *in vitro*. ES cells have been established in the mouse[1-2] and were shown to be capable of contribution to any kind of tissue when being combined with normal preimplantation embryos as chimeras.[5] Totipotent ES cells provide a powerful tool for the studies of early embryonic development,[6-8] gene targeting,[9-11] cloning[12-13] and regenerative medicine.[14-15] Because of their potential use for targeted gene manipulation, isolation of ES cells in livestock species could have numerous agricultural, biomedical and pharmaceutical applications. The use of ES cell technology in livestock may overcome current limitation on efficient gene transfer by providing an abundance of totipotent stem cells to be genetically manipulated by using conventional recombinant DNA techniques. However, efficiency toward establishment of ES cell lines from species other than the mouse was low.[16-18] Therefore, a thorough understanding of the factors or conditions for ES cell isolation and long-term maintenance is crucial.

Recent research studies attempted to use different kinds of feeder cells to support ES cell growth [19] and prevent cell differentiation.[20] Mouse ES cells were isolated on mouse embryonic fibroblasts[21] in medium supplemented with or without leukemia inhibiting factor (LIF).[22-23] This factor is a cytokine produced by a number of sources including mouse fibroblast cell lines (STO), buffalo rat liver (BRL) cells, Vero cells and human embryonic fibroblasts. It is considered an important factor to prevent ES cell differentiation.[22,24] LIF producing STO cells have been commonly used for isolation of mouse ES cell lines. BRL-conditioned medium combined with STO feeder cells was described for isolation of mouse ES cell lines.[25] Since bovine fibroblasts and oviductal cells have also been demonstrated to synthesize LIF,[26] the aim of this experiment was to use mixed feeder cells of mouse and bovine embryonic fibroblasts to increase the efficiency of bovine ES cell isolation and long-term maintenance.

MATERIALS AND METHODS

Mouse fibroblast feeder cells

Fifteen-day-old mouse fetuses were dissected from the uteri and separated from placental tissue. The carcass was washed in phosphate buffer saline (PBS) and transferred into a tissue culture dish containing 3 ml of 0.25% trypsin/ethylene glycol tetraacetic acid (trypsin/EGTA). It was then finely minced and incubated for 5 min at 37°C. The trypsin disaggregate was neutralized with 5 ml of Dulbecco's Modified Eagle's Medium (Gibco,Grand Island, NY) containing 10% (v/v) new born calf serum (NCS; $DMEM_{10}$). The suspension was then transferred to a conical centrifuge tube and large pieces of cellular debris were allowed to settle for 2-3 min. The supernatant containing the fibroblasts was transferred to 25 ml tissue culture flasks or 60 mm dishes and cultured at 37°C in 5% CO_2 humidified incubator. The fibroblasts were subcultured as previously described.[27]

Bovine fibroblast feeder cells

Bovine fetuses at about 45 days old were retrieved from a local slaughterhouse. The heads, bones and abdominal organs of the fetuses were removed. The remaining tissues were minced and digested using 5 ml 0.25% trypsin/EGTA for 15 min before neutralizing with $DMEM_{10}$. The minced tissues were put into a conical centrifuge tube and centrifuged for 1 min at 500 rpm. The supernatant was transferred to 25 ml tissue culture flasks or 60 mm dishes. The cells were observed every 12 hours under x20 inverted microscope. When over 50% of the cells had attached to the bottom of the containers, the media were changed within 24 hours and subculture was performed during cell confluence.

Isolation of feeder cell lines

When fibroblast layer was confluent, they were disaggregated with 0.25% trypsin/EGTA. The concentrations of feeder cells were determined and transferred into other tissue culture flasks or dishes with the concentration of $2x10^4$ cells/ml. After 3 days of mouse fibroblast culture and 5 days of bovine or mixed fibroblast culture, the colonies were separated with a blunt needle. The fibroblast colonies were disaggregated by transferring into four-well dishes containing 0.25% trypsin/EGTA for 3 min at 37°C. The glass pipettes were used to aspirate and expel the medium several times for cell separation and then neutralized with $DMEM_{10}$. These cells were cultured in 5% CO_2 incubator at 37°C. When the cells were confluent, they were collected and disaggregated again with the same method and were subcultured for 10 passages.

Production of mixed fibroblast feeder cells

After 10 passages of mouse and bovine fibroblast subculture, one half of bovine fibroblasts and one half of mouse fibroblasts were pooled. The final concentration of the mixed cells was $5x10^4$ cells/ml. When the cells were confluent, they were disaggregated with 0.25% trypsin/EGTA and neutralized with $DMEM_{10}$. The same passaging procedure was repeated for at least 30 passages. When the cell monolayer surface became very flat and smooth without morphological and pH changes for at least two weeks, it was well stabilized. These stabilized mixed feeder cells were used for long-term isolation and culture of bovine ES cells.

Isolation and culture of embryonic stem cells

The culture medium for bovine ES cells containing 20% NCS was called $DMEM_{20}$. The embryos were produced from the procedures of *in vitro* maturation, fertilization and development.[28] The ICM of hatched embryos was disaggregated with 3% protease for 10 min at 37°C. Then, they were transferred to $DMEM_{20}$ supplemented with 0.2 mM mercaptoethanol and 1000 iu/ml LIF (Gibco) in 75 ml flasks covered previously with 0.5% gelatin (Sigma,St. Louise, MO) in distilled water. The ICM was also separated into either single cells or pieces containing 5-15 cells. The cell suspensions were transferred into 75 ml flasks or four-well culture dishes coated with a single layer of mouse, bovine or mixed feeder cells. After culturing for 3 weeks, colonies with a morphology resembling ES cells were selected for further passage and alkaline phosphatase (AP) staining.

Alkaline phosphatase staining

AP activity was determined as previously described.[29] Briefly, culture medium was removed from the plates and ES cells were fixed with 4% paraformaldehyde for 20 min. Fixed cells were washed twice with PBS and stained in 200 µg/ml naphtol AS-MX phosphate (Sigma) and 1 mg/ml Fast Red TR salt (Sigma) in 100 mM Tris buffer, pH 8.2 for 30 min at room temperature. Staining was terminated by washing cultures in PBS. Positive AP staining is characterized by red color.

Long-term culture of bovine ES cells

The colonies of ES cells were passaged using 2%

protease to separate the cells and replated on fresh mouse, bovine or mixed feeder cells. The media were replaced every other day, and the cells were passaged every 7 days for mouse feeder cells and 12 days for bovine or mixed feeder cells. The ES cells were observed every day to evaluate their differentiation according to morphology and AP staining.

Vitrification of ES cells

Three kinds of cryopreservation solutions were prepared with DMEM. The two vitrification solutions were DMEM with 20% (v/v) ethylene glycol (EG) plus 25% (v/v) dimethyl sulphoxide (DMSO), and 20% (v/v) propanediol plus 25% (v/v) DMSO. The conventional freezing solution contained only 10% DMSO. Two-ml cryo-tubes were used for loading the ES cells at a final concentration of $3x10^6$ cells/ml. After equilibration at -4°C for 2 min, the cryo-tubes with ES cells were directly plunged into liquid nitrogen (LN_2). After storage for at least one week, the cryo-tubes were warmed rapidly in 25°C water. The ES cells in the vitrification or conventional freezing solutions were expelled into tissue culture dishes. Then, 0.5 M sucrose in DMEM was added and incubated for 5 min to remove cryoprotectants from ES cells. The ES cell suspension was transferred into a 10 ml centrifuge tube, and 4 ml $DMEM_{10}$ was added before centrifugation at 3000 rpm for 5 min. The supernatant was discarded. The pellet was resuspended with 2 ml $DMEM_{20}$ and the cell concentration was determined by hemacytometer. The final concentration of ES cells was $2x10^4$ cells/ml for the co-culture with feeder cells.

Statistical analyses

The effects of feeder cells on the time of attachment, differentiating inhibition time of bovine ES cells were analyzed by Students *t*-test. A chi-square analysis was performed to determine the effect of feeder cells on the rate of attachment and the effect of the vitrification solution on survival rate of bovine ES cells.

Results

Isolation and long-term establishment of bovine ES cell lines

As shown in Table 1, mouse and bovine feeder cells had different advantages in supporting bovine ES cells. Mouse fibroblasts have been proved superior over bovine fibroblasts for bovine ES cell attachment ($p<0.05$). The percentage of bovine ES cell attachment on mouse, bovine and mixed fibroblasts were 80, 55 and 78%, respectively. It took an average of 36 hours for ES cells to attach on mouse or mixed feeder cells and 72 hours on bovine feeder cells. Bovine ES cell lines could be maintained in culture on a feeder layer of mouse, bovine or mixed embryonic fibroblasts by passaging every 7 days for mouse fibroblasts and 12 days for bovine or mixed fibroblasts. Table 1 also demonstrates differences in the ability to inhibit ES cell differentiation by various types of feeder cells. Mixed fibroblasts appear to inhibit bovine ES cell differentiation better ($p< 0.05$) than mouse fibroblasts. The efficiency of bovine fibroblasts to maintain undifferentiated bovine ES cells was longer than mouse fibroblasts, however, no significantly different was observed. When the bovine ES cells were co-cultured with mixed feeder cells, the efficiency of bovine ES cell isolation was significantly increased.

ES cell colonies were classified as stem or differentiated colonies according to their morphology and AP staining. The ES cell colonies exhibited morphology characterized by prominent colonies and tightly packed with very small cells (Fig 1). The colonies of ES cells were disaggregated into single cells showing the morphological characteristics of a small cell and round shape with a small proportion of cytoplasm to nuclei. The shapes of ES cells co-cultured with mouse, bovine or mixed fibroblasts were not different. The AP activity was present in a number of ES cell lines (Fig 2) that culturing on all types of feeder cells. The occurrence of AP-positive cells was lost when the ES cells had

Table 1. Effect of different types of feeder cells on attachment and long-term culture of bovine ES cells.

Feeder cells	Time of attachment (hours)	No. of attachment (%)	Differentiating inhibition time (days)
Mouse fibroblasts	36±18[a]	40/50 (80%)[a]	196 ±49[a]
Bovine fibroblasts	72±24[b]	28/51 (55%)[b]	225±6[ab]
Mixed fibroblasts	36±18[a]	35/45(78%)[a]	258±19[b]

[a,b] Values with different superscripts are significantly different ($p<0.05$).

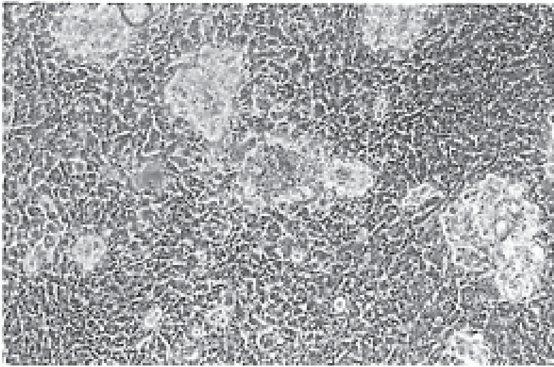

Fig 1. Bovine ES cell colonies are growing on stabilized mixed feeder cells. The colonies were tightly packed with very small undifferentiated ES cells.

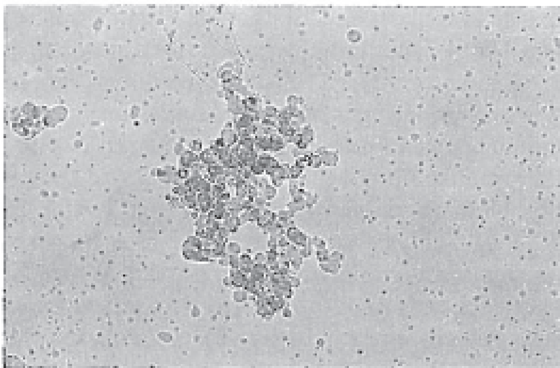

Fig 2. Alkaline phosphatase staining of bovine ES cells. Most of ES cells exhibit an AP-positive staining.

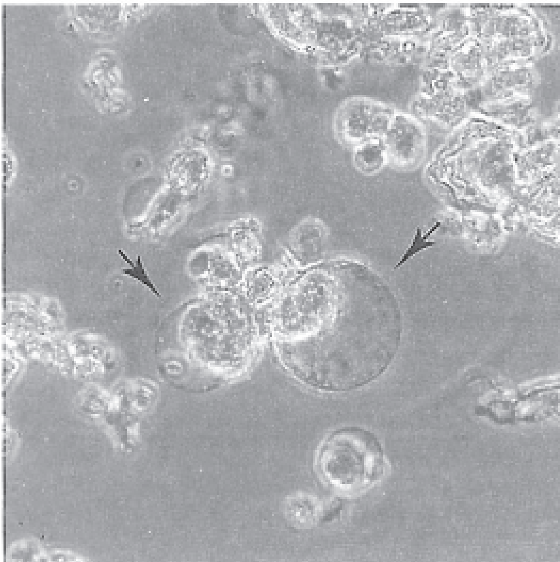

Fig 3. The differentiated ES cells were identified by the criteria of cell aggregation and forming embryoid body (arrows).

Table 2. Survival rates after 24-hour culture of vitrified-warmed bovine ES cells in various vitrification solutions.

Passage	Survival rate (%)		
	Solution 1[a]	Solution 2[a]	Solution 3[b]
2	50	50	30
4	50	50	30
6	50	50	30
8	50	50	30
10	50	40	30
12	50	40	20
14	40	40	20
16	40	40	20
18	30	20	10
20	30	20	10

[a,b]Values with different superscripts are significantly different ($p < 0.05$).

Solution 1 20% EG plus 25% DMSO

Solution 2 20% Propanediol plus 25% DMSO

Solution 3 10% DMSO

differentiated. The differentiated ES cells were identified by the criteria of cell aggregation and forming embryoid body (Fig 3).

Vitrification of bovine ES cells

After warming of the cryopreserved ES cells and culture for 24 hours, the difference of living and dead cells could be distinguished from their colors and shapes under the phase-contrast microscope. The dead cells shrunk after being warmed but the living cells could recover to their original morphology. The rates of ES cell survival after cryopreservation and warming decreased gradually with subsequent subcultures. At the first few passages the survival rates were 30 to 50% and decreased to only 10 to 20% after 20 passages (Table 2). The vitrification solution with 20% EG plus 25% DMSO, or 20% propanediol plus 25% DMSO provided a significantly ($p<0.05$) higher survival rate of ES cells than those thawed and cultured in the conventional freezing method using 10% DMSO.

Discussion

The present study has demonstrated the successful long-term culture of bovine ES cell lines using mixed feeder cells. Although the attachment of bovine ES cells to mouse fibroblast cell monolayer was more efficient than to that of bovine. ES cells remained undifferentiate longer on a bovine or mixed fibroblast cell layer. This means that the attachment

of ES cells on different feeder cells differs physiologically. Attachment of ES cells onto feeder cells occurs by the affinity of the molecules on both surfaces of ES cells and fibroblasts. The reason for the low affinity between bovine ES cells and fibroblasts of the same species remains unknown but there are few possibilities to explain higher differentiating inhibition by bovine fibroblasts. First, prevention of ES cell differentiation depends on the interaction between LIF and differentiation-inhibiting receptors on the surface of ES cells,[30] which supposedly transmit signals to ES cell nuclei to prevent gene activation for cell differentiation. Although mouse fibroblasts produce LIF, which maintains the undifferentiated state of mouse ES cells, it may be species specific. It may not effectively interact with bovine LIF receptor or require other cofactors to be able to respond with LIF receptors on the bovine ES cells.[31-33] LIF receptor may only be able to recognize homologous LIF produced by bovine embryonic fibroblasts. Nevertheless, human LIF was reported to maintain undifferentiated state of lower species as porcine ICM[29] and ES cells of rhesus monkey.[34] Second, the concentration of the differentiation-inhibiting factor released from bovine embryonic fibroblasts may be higher and the life span of the factor may be longer than factors secreted by mouse fibroblasts. Third, mouse fibroblasts may produce suboptimal quantities of LIF. Pease et al[22] have demonstrated that the amount of LIF present in culture medium conditioned by mouse feeder cells was as low as 500 units/ml, which is below the optimal concentration (1000 units/ml) necessary to culture ES cells. Our finding of bovine fibroblasts in inhibition of ES cells differentiation suggests that bovine fibroblasts have function similar to LIF in preventing bovine ES cell differentiation.

Mitomycin C has been used to block feeder cell division, but it has a side effect on ES cell proliferation even when it is washed out. In the present study, the stabilized condition of feeder cells was employed instead of using mitomycin C. The area of good contact of flat and smooth feeder cell monolayer forming on the bottom of culture dishes has a good inhibiting property for ES cell culture. Therefore, the establishment of stabilized mixed feeder cell lines in our study played an essential role in a long term culture of bovine ES cells. The AP-positive cells were found in tightly packed ES cell colonies and AP was lost when the differentiated cell formed embryoid body. AP was abundant in the most undifferentiated mouse ES cells, mouse embryonal carcinoma (EC) and most human EC cells.[35-36] AP activity was rapidly lost in bovine epiblast cells as they differentiated.[37]

There are very limited reports concerning vitrification of ES cells, in spite of many researches conducted on vitrified embryos of different animals.[38-39] DMSO was a common and an effective cryoprotectant used in either conventional or vitrified procedures. The common concentration of DMSO in freezing solution for cryopreservation of different kinds of cells is 10%. Recently, its concentration was raised up to 40% in order to vitrify embryos. EG has a higher permeability property and has been used for bovine embryo cryopreservation in our previous work.[40] We obtained a high pregnancy rate after the transfer of bovine embryos cryopreserved with EG solution. In the present study, these two cryoprotectants were combined in order to increase their permeability, shorten the exposure time, minimize their toxicity to ES cells and improve the efficiency of vitrification. Propanediol was also used to cryopreserve embryos and other somatic cells and showed lesser toxicity in bovine embryo cryopreservation[41] However, the present study revealed lower survival rate of ES cells vitrified with propanediol. The reason may be due to its lower permeability than other cryoprotectants. Nevertheless, the present study demonstrated that bovine ES cells could be cryopreserved without using a programmed machine. If a freezing machine was utilized to complete the procedure, it would require more time and be complicated. The vitrification method reported here could simplify the procedure and improve the viability of ES cells.

Acknowledgements

This work was supported by Thailand's National Science and Technology Development Agency. We are grateful to Dr C Pholpramool and Ms U Sanpatchayapong for their editorial assistance.

References

1. Evans MJ and Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154-6.
2. Martin GR (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cell. *Proc Natl Acad Sci USA* **78**, 7634-8.
3. Matsui Y, Zsebo K and Hogan BLM (1992) Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. *Cell* **70**,841-7.
4. Resnick JL, Bixler LS, Cheng L and Donovan PJ (1992) Long-term proliferation of mouse primordial germ cells in culture. *Nature* **359**, 550-1.
5. Bradley A, Evan M, Kaufman M and Robertson E (1984) Formation germ-line chimeras from embryo-derived teratocarcinoma cell lines. *Nature* **309**,255-6.

6. Robertson EJ (1991) Using embryonic stem cells to introduce mutations into the mouse germ line. *Biol Reprod* **44**, 238-45.
7. Wiles MV and Johansson BM (1997) Analysis of factors controlling primary germ layer formation and early hematopoiesis using embryonic stem cell *in vitro* differentiation. *Leukemia* **11**,454-6.
8. Rathjen J, Lake JA, Bettes MD, Washington JM, Chapman G and Rathjen PD (1999) Formation of primitive ectoderm-like cell population, EPL cells; from ES cell in response to biological derived factors. *J Cell Sci* **112**, 601-12.
9. Lohnes D (1999) Gene targeting of retinoid receptors. *Mol Biotechnol* **11**, 67-84.
10. Jakobs PM, Smith L, Thayer M and Grompe M (1999) Embryonic stem cell can be used to conduct hybrid cell lines containing a single, selectable murine chromosome. *Mamm Genome* **10**, 381-4.
11. Zheng B, Mills AA and Bradley A (1999) A system for rapid regeneration of coat color-tagged knockouts and defined chromosomal rearrangements in mice. *Nucleic Acids Res* **27**, 2354-60.
12. Modlinski JA, Michael AR,Thomas EW and Jolanta K (1996) Embryonic stem cell: developmental capabilities and their possible use in mammalian embryo cloning. *Anim Reprod Sci* **42**, 437-46.
13. Stice SL and Keefer CL (1993) Multiple generational bovine embryo cloning. *Biol Reprod* **48**,715-9.
14. Dinsmore J, Ratiff J, Deacon T, Pakzaban P, Jacoby D, Galpern W and Isacson O (1996) Embryonic stem cell differentiated *in vitro* as a novel source of cells for transplantation. *Cell Transplant* **5**, 131-43.
15. Brustle O, Spiro AC, Karram K, Choudhary K, Okabe S and Mckay RD (1997) In vitro- generated neural precursors participate in mammalian brain development. *Proc Natl Acad Sci USA* **94**, 14809-14.
16. Evans MJ, Notarianni E, Laurie S and Moor RM (1990) Derivation and preliminary characterization of pluripotent cell lines from porcine and bovine blastocysts. *Theriogenology* **33**, 125-8.
17. Notarianni E, Galli C, Moor RM and Evans MJ (1991) Derivation of pluripotent, embryonic cell lines from the pig and sheep. *J Reprod Fertil suppl* **43**, 255-60.
18. Stice SL, Strelchenko N, Keefer CL and Mathews L (1996) Pluripotent bovine embryonic cell lines direct embryonic development following nuclear transfer. *Biol Reprod* **54**, 100-10.
19. Piedrahita JA, Anderson GB and BonDurant RH (1990) Influence of feeder layer type on the efficiency of isolation of porcine embryo-derived cell lines. Theriogenology 34, 865-877.
20. Smith AG and Hooper ML (1987) Buffalo rat liver cells produce a diffusible activity which inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells. *Dev Biol* **121**, 1-9.
21. Wobus AM, Holzhausen H , Jakel P and Schoneich J (1984) Characterization of a pluripotent stem cell line derived from a mouse embryo. *Exp Cell Res* **152**, 212-9.
22. Pease S, Braghetta P, Gearing D, Grail D and Williams RL (1990) Isolation of embryonic stem (ES) cell in media supplemented with recombinant leukemia inhibitory factor (LIF). *Dev Biol* **141**, 344-52.
23. Moreau, JF, Donaldson DD, Bennett F, Witek-Giannotti J, Clark SC and Wong GG (1988) Leukaemia inhibitory factor is identical to the myeloid growth factor human interleukin for DA cells. *Nature* **336**, 690-2.
24. Smith AG, Heath JK, Donaldson DD, Wong GG, Moreau J, Stahl M and Rogers D (1988) Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* **336**, 688-90.
25. Handyside AH, O, Neill GT, Jones M and Hooper ML (1989) Use of BRL-conditioned medium in combination with feeder layers to isolate a diploid embryonal stem cell line. *Roux's Arch Dev Biol* **198**, 48-55.
26. Reinhart KC, Dubey RK, Mummery CL, van Rooijen M, Keller PJ and Marinella R (1998) Synthesis and reguration of leukemia inhibitory factor in culture bovine oviduct cell by hormones. *Mol Hum Reprod* **4**, 301-8.
27. Strelchenko N (1996) Bovine Pluripotent stem cell. *Theriogenology* **45**, 131-40
28. Pavasuthipasit K, Kitiyanant Y, Lhuangmahamongkol S, Tocharus C and Prempree P (1994) Porcine oviductal cells support *in vitro* bovine embryo development. *Theriogenology* **41**, 1127-38.
29. Moor K and Piedrahita JA (1997) The effect of human leukemia inhibitory factor (hLIF) and culture medium on *in vitro* differentiation of cultured porcine inner cell mass (pICM). *In Vitro Cell Dev Biol* **33**,62-71.
30. Williams RL, Hilton DJ, Pease S, Willson TA, Stewart CL, Gearing DP, Wayner EF, Metcal D, Nicola NA and Gough NM (1988) Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cell. *Nature* **336**, 684-7.
31. Smith TA and Hooper ML (1983) Medium conditioned by feeder cells inhibits the differentiation of embryonal carcinoma culture. *Exp Cell Res* **145**, 458-62.
32. Wilson TA, Metcalf D and Gough NM (1992). Cross-species comparison of the sequence of the leukaemia inhibitory factor gene and its protein. *Eur J Biochem* **204**,21-30.
33. Gearing DP, Thut CJ, VanderBos T, Gimpel SD, Delaney PB, King J, Price V, Cosman C and Beckman MP (1991). Leukemia inhibitory factor receptor is structually related to the II-6 signal transducer, gp 130. *EMBO J* **10**, 2839-48.
34. Thomson JA, Kalishman J, Golos TG, During M, Harris CP, Becker RA and Hearn JP (1995). Isolation of a primate embryonic stem cell line. *Proc Natl Acad Sci USA* **92**,7844-8.
35. Cotte CA, Easty GC and Neville AM (1981) Establishment and properties of human germ cell tumors in tissue culture. *Cancer Res* **41**, 1422-36
36. Benham FJ, Andrews PW, Knowles BB, Bronson DL and Harris H (1984) AP isozymes as possible markers of differentiation in human testicular teratocarcinoma cell lines. *Dev Biol* **88**, 279-87.
37. Talbot NC, Powell AM and Rexroad CE (1995). In vitropluripotency of epiblasts derived from bovine blastocysts. *Mol Reprod Dev* **42**, 35-52.
38. Dobrinsky JR (1996) Cellular approach to cryopreservation of embryos. *Theriogenology* **45**, 17-26.
39. Kasai M (1996) Simple and efficient methods for vitrification of mammalian embryos. *Anim Reprod Sci* **42**, 67-75.
40. Jiang G, Sun Y, Yang Z, Tocharus C, Kitiyanant Y and Pavasuthipaisit K (1997) The differences of osmotic behaviors of bovine embryos from *in vitro* and *in vivo* and their quality evaluation. *Theriogenology* **47**, 307 (Abstr).
41. Van Wagtendonk-De Leeuw AM, Den Daas JH, Kruip TA and Rall WF (1995) Comparison of the efficacy of conventional slow freezing and rapid cryopreservation methods for bovine embryos. *Cryobiology* **32** , 157-67.