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Isolation and primary culture of ES-like colonies from NMRI mouse embryos

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Abstract

Background: Embryonic stem (ES) cells are pluripotent cells conventionally isolated from early embryos. Studies have shown that ES cells serve as a practical model for biomedical studies.

Objective: The aim of the present study was to optimize culture conditions for establishment of ES-like colonies from NMRI mouse blastocysts as well as 2-cell stage embryos.

Materials and Methods: Both expanded blastocysts and 2-cell stage embryos were co-cultured on mouse embryonic fibroblast (MEF). Plating capacity and formation of Inner cell mass (ICM) were examined daily. The differentiation and growth behavior of ICM cells were examined with various procedures. ICMs derived from initially cultured 2-cell or blastocyst embryos were disaggregated either mechanically or enzymatically, and seeded onto MEF with or without leukemia inhibitory factor (LIF). The resulted colonies were disaggregated and reseeded onto MEF and the colonies that were morphologically similar to ES cells were evaluated for pluripotency using alkaline phosphatase (ALP) expression as a stem cell marker.

Results: No morphologically good ES-like colony was isolated from 2-cell embryos after passages, while 273 (79%) good-looking ICMs were isolated from 352 blastocysts. Four sets of colonies remained undifferentiated following passages. Enzymatic method of ICM disaggregation was superior to the mechanical method. Besides, all ES-like colonies were obtained from the ICMs cultured in presence of MEF and LIF.

Conclusion: Our results show that NMRI mouse ICMs could be isolated and cultured from blastocyst stage embryos with a suitable culture system and ES-like cell colonies remain undifferentiated when cultured with MEF and LIF.

Key words: *NMRI mouse, Inner cell mass, Embryonic stem cell.*

Introduction

Embryonic stem (ES) cells are pluripotent cells derived from preimplantation embryos (1). Under appropriate conditions, ES cells are capable of self-renewing *in vitro* and *in vivo*, and differentiating into various cell types (2, 3).

These properties of ES cells have led to their extensive use in developmental biology, genetics and biotechnology as well as *in vitro* models to study early stages of embryogenesis (4, 5). Of particular clinical interest is that these cells are useful in treatment of degenerative diseases such

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as Parkinson, diabetes mellitus, Alzheimer, as well as leukemia and cardiac infarction (6). Stem cell lines have been produced from chicken (7), mink (8), hamster (9), pig (10), rhesus monkey, common marmoset, and human (11).

However, techniques for the derivation of stem cells are not similar in all species. For example, leukemia inhibitory factor (LIF) seems to be most favorable for the maintenance of mouse ES cells, but possibly not in the human ES cells (12, 13), and non human primate ES cell lines (14). ES cells exhibit typically small, round, shiny cells with high nuclear/cytoplasm ratio. They pack tightly together in small nests so that it is difficult to discern the individual components of the cells (15). ES cells can be maintained undifferentiated if cultured on STO fibroblasts (16) or primary embryonic fibroblasts (17) or in the presence of LIF (3). Each method has advantages and disadvantages (18). Embryoid body (EB) formation is an evidence of pluripotency (19). EBs are differentiated cell aggregates first described arising in mice and contain a variety of cell types (20).

In mouse, ES cells were first isolated from strain 129 by Evans and Kaufman (1981). These cells share several morphologic characteristics including high levels of intracellular ALP and presence of specific cell surface membrane glycolipids and glycoproteins (21). Considerable difficulties has been reported in deriving ES cells from other inbred mouse strain (22). Ouhibi *et al* (1995) reported that although the best practical method for maintenance of pluripotent cells in tissue culturing remains the use of STO or MEF, some strains of rat ES cells grow better on rat uterine epithelial cells (RUEC) than STO or MEF. Whereas, Kim *et al* (2007) compared three types of feeder layers and found STO and MEF as the most appropriate feeder layers for initial culture of porcine ICM than porcine uterine epithelial cells (23).

Procedures leading to the establishment of ES cell lines had to be modified among the mouse strains due to the strain differences (22). In this study, we evaluated the establishment and maintenance of ES-like colonies from blastocyst and 2-cell stage NMRI mouse embryos. To obtain the appropriate conditions, we have examined various culture conditions and procedures for isolation and maintenance of ES-like colonies *in vitro*.

Material and methods

In this experimental study, chemicals were purchased from Sigma Company (Sigma-aldrich, St. Louis, Mo, USA) unless otherwise stated.

Embryo

Female NMRI mice were superovulated by injection of 7.5 IU pregnant mare's serum gonadotrophin (PMSG) followed 48 h later by 7.5 IU hCG (Seromed, Italy). Mice were caged overnight with the males from the same strain. The following morning, female mice with vaginal plug were considered pregnant and sacrificed by cervical dislocation 48 h and 92 h post-hCG injection to obtain 2-cell and expanded blastocyst stage embryos, respectively. The uterine horns were flushed with α-MEM supplemented with 5 mg/ml bovine serum albumin to obtain expanded blastocysts, or the uterine tubes were flushed with the same medium to obtain 2-cell stage embryos, as described elsewhere (24). Morphologically normal embryos were washed two times and kept in 25 µl drops of the same culture medium under light paraffin oil in groups of five embryos per drop until further use.

Mouse embryonic fibroblasts (MEF) preparation

MEF was prepared according to a common protocol described elsewhere (25) with minor modifications; 13-14 days old mouse embryos were removed from uterine horn under sterile conditions and rinsed several times in phosphate buffered saline (PBS). Embryos were decapitated and all the soft tissues inside the body cavity were detached and removed carefully. Remaining tissues were cut into small pieces and digested with 0.5 g /L trypsine and 0.2 g/L EDTA in PBS for 30 minutes at 37 ºC. Tissue dissociation was aided by repeated pipetting. After centrifugation at 300 g for 10 minutes, the cell pellets were harvested in α-MEM supplemented with 15% FBS (Gibco), 100 IU/ml penicillin G, 60 µg/ml streptomycine sulphate and 50 µg/ml fungizone in 25 cm^2 culture flasks (Falcon®, USA). When the cell confluence exceeded 90%, cells were detached and subcultured in the same medium. Cells at passage 2-5 were used as feeder layer in the following experiments. Passage two MEF were equilibrated in 10% DMSO and 20% FBS in culture medium for 4h, were stored for one night in liquid nitrogen

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vapor, and were immersed in liquid nitrogen and stored there frozen for further use.

MEFs inactivation

MEFs were mitotically inactivated with 10µg/ml mitomycine-C in culture medium for 2 h at 37ºC. Mitotically inactivated cells were trypsinized and harvested at density of 5×10^4 cells/ml, either in drops of medium under paraffin oil or in 12 well culture plates (Falcon®, USA).

Embryo co-culture and assessment of development

Two-cell stage and expanded blastocyst embryos were seeded onto MEFs, cultured in α-MEM, supplemented with %10 FBS, 100 IU/ml penicillin and 60 µg/ml streptomycin, at 37˚C humidified atmosphere of 5% CO_2 in air, seven and three days for 2-cell and blastocyst embryos, respectively. Embryos were carefully observed every 24 h under an inverted microscope (Nikon TS100, Japan), and the development to higher stages was recorded. Embryos which attached to the culture dish and developed extensive ICMs were selected for the next steps of experiments.

ICMs disaggregation

Morphologically normal ICMs with distinct border and highly packed cells derived from attached embryos were picked with a finely polished capillary pipette. ICMs were disaggregated either mechanically or enzymaticaly. Mechanical disaggregation was performed by serially passage of inner cell mass through micropipettes with reducing diameters in PBS with 10% FBS and 10 µM EDTA. While, for enzymatic disaggregation, the ICMs were put in PBS containing 0.5 g/L trypsin and 0.2 g/L EDTA followed by gentle pipetting until clumps of about 10 cells were formed.

ICMs cultivation

Cell clumps resulted from disaggregated ICMs were harvested in ES medium (ESm) consisting of α-MEM supplemented with 20% FBS, 1 mM 2-β mercaptoethanol, 2 mM L-glutamine and 2 g/L glucose.

The medium was refreshed by replacing half of the medium with fresh medium every other day. All the culture dishes were incubated at 37˚C humidified air with 6% CO_2. Morphologically normal ES-like colonies with distinct border and no sign of differentiation were disaggregated and re-seeded onto newly inactivated and harvested MEFs.

Experimental design

Disaggregated ICM clumps were cultured in; ESm with 1000 IU LIF (group I), ESm with MEF (group II), ESm with MEF and 500 IU LIF (group III) and ESm with MEF and 1000 IU LIF (group IV). Inactivated MEF monolayer was prepared 24 h prior to ICM cultivation. Formation of cell colonies with highly packed, shiny, round cells was recorded after 3 days and the presence of ALP activity was examined in colonies on day 7. Colonies were examined daily under inverted microscope and any sign of differentiation (outgrowth of colonies, formation of differentiated cells at the periphery of colonies, and formation of morphologically different cells next to the colonies) was noted. Morphologically normal ES-like colonies were partly disaggregated and subcultured approximately 7 days after initiation of the culture. Culture medium was stored in a humid 37˚C incubator with 6% CO_2 for at least 2h prior to use. Half of the ESm on growing colonies was replaced with fresh medium every 2 days.

Alkaline phosphatase (ALP) staining

At day 7 of passage 4, ALP activity was determined in ES-like colonies using a commercially available kit (86R Sigma) according to the manufacturer. Briefly, cells were fixed in a solution of %67 acetone, %8 formaldehyde and %25 sodium citrate and stained with napthol/FRV-Alkaline AP substrate. After staining, colonies which colored dark-red were considered positive for ALP activity (26).

Statistical analysis

Experiments were repeated at least 5 times. The number of colonies and ALP positive colonies in each group was recorded. Data are expressed as percentage of colonies formed in each group. Data were statistically compared using χ^2 test. $p<0.05$ was considered significant.

Results

Development of 2-cell and expanded blastocyst embryos

In the first experiment, 75 morphologically normal 2-cell and 334 expanded blastocyst embryos were put into drops of medium with MEF. The rate of development to plated embryos and the rate of ICM formation in 2-cell embryos and blastocysts are shown in table 1.

More expanded blastocyst embryos attached and plated to the substratum than 2-cell embryos ($p<0.05$). Furthermore, 2-cell-derived ICMs (n=10)

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were not suitable for further steps of experiments because of their poor growth and abnormal morphology (small size of ICMs and a mixture of cells with different morphology), but majority of ICMs (253; 79%) derived from expanded blastocyst embryos were appropriate for further experiments.

ES- like colony formation

We examined four methods for cultivation of ICM-derived cell colonies (Table II, III). Neither group I, nor group II could maintain the cell clumps at undifferentiated state after 7 days. However, feeder layer appeared to be more essential for support and maintenance of cell colonies (Table II). In addition, differentiation of cell clumps occurred more rapidly in group I (LIF alone), when compared to group II (MEF alone) (data not shown). Nearly identical number of ES-like colonies remained at undifferentiated state in group III and IV; 22% and 20% colony formation for group III and IV, respectively. Disaggregation of colonies with trypsin/EDTA yielded higher results and the percentage of ES-like colony formation was greater than mechanical method (Table III). The combination of LIF and MEF comprised better culture system for maintenance of ES-like colonies. However, no statistically significant difference was detected when different doses of LIF (500 and 1000IU) were applied for prevention of differentiation (Table III). A small proportion (1-5%) of colonies spontaneously generated embryoid body-like structures when cultured onto MEF with LIF. Colonies at passage 4 were stained with ALP kit and the dark-red clumps were observed in both group III and group IV (Figure 1, d).

Table I. Development of embryos to plated blastocyst and ICM formation in 2-cell and blastocyst stage mouse embryos.

Embryo stage	No. of embryos	Replica	No. of plated blastocysts (%)	No. of embryos with normal ICM (%)
2-cell	75	5	45 (60)	10 (22)
Blastocyst	352	10	321 (91)*	253 (79)*

Within the same column, values with * superscript were significantly different ($p<0.05$).

Table II. Effects of culture conditions on maintenance of ICM colonies at undifferentiated state.

Culture condition	No. of ICM	% Undifferentiated colonies				
		Day 1	Day 2	Day 3	Day 4	Day 5
ESm + MEF	10	100	50	30	20	0
ESm + LIF	10	10	0	0	0	0

Table III. Colony formation and maintenance of ES-like colonies at undifferentiated state in presence of MEF and different doses of LIF after different disaggregation methods.

Disaggregation method	LIF concentration	No. of ICMs	Undifferentiated colonies (%)		
			Day 3	Day 5	Day 7
Enzymatic	1000	81	81 (100)	54(66.6)*	18 (22.2)*
	500	78	78 (100)	47(60.2)*	16 (20.5)*
Mechanical	1000	46	46 (100)	9(19.6)	2 (4.3)
	500	18	18 (100)	3(16.6)	1 (5.5)

* values from enzymatic method at day 5 and 7 were significantly different from values from mechanical method ($p<0.01$)

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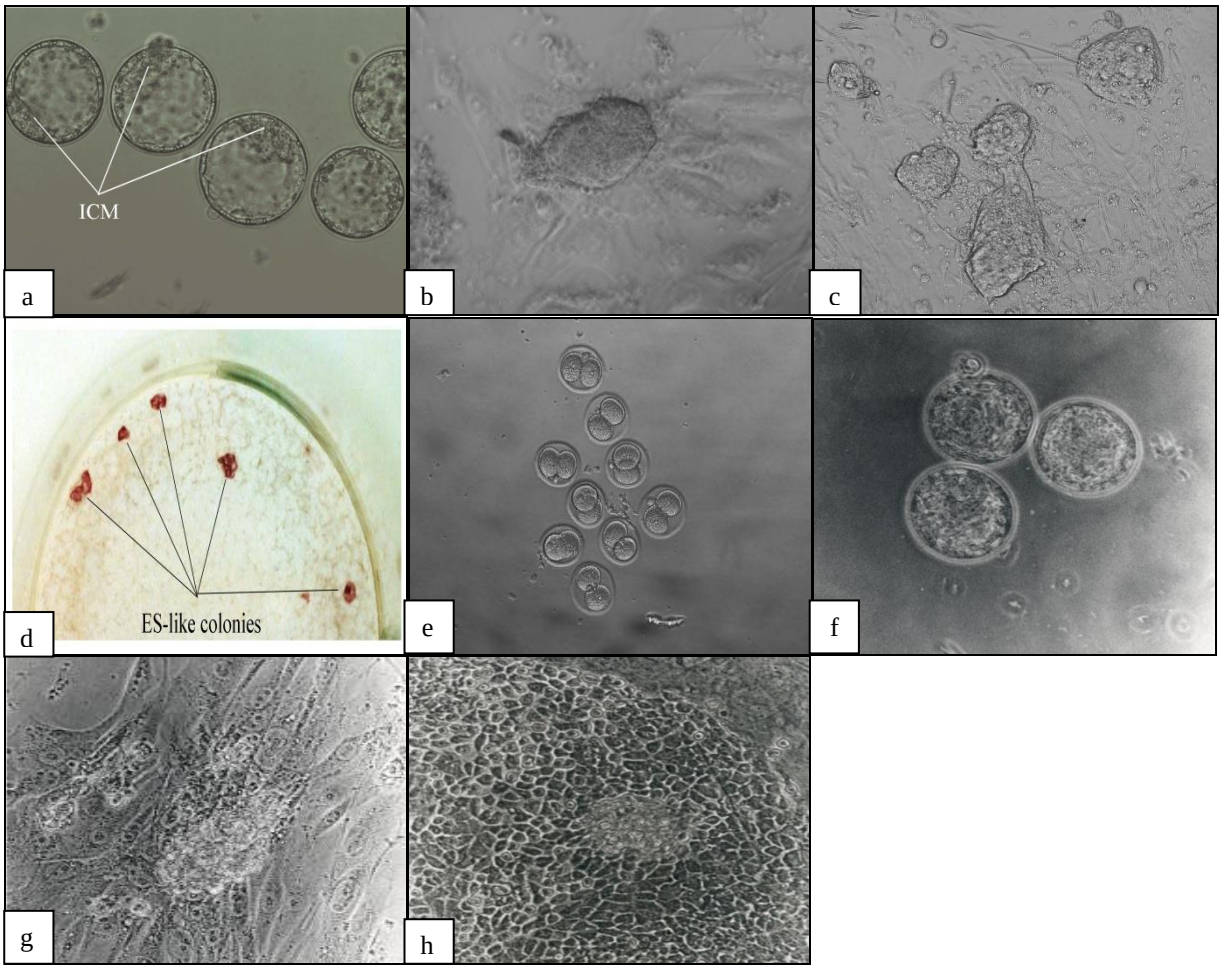


Figure 1. Expanded blastocyst and 2-cell stage embryos were cultured at appropriate conditions to achieve ES-like colonies. Expanded blastocysts (a) were flushed from uterine horns 96 h after mating. Hatching blastocysts were cultured on mitomycin treated MEFs layer and after 6-7 days, good quality ICMs (b) were disaggregated and cultured on fresh MEFs (c). ES-like colonies at passage 4 were positive for ALP activity (d). Two-cell embryos (e) were cultured to blastocyst stages (f). These blastocysts did not produce good quality ICMs (g) and spontaneous differentiation (h) was initiated in most of the ICMs.

Discussion

The present study was carried out to optimize the conditions that could support isolation and primary culture of NMRI mouse inner cell masses derived from both 2-cell and blastocyst stage embryos. Our results demonstrate that *in vivo* produced blastocysts have greater potential to generate morphologically normal ICMs. Besides, enzymatic disaggregation of ICMs is more appropriate and gives much higher results when compared to mechanical method. Although most ES cell lines have been derived from ICM or a further subset of ICM cells known as the epiblast, but the possibility exists that mouse strain may affect ES production during the period of development that readily yields ES cell line. Cell lines have been derived from 129s2/sv Hsd mouse morula and earlier cleavage stages embryos (4cell) cultured onto MEF with high efficiency (27). Further putative ES cells have also been obtained from mouse (28) and mink (8) by initially culturing the 8 cells and morula on top of a feeder layer. In comparison to mouse, using morula in buffalo no primary cell colonies were formed (29). Adequate numbers of 2-cell embryos plated successfully, but the ES-like colony formation capacity of the plated embryos was very low, and none of the ICMs generated from 2-cell embryos remained undifferentiated during subcultures. Differences in developmental capacity of the in vitro produced embryos comparing to the in vivo produced embryos may explain the lower potential of 2-cell embryos to generate normal ICMs (30). In addition, species variations should not be ignored (22).

Co-culture of embryos with various somatic cells has been suggested as a promising approach to improve embryo development (23, 31, 32). The beneficial effect of co-culture systems is either through regulating secretion of embryotrophic substances into the culture media or removing

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embryotoxic materials from it (33). In the present study, MEFs as a source of growth factors production failed to stimulate *in vitro* cleavage of 2-cell embryos to the same degree as *in vivo* environments. In other word, *in vivo* produced blastocycts had greater potential towards plating stage and formation of high quality ICMs, probably due to the more time spent in oviduct. In fact, when the embryos are developing *in vivo,* the cells and the fluid of fallopian tube as well as the uterine epithelial cells support them. The level of nutrients such as ions, glucose, amino acids and hormones is most likely controlled *in vivo* in comparison to *in vitro* conditions (34).

To date, utilizing epiblast from delayed-implanting blastocysts in conjunction with MEF rather than STO feeder cells has enabled researchers to obtain ES cell lines reproducibly from several strains of mice including pure CAB/ca (35). According to accumulating knowledge, various techniques have been used to expand established ES cells. Some laboratories have employed mechanical disaggregation techniques, whereas others have used different enzymes such as collagenase, trypsin and proneas for disaggregation of ES colonies (13). Usually, ES cells are reported to be trypsinised routinely without problems, but Kawase *et al* (1994) indicated that ES cells of the BXSB mouse were very sensitive to trypsinization in subsequent sub-culturing. We used both mechanical and enzymatic methods to isolate and sub-culture colonies grown on MEFs feeder layer. Usually ES cells are propagated after enzymatic disaggregation of colonies and smaller clumps are harvested for new colony formation. Our enzymatic method was encouraging, most likely because the size of cell clumps obtained from ICMs was smaller than mechanical method and the rate of differentiation after cultivation onto MEFs was lower in smaller colonies when compared to larger ones. Although trypsin is the most commonly reported enzyme that disaggregates ES cells, it may cause a failure of ES cells to self-renewal (36). However, after the initial adaptation of the lines to trypsin, this procedure can be used, and yield large quantities of human ES cells that exhibit all properties of pluripotent cells (35). Mechanical approach has also been reported to be a simple and quick procedure for producing ES cells from ICMs (18). Use of these procedures has been confirmed by other researchers as well (9, 37). Our data indicate that similar to human (38), enzymatic procedure is ideal for isolation and disaggregation of cell colonies from NMRI mouse embryos.

Culture of colonies on MEFs or in the presence of LIF showed that maintenance of ES-like clumps at undifferentiated state is not well supported by LIF or MEF alone. However, our daily observations showed that ES-like colonies differentiate spontaneously on MEFs feeder layer more slowly than those ES-like colonies cultured in presence of LIF alone. The effect of LIF on isolation of porcine (23), primate (14) and human's ES cells (13) have been investigated and the results showed that LIF did not delay spontaneous differentiation of ICMs. LIF acts via activation of transcription STAT3 signaling (12). Unlike many mouse ES cells, addition of LIF and activation of the gp130/jak/STAT3 signaling pathway (39) failed to block spontaneous differentiation of cell clumps from NMRI strain. Besides the STAT3, expression of transcription factor oct4 (40, 41) and wnt-signaling (38) are critical for the maintenance of ES cell plouripotency.

It is important to identify factors that may facilitate growth and inhibit differentiation of ES cell colonies. Although adding LIF to the growth medium can be replaced with culture of ES cells from known strains on feeder layers, of course the beneficial effects of exogenously added LIF in presence of fibroblasts is still unclear (6). Our comparative study using LIF with MEF revealed that MEF by supportive action of LIF is essential to maintain and expand undifferentiated ES-like cells from NMRI mouse embryo. This finding is supported by a study that showed in CBA/ca inbred mice, LIF in combination with MEF not only enabled derivation of cell colonies from blastocyst but also improved their success rate (35).

Generation of cell colonies initially require a monolayer of inactivated MEF, therefore it seems likely that juxtacrine factors are critical to cell colony growth (42). However, the isolation and maintenance of ES cells in the absence of feeder layers will greatly facilitate studies on ES cell properties. Our results demonstrated that the amount of LIF present in the culture medium, when conditioned by MEFs feeder cells, can be as low as 500 units, which is below the optimal concentration (1000 IU) usually used to culture somatic ES cells.

These data are in accordance to the results reported earlier (9). Our ES-like colonies were similar to the established mouse ES cells, not only in morphology (24) but also in the expression of specific cell markers for pluripotent cells such as ALP enzyme. In our experiments, ES-like colonies showed positive ALP activity and spontaneous

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formation of EB-like structures similar to those generated from the other mouse strains; S2/sv129 (27). The findings presented here consent that in NMRI mice, the efficiency of generating ES cells is influenced by the genetic strain of laboratory mouse and individual factors that affect pregnant females (38, 43). In a few strains of laboratory mice, notably 129, C57BL/6 and a hybrid strain, the result of ES cell production was encouraging. However, ES cells derived from C57BL/6 blastocysts do not behave as reliably as do ES cell from the 129 strain (22). For these reasons, and despite the undoubted need of ES cells as experimental models and because ES cell derivation from mouse blastocysts is not a fully established method especially in different species (43), further studies are required to establish proper designs and cell free systems for maintenance of ES cells from various strains of mouse.

In conclusion, NMRI mouse ICMs can be derived from both 2-cell and expanded blastocyst embryos. However, ES-like colonies are produced more efficiently from blastocyst embryos compared to the 2-cell embryos. Enzymatic method for disaggregation of ICMs gives higher results and the concentration of LIF for maintenance of pluripotency properties of ES cells can be lower than optimal concentration reported in other studies.

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