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# Systems for Production of Calves from Cultured Bovine Embryonic Cells

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# 우 수정란의 배양세포들로부터 송아지 생산을 위한 체계

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## 요 약

전능성을 지닌 우 수정란 세포배양기술 체계 확립은 가축육종에 중요한 의의를 지닌다. 이러한 체계는 1) 핵치환에 의한 다수의 클론동물 생산에 대한 기전, 2) 형질전환세포를 선발하기 위한 marker의 사용으로 효과적인 유전자 전이체계와 3) site specific 유전자 전이에 대한 기전 또는 homologous DNA서열 재조합에 의한 결손 등에 대한 기전을 이해하는데 이용될 수 있다. 우 수정란세포의 배양은 배반포 내부 세포괴, 상실배와 16~20세포기로부터 확립하였다. 이들 모든 세포들은 생쥐 배아간 세포 형태와 유사하였으며 배양시 분화와 증식에서 다능성을 나타내었다. 배양체계는 미세소적이나 배양용기, 우 또는 설치류 섬유아 세포주를 단기간 배양 또는 장기간 배양방법을 이용하였다. 유사분열시 요구되는 배양체계나 배양액 그리고 분화 억제에 대한 괄목할만한 장점은 아직 밝혀지지 않고 있다. 현재 16~20 세포기의 배양세포의 전능성에 대해서는 알려져 있지 않다. 배양된 ICM세포 전능성은 27일간 배양한 ICM 세포로부터 4마리의 산자 생산에 의해 입증되었다.

## I. INTRODUCTION

Embryonic stem cells have been isolated from the late stage inner cell mass of mouse blastocysts (Evans and Kaufman, 1981) or morulae (Eistetter, 1989) and cultured on differentiation inhibiting mouse fibroblast feeder layers to large cell numbers. These ES cells exhibit pluripotency. When chimerized with normal embryonic cells, they result in ES cell chimeric offspring and pure ES cell germ line descendants (Evans and Kaufman, 1981; Stewart, 1991). In mice ES cells have been used to study embryo development, cell fate and lineage (Joyner, 1991). More important to livestock production ES cells could provide, as they do in mice, a mechanism for gene transfer by transfection, infection or injection of genes into the cells (Evans and Kaufman, 1981 ; Gossler et al., 1986; Lovell-Badge et al., 1987; Joyner, 1991; Stewart, 1991; Anderson, 1992). Using a selective marker, the trans-

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genic cells could be separated and used by chimerization into a cleavage stage embryo or blastocyst, as in mice to produce transgenic offspring (Hooper et al., 1987; Joyner, 1991; Stewart, 1991). Additionally, homologous recombination techniques can be used with cultured ES cells to add or delete genes specific sites in the genome (Koller et al., 1989; Capecchi, 1989; Stanton et al., 1990; Yagi et al., 1990). The ES cells also provide a large population of totipotent cells potentially useful for production of clonal offspring by nuclear transfer (First and Prather, 1991). This also provides a possible way for production of offspring from transgenic ES cells. Until recently as published in this paper and those of Wheeler (1994) and Sims and First(1993, 1994) evidence of ES cell totipotency and use of ES cells for the above purposes have been achieved only with mouse ES cells (Hooper et al., 1987; Koller et al., 1989; Capecchi, 1989; Stanton et al., 1990; Yagi et al., 1990; Stewart, 1991; Anderson, 1992). Cultured ES cells have great value for use in domestic animals to: 1) make large numbers of clonal offspring by nuclear transfer, 2) provide efficient gene transfer, with use of selectable markers for transgenes, and 3) as a mechanism for site specific gene transfer or deletion via site specific homologous sequence recombination. Development of a totipotent ES cell system depends on: 1) identification and isolation of ES cells, 2) the ability to inhibit differentiation and commitment to differentiate, 3) the ability to culture and passage, 4) demonstration of pluripotency on removal of differentiation inhibition, and 5) demonstration of totipotency as evidenced by ES cell offspring or offspring form ES cell chimeras.

This paper will focus on some of the variables affecting the isolation, derivation, culture, pluripotency and totipotency of bovine putative ES cells. These variables include : stage of the embryo supplying putative ES cells, methods of ES cell isolation, culture methods and differentiation prevention.

## Ⅱ. ESTABLISHMENT OF ES CELL CULTURES

Most attempts to isolate and culture inner cell mass cells have been based on or adapted from the original methods of Evans and co-workers for mice(Evans and Kaufman, 1981). These methods have involved placing either the entire day 4 hatched mouse blastocyst or its ICM after immunosurgical removal of the trophoblast cells onto a murine mitomycin C and LIF treated fibroblast feeder layer. The ICM is allowed to out grow for 3~4 days and is then picked off and briefly incubated in trypsin to disaggregate the cells.

In bovine there is no discrete epiblast so some workers have allowed outgrowth for as long as 7 days. Conceptually, there may be problems associated with this method. First cells undergoing prolonged association with each other may transcribe information and some become committed to differentiation before isolation or influence of LIF. Second the trypsin treatment may be damaging to the cells and their lineage. It is known that mouse ES lines passaged with

**Table 1. Use of loose suspension cultured inner cell mass cells as donors of nuclei in nuclear transfer to produce blastocysts**

| | |
|---|---|
| Cell lines | 5 |
| Culture duration | |
| mean | 37 days |
| maximum | 101 days |
| Nuclear transfers | 659 |
| Blastocysts | 109(15%) |

adapted from Sims and First (P.N.A.S., 1994)

**Table 2. Use of loose suspension cultured inner cell mass cells as donors of nuclei in nuclear transfer to produce blastocysts and calves**

| | | | |
|---|---|---|---|
| Cell lines | | 5 | |
| Cultrue duration (× days) | | 34 | |
| Nuclear transfers | | 239 | |
| Blastocysts | | 42 | (14%) |
| Blastocysts (n=34) transferred into cows (n=27) | | | |
| | Pregnant at 42 days | 13 | (49%) |
| | Fetuses at 56 days | 10 | (37%) |
| Calves born | | 4 | (12%) |

adapted from Sims and First (P.N.A.S., 1994)

**Fig. 1. Cell population in loose suspension microdrop culture on proliferation of cultured bovine ICM cells.**

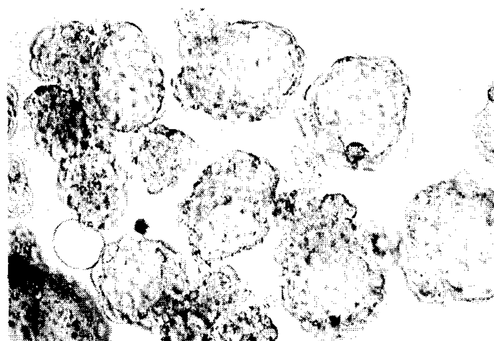

**Fig. 2. Bovine embryoid bodies from microdrop culture of ICM cell in loose suspension microdrop culture.**

the use of trypsin can exhibit abnormal karyotypes with frequencies as high as 30 to 50% (Stewart, 1991). These methods also presume that the technician knows exactly how to distinguish an ES cell from other cells.

Because these mothods had not produced totipotent ES cell lines in domestic animals (Anderson, 1992), we chose in the first experiment to physically disassociate the ICM cells by 4 days after immunosurgery and to culture in a loose suspension culture of cells in media (Sims and First, 1993, 1994; Tables 1 and 2). The idea being that cell-to-cell contact is required for differentiation and preventing contact should prevent differentiation. There was no apparent differentiation unitl the ICM cell population growth forced ICM cell contacts in a microdrop at about 1,500 cells/microdrop (Fig. 1). The result being formation of embryoid bodies (Fig. 2). With precompaction morulae, there is no need for immunosurgery of trypsin because the cells are easily and immediately separated by pipetting through a small bore pipette and by micro neelde separation. In our experience, ICM or morulae derived cell lines have not usually become established without initially disaggregating the cells.

## Ⅲ. CULTURE METHODS AND MEDIA

Most attempts to culture bovine ES cells have been patterned after the ES cell isolation and culture methods employed for culture of mouse ES cells and these are essentially the original methods of Evans and Kaufman (1981). The ES cells are cultured on mouse fibroblast cells, usually STO cells which are mitomycin C treated to prevent outgrowth of the feeder layer. To this is added murine LIF or STO cells transfected with the gene for murine LIF which promotes mitosis of ES like cells while preventing their differentiation (Stewart, 1991). Most attempts to culture bovine ES cells in this manner have resulted in demonstration of pluripotency, but not totipotency (Evans et al., 1990; Strelchenko et al., 1991; Saito et al., 1992; Anderson, 1992; Stice et al., 1994; Strelchenko and Stice, 1994) except for presence of bovine ES cells in a chimeric fetus (Stice et al., 1994). However, use of the mouse culture system has resulted in extensive proliferation and extensive passage of the cultured cells. It has been suggested that muring LIF and the mouse culture system are not effective in preventing the initiation of differentiation in cultured ES cells of domestic species (Anderson, 1992). It is known that the base sequences of DNA of human and murine LIF have 75% homology (Stahl et al., 1990). It is also known that the base sequences of ovine and probably bovine LIF are more like human LIF than mouse LIF (Williarms, personal communication).

It may be that bovine specific culture systems need to be developed. Our first attempt was the use of a loose suspension culture. Mouse ES cells had already been cultured without feeder layers by use of fibroblast conditioned media or cell-free media with LIF added (Pease et al., 1990; Stewart, 1991). Totipotent embryonal carcinoma lines have also been isolated and cultured in cell-free media without added LIF (Mintz and Cronmiller, 1981; Stewart and Mintz, 1981).

To develop a loose suspension culture, several differentiation inhibiting and mitotic factors including LIF and BRL cell conditioned media were tested in various media combinations for their ability to promote prolonged mitotic activity of ICM cells cultured in loose suspension. Only media consisting of CRlaa (Rosenkrans and First, 1994) plus SIT and either glucose, rifampicin, laminin, or 10% FCS supported mitosis through two weeks of culture. Of these, only CRlaa plus SIT plus 10% FCS allowed mitosis and continued proliferation of ICM cells through week four. ICM cells from day 8 and 9 bovine blastocysts multiplied in culture when cultured in CRlaa plus SIT and 10% FCS with some lines reaching 2,000 cells after two weeks of culture. These cells have the appearance of mouse ES cells; small cells with large nuclei, little cytoplasm and prominent nucleoli. When removed from nondifferentiating conditions and allowed

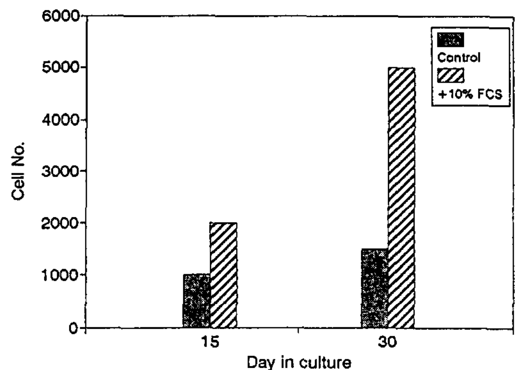


**Fig. 3. The population growth of loose suspension cultured ICM cells following the addition of FCS on Day 4 embryo culture before establishment of ICM cell culture.**

to aggregate, the cultured cells formed embryoid bodies. These embryoid bodies do not appear to differ morphologically from mouse embryoid bodies.

Interestingly the inclusion of fetal calf serum to the last four days of the culture of the embryos making up a cell line considerably improved the proliferation of the resultant cultured ICM cells (Fig. 3).

Initial loose suspension culture studies with pooled embryos produced cell lines which proliferated slowly, but usually lost life by five weeks of culture. Live dead staining at three weeks with calcein AM (live) or ethidium homodimer (dead) showed approximately 80% live cells (green) and 20% dead (red) or dying (orange to yellow) cells whereas at five weeks, nearly 80% were dead or dying. When single embryo lines were attempted with this culture system, only a few (Table 3) of none (Table 4) survived for more than one week. Although nuclear transfer blastocysts and offspring were produced from pooled embryo short-term, loose suspension cultures, this method was clearly inadequate for extensive cell proliferation or long-term culture.

We have recently replaced this method with the use of bovine fetal fibroblast cells derived from a 30-day bovine fetus as feeder layer in a microdrop of the same CRlaa SIT, 10% fetal calf serum media previously decribed. Both ICM and precompaction morulae cells proliferate rapidly and tend to overgrow the feeder layer to form embryoid bodies unless new feeder cells are added at least once per week. Data for establishment of cell lines and early cell prolifer-

**Table 3. Effect of number of ICM cells starting a loose suspension culture on ICM cell survival**

| No. inner cell masses starting cell line | No. cell lines | |
|---|---|---|
| | Started | Surviving at 1 month |
| 1 | 155 | 37(23.9) |
| 3 | 231 | 172(74.5) |

* P<0.01

**Table 4. Effect of number of 16~20 cell precompaction morulae starting a culture on ES cell survival and proliferation**

| No. embryos in cell line | Initial | | Cultured 10 days | | |
|---|---|---|---|---|---|
| | # Lines | # Cells | # Lines | # Cells | # Cells /embryo |
| 1 | 65 | 18 | – | – | – |
| 3 | 46 | 52 | 14 | 5,539 | 1,846 |
| 5 | 32 | 86 | 17 | 25,670 | 5,134 |

**Table 5. The effect of single vs. multiple 16~20 cell bovine embryos in feeder layer microdrops on establishment of embryonic stem cell lines**

| No. embryos per line | N | Cells /drop | Cells /embryo | Lines |
|---|---|---|---|---|
| 1 | 65 | 18 | 18 | 0 /65 (0) |
| 3 | 46 | 52 | 17.3 | 14 /46(30.4) |
| 5 | 32 | 86 | 17.2 | 17 /32(53.1) |

ation data from media are shown in Tables 4 and 5. Totipotency of ICM or 16~20 cell-stage cells cultured on this feeder layer is as yet untested. These culture systems represent a primitive attempt to understanding how to culture bovine ES cells and to understanding the requirements of bovine ES cells mitosis, differentiation or prevention of differentiation. Much research is yet needed to identify optimum culture conditions for bovine ES cells.

## Ⅳ. STAGE OF EMBRYOS USED TO MAKE ES CELL LINES

In mice successful ES cell lines, as evidenced by chimeric offspring, have usually been derived from outgrowth of epiblast cells of the inner cell mass of day 4 blastocysts as described originally by Evans and Kaufman (1981). There is evidence that late stage ICMs with higher cell counts, more consistently from cultures and totipotent cell lines. Mouse ES cell lines have also been formed from morulae (Eistetter, 1989). Establishment of ES cell lines have also been attempted from murine primordial germ cells. Interestingly when used in nuclear transfer 2-cell, 8-cell, ICM derived cells and male, but not female, primordial germ cells resulted in some blastocyst but only 2- and 8-cell nuclei were totipotent and resulted in offspring (Kato and Tsunoda, 1993).

When totipotency was tested by chimera, offspring resulted from mouse ICM cells and cultured mouse ES cells but not 12.5~17.5 day fetal mouse germ cells. There was a linear effect of the number of ICM cells used to make chimeras on the frequency of offspring derived in part or whole from ICM cells (Kato and Tsunoda, 1993). We reported recently the birth of 4 calves from the use of 16~27 day loose suspension cultured bovine ICM cells in nuclear transfer (Sims and First, 1993,, 1994). These data are shown in Tables 1 and 2. This and the previous evidence that offspring results from use of ICM cells in nuclear transfer in cattle (Keefer et al., 1993), sheep (Smith and Wilmut, 1989) and rabbits (Collas and Robl, 1991) suggest that cultured ICM cells should be totipotent.

Compact morulae are totipotent when used in nuclear transfer (Bondioli et al., 1990). However, the totipotent cells are principally inside nonpolarized cells (Navara et al., 1991). ES cell lines formed from compact morulae have demonstrated prolonged culture and pluripotency. However, except for one chimeric fetus thus far, their use in nuclear transfer or chimera have not resulted in offspring of ES cell parentage (Strelchenko and Stice, 1994). Precompaction bovine morulae of the 16~20 cell stage have been used to form cell lines grown on fibroblast feeder layers (Strelchenko and Stice, 1994). In one report, these lines were derived from single embryos (Strelchenko and Stice, 1994). However, in our lab, three or more 16-cell embryos have been needed to reliably form a cell line as shown in Tables 4 and 5. These cells should be totipotent because they are derived prior to commitment for the first differentiation events, cell polarization and compaction (Navara et al., 1991). Their totipotency is as yet untested. However, in our laboratory, they proliferate rapidly (Tables 4 and 5) and show evidence of pluripotency when feeder layers are overgrown of removed.

## Ⅴ. EFFECT OF NUMBER OF CELLS ON ES CELL CULTURES

In mice use of more advanced ICM stages with higher cell numbers results in a higher frequency of established cell lines. Our laboratory has compared the efficiency of establishing bov-

ine ICM lines in loose suspension-microdrop cultures from 1 or 3 ICMs of hatched blastocysts (Table 3). Seventy five percent of the lines started from 3 embryos were established and survived to one month whereas twenty four percent of those started from one ICM survived to one month. Others have started cell ilnes from single ICMs with culture on a STO mouse fibroblast feeder layer (Strelchenko et al., 1991; Satio et al., 1992; Anderson, 1992; Stice et al., 1994). The use of a feeder layer in a dish may more efficiently provide for cell growth than loose suspension or feeder layer microdrops.

Using a bovine fetal fibroblast feeder layer with microdrop culture, we tested the effect of 1, 3 or 5 embryos from the 16~20 cell stage on the establishment and maintenance of bovine presumptive ES cell lines (Tables 4 and 5). As shown in Table 5, 53% of the attempts gave cell lines when started with 5 embryos, 30% from 3 embryos and none from a single 16~20 cell embryo. Across all three experiments it appears that a critical cell mass of approximately 50 to 100 cells is needed in microdrop culture to establish ES cell lines reliably. The increase in cell number at 10 days by lines from 3 or 5 embryos is exponential. The increased proliferation may be due to synergisms of embryos of a critical mass of like cells, synergism of embryos of different genotypes, inhibition of cell cycle when only a few cells are present or the take over of the culture by cells of a dominant embryo. Pooling embryos to start a cell line provide a reliable way of establishing cell lines for research needs such as definding and optimizing culture conditions. However, in commercial use single embryo lines will be required. This should be possible when the variables affecting ES cell establishment and proliferation are understood.

## Ⅵ. SUMMARY

The goal of cell stem cell technology is to produce a viable and genetically normal animal. To achieve this goal various laboratories have followed 2 different pathways beginning with either the culture of 1) single or pooled ICMs grown with or without a feeder layer or 2) single or pooled 16~20 cell stage embryos grown with a feeder layer. Also, thus far embryonic cell cultures or lines have been established by several methods including loose suspension culture for short-term cultures and more commonly murine or bovine fibroblast feeder layers for long-term culture. Pluripotent lines have been derived from 16-cell through blastocyst inner cell mass stages. The efficiency of establishing cell lines and cell proliferation apper to be affected by the number of cells or embryos starting the line. Most attempts to produce offspring from long term STO cell feeder layer cultured ICM or morulae derived ES cells have resulted in pregnancy failure in the first trimester when ES cells were used in cuclear transfer or have failed to retain ES cells in the progeny produced by chimerization. The exception is 1 chimeric fetus from use of morula ES cells in the chimerization with early embryonic cells. There is much to be learned yet about ES cell culture requirements for maintenance of totipotency. If bovine ES cell lines loose imprinting pattern and totipotency with long-term culture and passage as suggested for mouse ES cells, we may be limited to the use of short-term cultures for multiplication of embryos and efficient production of transgenic animals. No bovine ES cell system has yet met all of the criteria indicated for a totipotent ES cell line.

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