Iranian Journal of Reproductive Medicine Vol.3. No.1 pp: 42-46, 2005

Isolation and differentiation of mouse embryonic stem cells

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Abstract

Background: Recently, embryonic stem (ES) cells have become very important resources in basic medical researches. These cells can differetiate into derivatives of all primary germ layers.

Objectives: In order to isolate embryonic stem cells in vitro, the blastocyst were cultured and the morphological aspects, population doubling time, alkalin phosphatse and differentiation properties of the cells were investigated.

Materials and Methods: The balstocysts from NMRI mice were cultured for 3 days up to time that inner cell mass (ICM) reach to the outgrowth stage. The cells were disaggregated and trypsinized every 3 days until the appearance of the colonies of ES cells. The colony positive cells were fixed and stained for alkaline phosphatase. The ES cells were cultured in suspension state for 5 days, at the same time Leukaemia Inhibitory Factor (LIF) was removed from media to form embryoid bodies(EBs). The EBs were cultured for 8 - 20 days on collagen coated dish to induce the spontaneouse differentiation.

Results: During the 6-9 days after the disaggregation of ICM in the expansion stage, the colony of ES cells appeared as a flat monolayer mass with strike boundaries and nondistinguish cytoplasm including a few nuclei. In colony formation stage, the morphology changed from flat monolayer to round multilayer with strike define boundaries. Undifferentiated cells were seen as intensely small cells attached together compactly with high nucleus/cytoplasm (N/C) ratio. The cells of colonies tend to differetiate by separation from each other and became larger and diffused on substrate by attaching to dish. The positive alkaline phosphatase cells were seen in typical morphology of ES colonies. The EBs cells were seen in culture after 5 days in suspension and began to spontaneously differentiate into various types of cells such as nerve and hematopoitic lineages.

Conclusion: Despite strike morphology of ES colonies, it is difficult to distinguish the differentiated from undifferentiated cell colonies in the colony formation stage. New ES cells are capable to give rise into EBs and are susceptible of spontaneously differentiation in various type of cells.

Key Words: *Embryonic stem cells, Embryoid bodies, Differentiation, Mice.*

Intruduction

The embryo develops from a group of undifferentiatiated cells that during the embryogenesis, the fates of them become gradually restricted as they enter new developmental pathways. As the development proceeds, the pluripotent ICM must choose one of two fates. First entails the formation of extra- lineage from primitive endoderm sources . Second contribute in formation of all three embryonic germ layers derivities from primitive ectoderm. The later contains uncommitted cells that are embryonic cell capable to produce ES cells. Both population acquier a signaling factors to differentiate into a vescicular cystic apperance like primitive endoderm or pre-amniotic cavity (1). Mouse ES cells can grow to mouse embryonic fibroblast (MEF) or STO cell line. The feeder layer by releasing agents with differentiation inhibitory activity encouraged ES cells to undergo spontaneously differentiation (2,3). The medium conditioned by buffalo rat liver (BRL) or supplimented by myeloid leukaemia inhibitory factor (LIF) are also used to maintain the stem cells undifferentiation (4,5). ES cells can prolifeate indefinitely in vitro and are unique in their ability

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by growing as immortal cells and express high telomerase (6) as well as to retaining a normal karyotype during multiple passages (7). The self renewing properties of ES cells permite them to generate many types of cells in the body. If ES cells were injected into blastocyst cavity, thereafter they may contributed in germ layer transmission and form a chimeric mouse(8). Either they are able to differentiate into a very large variety of cell types by injecting them into muscle or testis of immunodificency mice to produce embryonic teratoma (9) or they can spontaneously differentiate in vitro into EBs composed of three embryonic germ layers (10).

Mouse ES cells are a rigerious componet in the biomedical engineerring. The genitically manipulated mouse ES cell lines can carry a specific gene into embryonic genome and consequently making a transgenic animals as a model of knock-out or knock-in mice (11). The aim of this study was the isolation of ES cells in vitro from the mouse blastocysts that show a good breeding rate. The morphology, population doubling time, alkalin phosphatse properties were studied during the outgrowth, disaggregation, expansion and colony formation stages. Also their differentiation properties were investigated throughout formation of EBs and spontanously differentiation.

Materials and Methods

blastocysts preparation

Natural mating between 20 female superovulated (PMSG 7.5 IU, HCG 10 IU) and male NMRI strains mice were done to provide blastocysts. The blastocysts were flushed to form uterus by M2 media 5 days after mating.

The feeder layer preparetion

Rat embryonic fibroblast (REF) in a natural cycle or mouse embryonic fibroblast (MEF) in a superovulated cycle were prepared to form embryos in a midgestation age according to protocols discribed by Abbondonzo et al. , 1993 (12) .

ES cells establishment

The blastocysts were transfered to 35 mm dishs on the REF feeder group or MEF feeder layer group which perviously inactivated with mitomycin C (kyowa, Japan) 10µg/ml for 2h in CO2 incubator. The ES media containg DMEM high glucose (Sigma) + 20%FBS (Gibco) + LIF 1000IU/ml (Sigma) + 2-mercaptoethanol 0.1mMol (Sigma) + L–Glutamin 2mMol (Sigma) and Penicillin/Streptomycin 100mg/100IU/ml (Sigma) for 3 days. Disaggregation was carried out according to method discribed by Bongso et al. (1994) with some modification. Briefely, the outhgrowth ICM were disaggregated mechanically by hand pulled pasteur pipette in different size in 50 µl DMEM media under mineral oil (sigma). Then, the disaggregated ICM was transfered to one well of 96-well dish (NUNC) and cultured for 3 days (13). An alternative procedure for disaggregation of ICM was culturing of blastocysts on 96-well dish and trypsonizing the outgrowth ICM in situ with trypsin/EDTA 0.1% /1mM(sigma) in PBS (14). It was possible to trypsonize the cells in 96-well dishs up to 3 more passages every 3 days until the ES colony morphology appear in the expansion stage. It is essential to monitor microscopically the formation of ES colonies in this stage daily. The colony positive dishes have to subculture 2 time more in the colony formation stage untill the cells became confluent enough for passage in 4-well dish (NUNC). Depending to doubelling time the cells must be trypsonized up to 4 further passage every 3 days. Then the confluent cells were passaged into 35 mm dish (NUNC) as the passage number one. The first frozen cells were carried out in passage number two (60mm dish) using DMSO 10%, FBS 20% and DMEM media.

Alkalin phosphatase assesment

The ES cells were cultured in 35 mm dish for growing, then the ES colonies were fixed by 4% formalin in PBS buffer and naphtol AS-MX (Sigma) was used according to manufactures instraction for alkalin phosphatase staining.

Formation of EBs and spontanously differentiation

The ES colonies were cultured for 5 days on 24-well dish (Cellstar) in suspention state by adding 1% trypsine to ES media and removing LIF. Then, the EBs were trypsonized with mild 0.5% /0.5mM trypsin/EDTA (sigma) in PBS and then the media removed and transfer into centrifuge tube for a few minutes. The sedimentary EBs were transfed on the collagen coated 4-well dish and cultured for 20 days to induce the spontanously differentiation. For detection of hematopoeitic cells, the differentiated cells were fixed by carrnoy's fixative(glacial acetic acid and metanol 1:3) and stained by Wright-Gimsa method.

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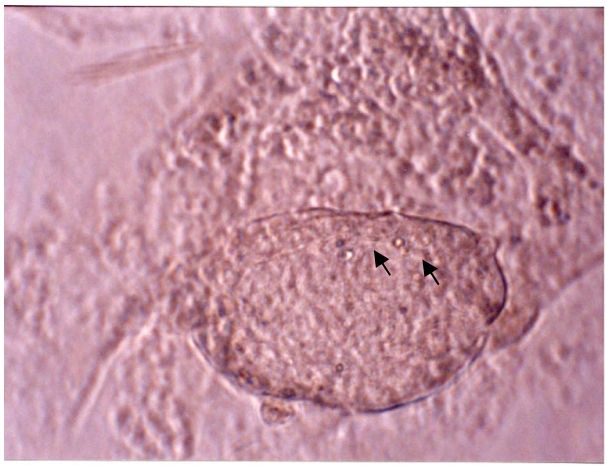


Figure1. Zona hatched blastocysts that were cultured on inactivated embryonic feeder layer. Arrows indicated the ICM that became bulge.

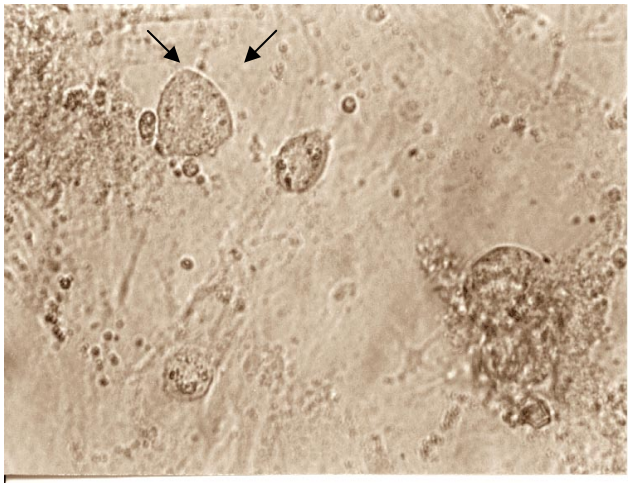


Figure 2. ES colonies morphology in expansion stage. Arrows indicated flat ES colony cells with few neclei.

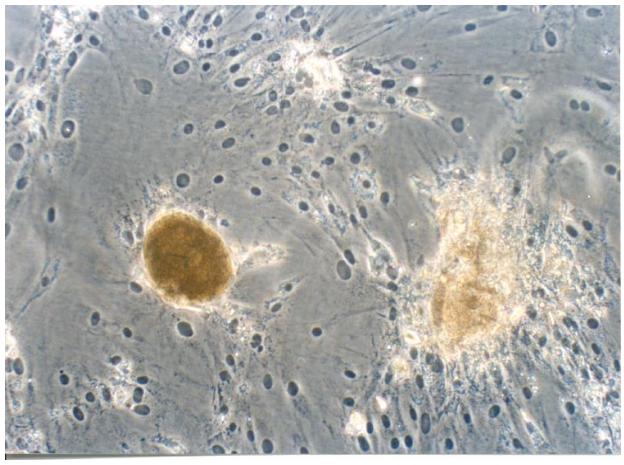


Figure 3. Alkaline phosphatase staining. The colony in left show a positive reaction in a typical ES colony. A differentiated diffused ES cells appear in right side of picture with a medorated positive reaction.

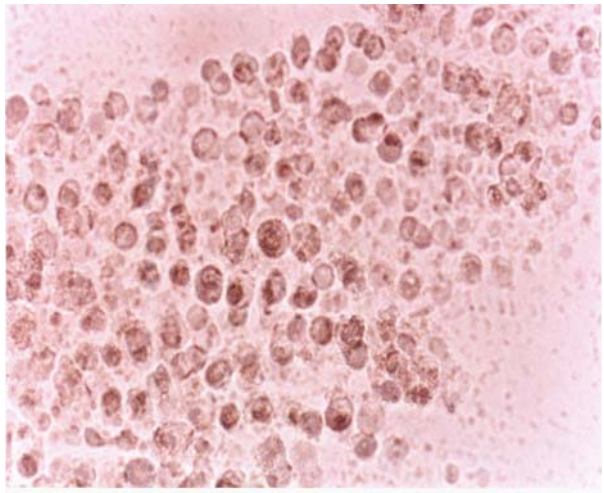


Figure 4. Culture of mouse ES cells in suspention and absence of LIF to induce EBs formation. Numerous cystic caviated EBs were formed after 5 days.

Results

ES cells establishment from NMRI mouse

In total 25 blastocysts obtained from superovulated mice. From them 15 blastocysts transfered on the REF feeder layer and other 10 blastocysts cultured on MEF feeder. The blastocysts in both groups became to leave zona pellucida and the ICM appear as a bulge outgrowth mass during 3 days (figure 1). There were 8 outgrowth of ICM in REF group and 4 in MEF group. Non of disaggregated ICM in REF group have shown the colonies with ES morphology, while there were 3 colonies with strike morphology of ES cells in MEF group.The disaggregated ICM in MEF group start to form colonies in a period of 3 to 9 days after the blastocyst culturing in expansion stage, means it may need 2 to 3 more passages after disaggregation of ICM to form colony. The strike morphology of ES cells in expansion stage is as a flat monolayer colony with smooth line boudary and many obvious nuclei with integrated cytoplasm (figure 2). Both mechanically and trypsonizing disaggregation have been shown in ES colonies. The next stage was formaition of typical ES colonies. The morphology of ES colonies in this stage change from flat monolayer to round multilayer with strike define boundaries. It was composed of small cells attached to each other with high N/C ratio where a single cytoplasm was not detectable. The ES cells colony tend to differetiate by separation from each other and becoming larger in size and attaching to bottom of dish. Alkaline phospatase staining have shown a extensive positive reaction in undifferentiation ES

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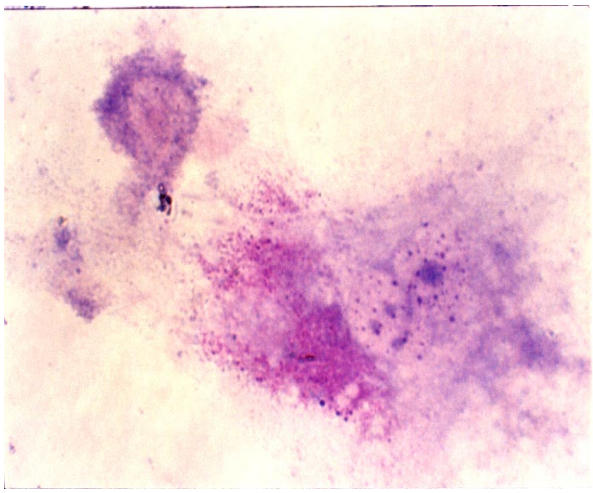


Figure 5. Differention of EBs into specialized cells with different morphology. The acidophilic granules can seen in left bottom and basophilic in left up. Two azorophilic cells with typic morphology of early nutrophilic cells appear in right. Wright- Gimsa staining

cells with strike ES colony morphology and a medorate patchy positive reaction was shown in differentiation ES cells that became to sperad on the bottom of dish (figure 3).

Mouse EBs formation and spontanously differentiation

The ES cells start to differentiate into mouse EBs cells almost 5 days after culture in suspention and remove of LIF. The EBs cells were largely composed of densely packed mouse ES cells. Soon after the center of these bodies became cavitated, the bodies began to accumulate fluid and turn into cystic EBs (figure 4). More than 20% of cells appeared as a vescicular morphology. Pipetting of cells in days 2 and 4 of cultre in suspention will increase the percent of cavitated EBs. The EBs continue to develope up to 14 days in suspention stage and during this period the EBs became to pack dens mass of cells by reducing the size of cavity. By plating of EBs cells for further days on collagen coated dish the EBs begans attaching to dish and differentiating into various types of specialized cells while some of them contain a few granules. The wright-gimsa stainning, demonestrated cells containing of acidophilic, basophilic and azophilic granules which probably indicated on vourious types of hematopoitic lineages (figure 5).

Discussion

A neccesity for isolation of ES cells is information about the behavior of disaggregated ICM on inactived embryonic fibroblast feeder layer and cognition of their morphology in vitro. Therefore we evaluated the morphological criteria for approval of ES cells early in expansion stage and later in colony formation stage. We demonestrated that using morphology index in expansion stage approve the first sign in isolation of ES cells. Almost 9 days after disaggregation of ICM in the expansion stage, we can diecide whether to go on or discart the cells to save time and money. It was previously reported that mouse ES cells in contrast to human ES cells only require LIF and are feeder layer independent (15). While in the present study adding LIF to media, there is some evidence in our results that show the ES cells harvesting is dependent to the type, source and quality of feeder layer. The best results obtain by mouse superovulated embryonic fibroblast in a young passage. The results also show that disaggregation is a critical stage in ES cells production. Delay in proceeding of mechanically disaggregation or any increasing in trypsin concentration will cause failure in ES colony formation.

The ES cells are capable to diferentiatie into various lineages of specialized adult cells in two different procedure: in suspention culture by mediation of EB formation and directly by responding to growth factors (16) and transcription factors or without formation of EBs such as hemotopoeitic lineage in a co-culture system. It is more likely that the fate-determination of cells making EBs is detected in a period of almost 5 days, so this is the optimum time for plating of EBs and allowing them for attaching to collagen or fibronectin coated subestra and dissociation to specialized cells. The EB formatiom is a process of two steps: aggregation step that allow the ES cells compact together and exerts a complex signaling in somewhat resembling the gastrolation process and dissociation step that allow cavitation of EB after 5 days. In this period the EB cells can response to growth factor and transcription factors by expression various receptors. To study the pluripotency of new produced ES cell lines, we assess their differentiation potential by continuing culture of one ES line and inducing them into EBs formation.

The present study demonestrate, that density of mouse ES cells are very important in their differentiation into EBs. In the case crowd ES cells they tend to attach each other and to make a cord or cluster that may inhibit their development. The length of growing ES cells in suspension is another important factor. In a long period of suspension culture the EBs change their morphology from translusent vesicle with large cavity to opaque

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spherical mass full of specialized cells with small cavity. This finding is emphasized by Itskovitz-Elder et al. (2000) who studies the EBs stracture by processing of EBs for routine histological examination and in situ hybridization using labeled monoclonal antibodies against α-fetoprotein, δ-globin and neurofilament for detection of tissue derived of endoderm, mesoderm and ectoderm respectively. They have been demonestred that in the case of endodermal marker for example α-fetoprotein was primarly expressed in interior part of some EB while expressed in exterior part of some others. This means that the EBs like a teratoma are capable to produce various types of cells after dissocision in the surface of dish (17).

Mouse ES cell derived hemotopoietic precursors, cardiomyocyte, neural precursors or insuline producing cells have been perviously transplanted successfully into recipent animals. A similar potentitial may be applied in the near future to patients with various disease. Simillary in vitro generated tissue from mouse or human ES cells may take the place at least in part of organ transplantation (18). So, our aim in isolation of new mouse ES cells was testing their capability in differentiation to various pure adults cell types and their capability in transplantation medicine in near future.

Conclusion

Using morphological criteria, is an important step in isolation of ES cells. There is some evidence in our results showing that ES colony formation is dependent to the source and quality of feeder layer. It is so difficult to distinguish the differentiated from undifferentiated cell colonies. Therefore, we used the capability of new mouse ES cell line by differentiating them into EB formation and then by testing their potentiol in sponatanouse differentiation. The new ES cell lines may be a useful sourse of mouse pluripotent cells that is capable for directed differentiation and producing pure reservious source of adult specialized cells for transplantation in numerouse pathologies in further workes.

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