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Establishment of Embryonic Stem Cell Line from ICR Mouse Blastocyst

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ICR 생쥐 배반포로부터 배아주세포계통 확립

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적 요

생쥐 배반포로부터 내부세포괴(inner cell mass, ICM)를 outgrowth로 분리하여 증식시킴으로써 배아주(embryonic stem, ES)세포를 확립하고자 본 실험을 실시하였다. 과배란처리와 교미에 의해 생산된 ICR 생쥐의 3.5일 배반포를 sDMEM내의 배아성 섬유아단층배양층에 배양하여 ICM세포의 증식을 조사한 결과, 3.5일부터 분리한 ICM세포들은 배양 7, 8일에 각각 1,500 및 3,200세포의 미분화세포로 증식하였다. 이들 세포의 계대배양에 의해 잠정적인 ES세포 colony를 얻었으며 10회의 계대배양후에도 그 형태가 변하지 않았다. 이들 세포는 다능성의 분화능을 보여 전형적인 ES세포 형태를 보였다. 이같은 결과는 ICR 배반포에서 outgrowth로 분리한 ICM으로부터 ES세포 확립이 가능함을 보여준 것이다.

Key words: ES cell, mouse

INTRODUCTION

Study of mammalian embryogenesis have extended to elucidate specific gene functions in whole organism via transfected embryonic stem(ES) cell. This significant advance have not been realized when the first ES cell was reported. With the development of various recombinant DNA techniques, it is now possible to look at the function of a specific gene in not only embryonic development but growth of the neonates carrying the manipulated gene. In this major achievement, establishment and maintenance of ES cell line are the core elements for the animal transgenenesis and the study of gene functions. We have already reported the development of presumptive ES colony(Park *et al.*, 1993). In this report we demonstrate established ES cell lines and their pluripotency in the mouse.

MATERIALS AND METHODS

1. Sources of embryos

ICR blastocysts and fetuses were recovered 3.5 and 13~14 day of pregnancy, respectively after superovulation(Lee *et al.*, 1993).

2. Culture of ES cells on feeder layer

Culture media and primary embryonic fibroblast cells were prepared as described previously(Park *et al.*, 1993)

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3. Growth of ICM on feeder layer

To analyse the cell proliferation of ICM cells on the feeder layer, the outgrowing blastocysts were removed with a Pasteur pipette at 3.5, 5.6, 7 and 8 day dpc. They were stained with Hoechst 33258 to count the cell number as previously reported(Lee and Byun, 1989). At least 10 embryos were used at each stage.

4. Preservation of ES cell colonies

ES cell colonies after 10 passages were recovered with trypsin-EDTA. After extensive washing in sDMEM + 15% FCS containing 0.4 M dimethyl sulfoxide cells were frozen and stored in liquid nitrogen gas.

RESULTS AND DISCUSSIONS

The attached blastocysts proliferated rapidly in the presence of mitomycin C-treated fibroblast cells. After the individual proliferating blastocysts were stained with DNA-specific Hoechst 33258 dye the average number of cells was determined in at least 10 embryos at various time points. As shown in Fig. 1, 3.5 day blastocyst became a mass of cells during 4.5 day of culture on the feeder layer. This growth of ICM colony indicates that our culture system is appropriate for the proliferation of ICM cells although culture without passage brought to partial differentiation of ICM cells. The number of

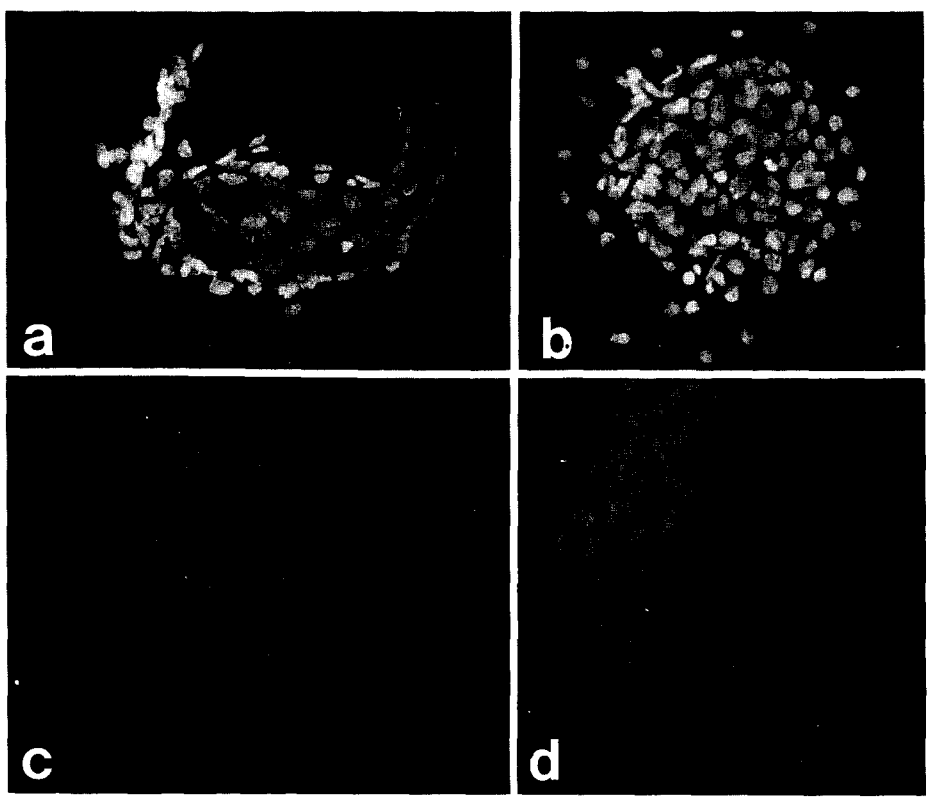

Fig. 1. Proliferation of ICMs attached on feeder layer. Cell clumps were isolated and stained with Hoechst 33258. 3.5 day blastocysts(a), 5(b), 6(c) and 7day ICM cells(d).

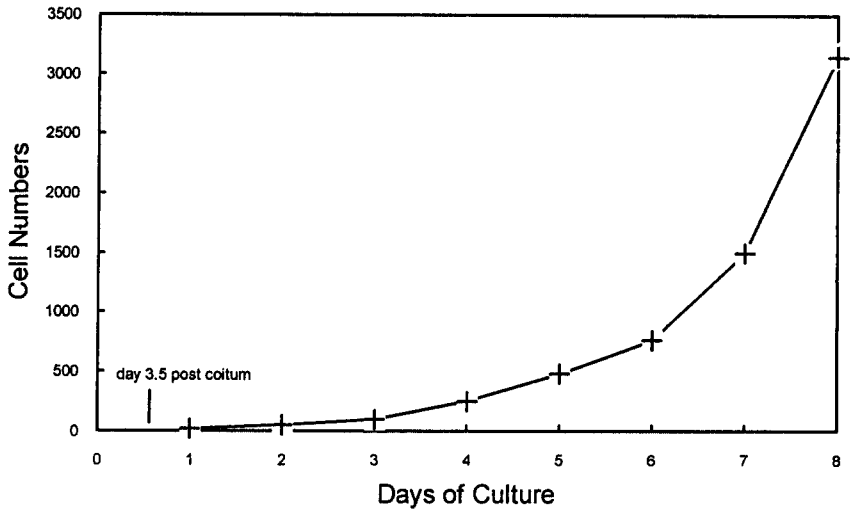

Fig. 2. Yield of ICM cells during trophoblast outgrowth and passage.

cells were about 1,500 and 3,200 cells at 7 and 8 days of culture(equivalent to 10.5 day dpc embryo), respectively(Fig. 2).

In the appropriate culture system ICM colonies were divided into several clumps by trypsin-EDTA for the subsequent passage. When four days have passed after the isolation of ICMs, proliferating ICMs were transferred to fresh feeder layer by simple detachment with hypodermic needles. This process was repeated as far as cells can undergo proliferation without extensive differentiation. During passages large clumps of cell clones were cut into several pieces before reseeding into fresh-made feeder layer. So did increased the number of cell clones as well as the number of cells in rich clones. By doing so, cells were maintained for considerable period without apparent cell differentiation. Such transferred ICMs were shown in Fig. 3a. Although the differentiated cells were found in the vicinity of the ICM, this was still positive growth of the ICM showing increasing size(Fig. 3b and c).

Some of the isolated ICMs propagated in this manner were presented in Table 1. This method provide an alternative way of propagation of

Table 1. A representative propagation of presumptive ES cell clones of ICR mouse *in vitro*[1]

No. of blastocysts at initial culture (Day 0)	No. of presumptive cell clumps at the following passages (day after blastocyst recovery)									
	1st (4)	2nd (8)	3rd (12)	4th (16)	5th (20)	6th (24)	7th (28)	8th (32)	9th (36)	10th (40)
30	20	17	17	27	48[2]	30	30[3]	18	18	18

[1] ICM clumps were transferred into freshly prepared feeder layer on each passages. Differentiated or less viable cell clumps were discarded during passages.

[2] and [3] Eighteen and and 12 clumps were used for dissociation into single cells for different purposes, respectively.

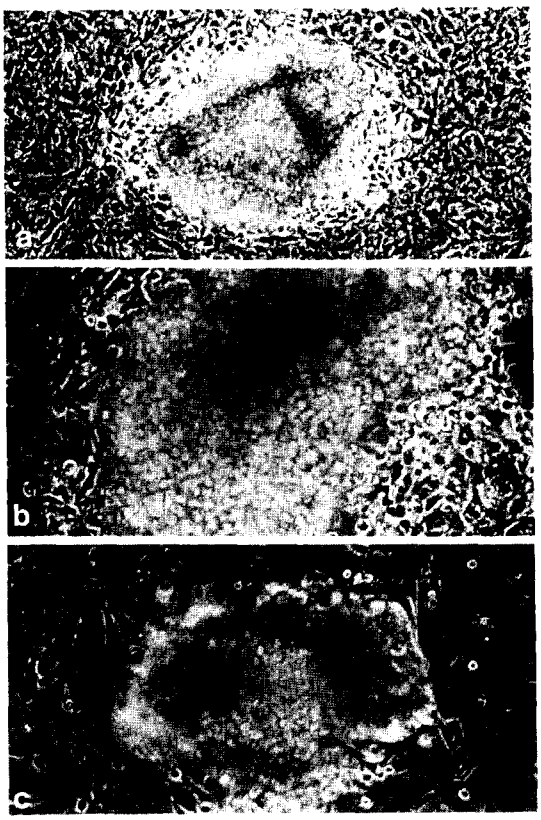

Fig. 3. Active proliferation and differentiation of physically isolated ICMs during passages. Probably endodermal cells attached on the vicinity of the cell mass(a). There was still a large proportion of cells showing pluripotency without extensive differentiation in the center of cell mass(b). Relatively suitable ICM clone showing no clear differentiation (c). Magnification are ×150 in a, and × 375 in b and c, respectively.

ICM cells as an effective means. The appearance of the cells in this presumptive masses was different from that of cells found in primary ES cell colonies(discussed below). However, the morphology of the cells was consistent after 10 passages. There is possibilities that they may represent other cell clones of endodermal cells. Similarly active proliferation of EC cells has been demonstrated(Rudnicki and McBurney, 1987).

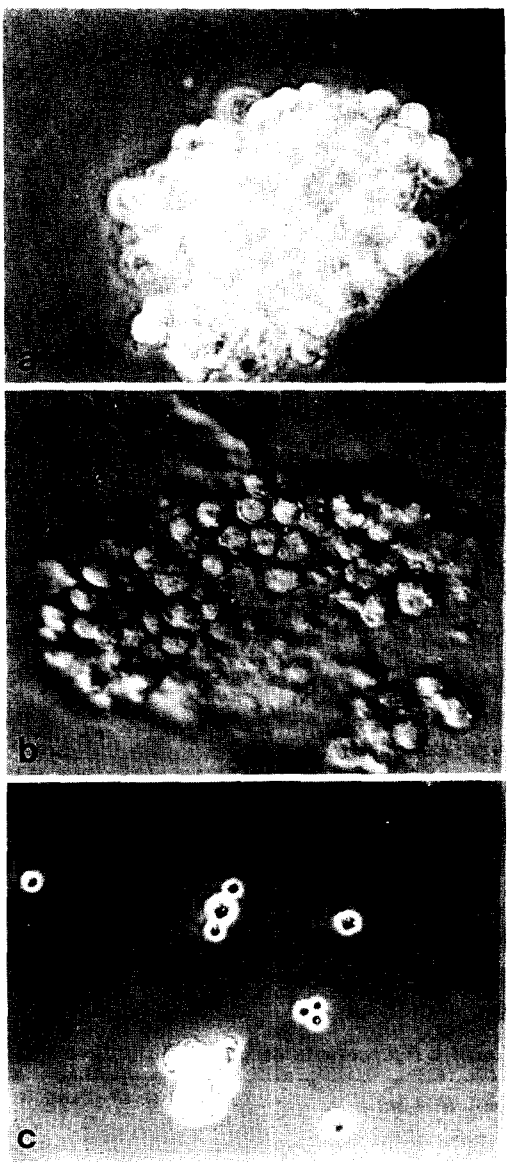

Fig. 4. Isolation of ICM cells by trypsinization. ICM was separated from 8 day of culture(a), and trypsinized before blowing off to prepare cells or clumps(c). Magnifications are ×600 in a and b, c and ×375 in c, respectively.

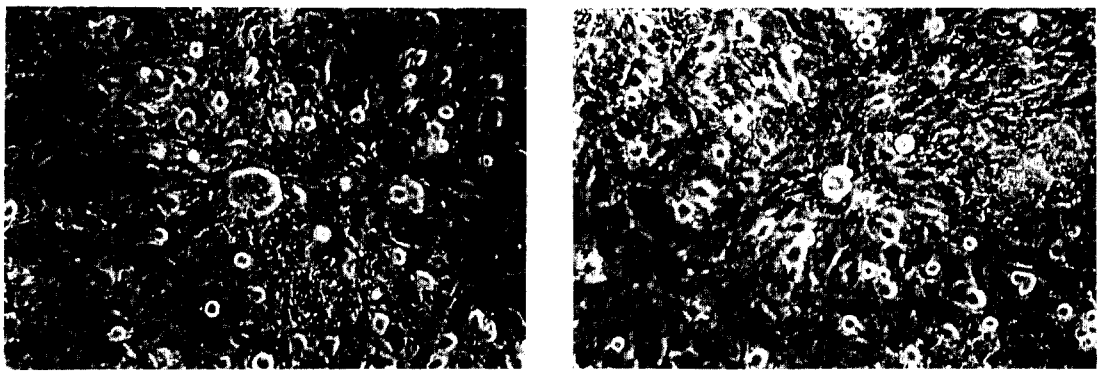

Fig. 5. Initial colonization of presumptive ES cell. Extensive colonization of dispersed ICM clones was shown in a and b. Large clones(arrows) are probably from clumps of cells. Smaller clones(arrowheads) were also formed. They proliferate and make network of ES clones. Magnification is ×375.

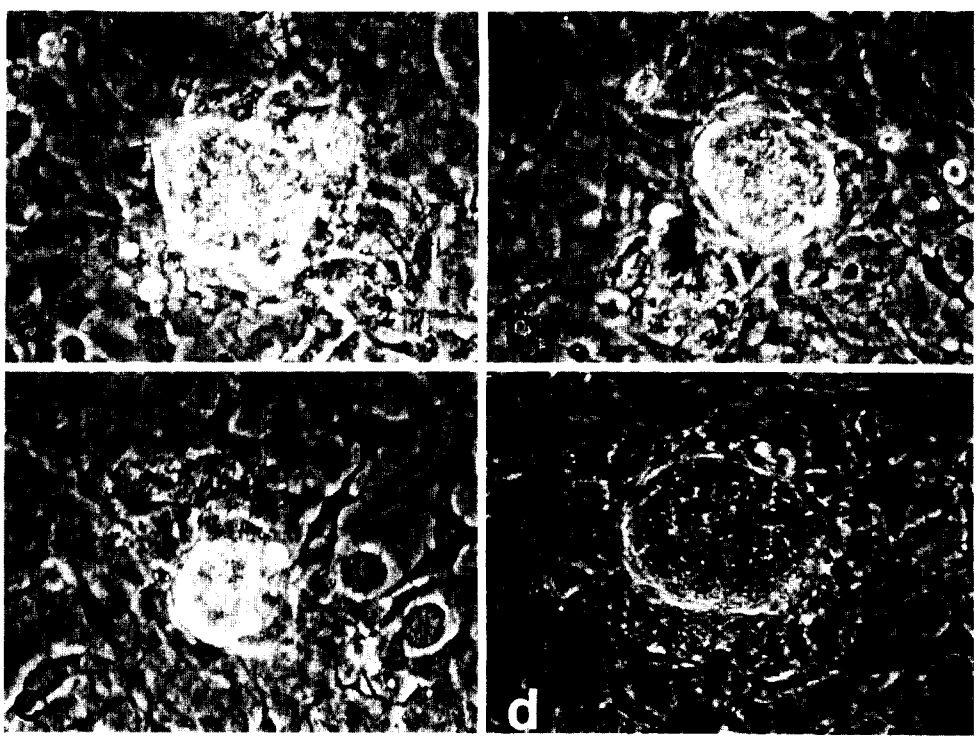

Fig. 6. Appearance of ES cell clones established. Clear outline is characteristics of ES cell colony. Different sizes and shapes are seen on the feeder layer. Magnification is ×250.

Isolated ICMs from 8 days of culture were processed by trypsinization to propagate putative cell clones. When ICMs were incubated in drop of trypsin-EDTA, the whole cell mass appeared loose(Fig. 4a), showing minimal connections between cells(Fig. 4b). This properties

one of the ICM clones as described elsewhere (Robertson *et al.*, 1986). Under higher magnification characteristics of ES cell can be seen. i. e., large translucent nuclei, prominent nucleoli, and relatively little cytoplasm(Evans *et al.*, 1990). These cell mass were rapidly passed through a flame-polished, small bored Pasteur pipette, breaking the cell masses into smaller clumps or individual cells(Fig. 4c) in sDMEM. The cells directly blown off on the fresh feeder layer rarely formed ES clones in this experimental condition.

By passing trypsinized cell fragments or cells, presumptive ES cell colonies a lawn of presumptive ES colonies was initially formed with individual ICM cells or ICM fragments, showing characteristic outline of the colony(Fig. 5). As the cells proliferated they formed larger colonies with sister ES cells. Among these small clones were demonstrated in Fig. 6. The characteristics of ES cell colony was clear outline formed by dividing sister cells with no individual cell boundary. The colonies had varying sizes, probably depending on the inital numbers of cells.

The proliferation of ES cells without extensive differentiation in a colony seemed to depend mainly on the state of mitomycin C-treated feeder layer. Some colonies often showed varying extents of differentiation into various cell types(Fig. 7a,b and c). Eventually the colonies deteriorated without further proliferation(Fig. 7d).

In the early phases of these experiment many contaminations came across. The routes of contamination were tested to pinpoint the source of contamination. Embryo and fetus-mediated contaminations often occurred at the initial stage of cell preparation. In particular, embryonic fibroblast cells a major source of contamination. Yeast and bacteria were common contaminants found in primary embryonic fibroblast cells(Fig. 8a and b). Medium-mediated contamination was

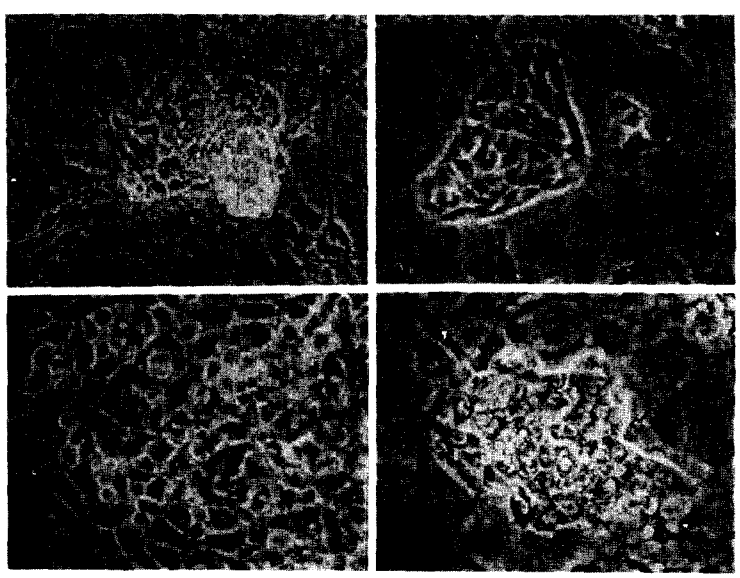

Fig. 7. Differentiation and deterioration of established ES clones. A colony starting differentiation(a). All cell in the became differentiated(b). Extensive differentiation occurred in c and eventually deteriorated(d). Magnification is ×375.

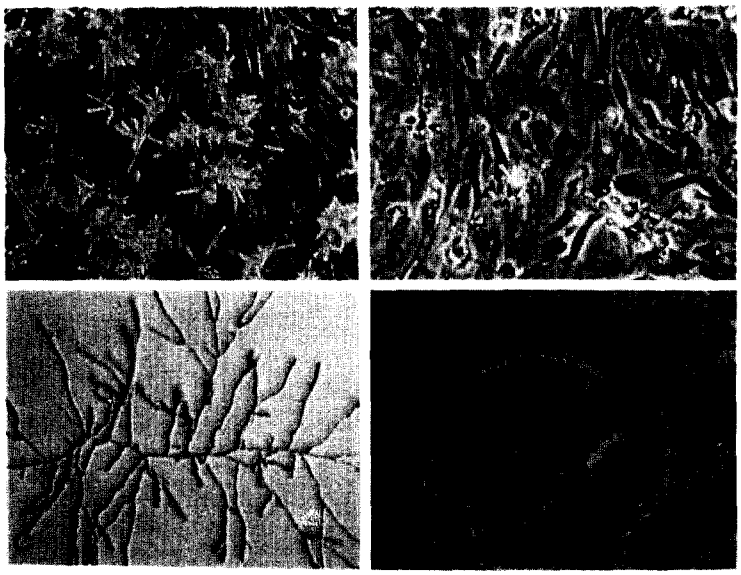

Fig. 8. Types of contaminations with microorganisms during culture. Yeasts(a), bacteria(b) and mold(c) were found in culture of embryonic fibroblast cells. Fungus was also found on the zona pellucida (an arrow in d) and can be removed by dissolving the zona pellucida (an arrowhead). Magnifications are ×150 in a, ×375 in b and d, and ×60 in c, respectively.

also found as form of mold growth(Fig. 8c). Unidentified fungus was found on the zona pellucida(Fig. 8d) although it rarely occurred. However, extensive examination and good practice in sterile technique can eliminate all the possible contamination.

In summary, the ES cells should be useful for the tests of pluripotent contribution to the chimaeric mouse using by embryo and ES cell aggregation, totipotency of ES cells by nuclear transfer, and the ES cell-mediated transgenesis by DNA transfection.

ABSTRACT

The aim of this study was to isolate inner cell masses (ICMs) and maintain them *in vitro*. ICR blastocysts at 3.5 day of pregnancy were recovered and cultured in sDMEM containing primary embryonic fibroblast. Isolated ICM from the outgrowth of blastocyst increased upto 1,500 and 3,200 cells at 7 and 8 days of culture *in vitro*. During subsequent passages putative ES cell colonies were recovered and examined for morphology and pluripotency. They should normal characteristic and pluripotency of ES cells. The results demonstrated that ICR ES cell line can be established through the isolation of ICM from the outgrowth of the blastocyst.

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