

# Study of several factors affecting on preparation of mouse embryonic stem cells<sup>☆</sup>

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Received September 20, 2008

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## Abstract

The several factors were studied such as the producing methods of embryos, the development stages of embryos and the culture media in order to provide some academic and practical data for the preparation of mouse embryonic stem (ES) cells. Fibroblasts were acquired from 13.5 d embryos of KM mouse. The feeder layer was made by fibroblasts of passage treated with mitomycin C. 3.5-day embryos were cultured on the feeder layer for 4 – 5 days, ICM was separated from the embryonic cells, which was in a good growth status, obvious eminence and without morphological differentiation. The first generation ICM was separated into small pieces of cell masse and then was cultured on the feeder layer in ES culture medium. The passages were carried out according to the appearance of new ES colonies, which grew well and had no differentiation. ICM and different generation of ES cell were identified by morphological observation and alkaline phosphatase staining. The results were showed as following: development ability of embryos from natural mating was better than from superovulation, blastulas had more developmental potential than morulas in the establishment of ES cell lines. DMEM with both low sucrose and high sucrose could be used to culture ES cell, and the medium supplemented with 15% serum was better in culture of ES cell than 10% and 20% group. [Life Science Journal. 2009; 6(1): 1 – 4] (ISSN: 1097 – 8135).

**Keywords:** mouse; embryonic stem cell; *in vitro* culture

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## 1 Introduction

Mouse embryonic stem cell lines have been established since 1981 and embryonic stem (ES) cells of many animals were isolated successively, such as golden hamster, marten, pig, chicken and monkey. ES cells provided extensive space for study of cell differentiation, animal development, establishment of study model and interpreting gene function (Kagnew *et al*, 2007; Kunarso *et al*, 2008). The premise and basis of various study was establishing and maintaining stem cell lines with differentiation potentiality and normal diploid karyotype (Huang *et al*, 2007). However, there was a low ratio in successful ES cell line's establishment. In this experiment with Kunming mice as experimental animal,

the several influencing factors on isolation and culture of ES cells were studied in order to provide theoretical and practical basis for establishment of stable and efficient system of isolation and culture of ES cells.

## 2 Materials and Methods

### 2.1 Experimental animals

Kunming strain mice (6 – 8 weeks) were offered by Medicine Factory of Heilongjiang.

### 2.2 Major reagent

The major reagents were trypsin (1 : 250, Amresco), fetal bovine serum (FBS, Gibco), mitomycin C (Sigma), PMSG (Ningbo hormone factory), HCG (Ningbo hormone factory), ethylenediamine tetraacetic acid (EDTA, Amresco).

The culture media were as follows:

Embryo culture medium I: DMEM (high sucrose)

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\*Supported by Fund of Heilongjiang Education Bureau. China (No.10531008).

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+ 15% serum + 2 mmol/L L-glutamate + 100 IU/ml penicillin + 100 µg/ml streptomycin.

Embryo culture medium II: DMEM (high sucrose) + 20% serum + 0.1 mmol/L β-mercaptoethanol + 2 mmol/L L-glutamate + 1000 IU/ml LIF + 100 IU/ml penicillin + 100 µg/ml streptomycin.

Embryo culture medium III: DMEM (low sucrose) + 15% serum + 0.1 mmol/L β-mercaptoethanol + 2 mmol/L L-glutamate + 1000 IU/ml LIF + 100 IU/ml penicillin + 100 µg/ml streptomycin.

### 2.3 Method

Fibroblast was acquired from 13.5-day embryos of Kunming mice. The feeder layer was made by fibroblast treated with mitomycin C (Lang *et al*, 2006). Mice were superovulated with 10 IU of PMSG and 10 IU of hCG, the embryos of 3.5 days after mating were flushed out from uterus in sterile environment, transferred into inactivated MEF dish and cultured for 4 – 5 days in 5% CO<sub>2</sub>, 37 °C. ICM colonies with well growing status, obvious eminence and without morphological differentiation were passaged. ICM were collected by glass pipette, washed in PBS, incubated individually in the drops of digestive enzyme buffer for 1 – 5 minutes and were dispersed into small pieces of mass by glass pipette. Then they were transferred into MEF dishes and cultured under 5% CO<sub>2</sub>, 37 °C. Observation was taken everyday. Passages were carried out according to the appearance of new ES colonies which grew well and had not differentiated. ES cells were identified by means of alkaline phosphatase staining.

### 2.4 Statistical analysis

All data were analyzed by SPSS, the value less than 0.05 was considered to be significantly different.

## 3 Results

### 3.1 Culture of embryos (Table 1)

**Table 1.** Comparison of culture of embryos between the natural mating and superovulation ( $n = 6$ )

Method of acquiring ovum	Number of embryos	Ratio of attaching wall cultured 3d (%)	Ratio of ICM growing (%)	Ratio of growth ICM after passage (%)
natural mating	60	90.0 ± 3.65 <sup>a</sup>	81.66 ± 3.07 <sup>a</sup>	50.0 ± 4.83 <sup>a</sup>
super-ovulation	60	83.33 ± 2.11 <sup>a</sup>	73.33 ± 3.33 <sup>a</sup>	35.0 ± 2.24 <sup>b</sup>

<sup>a,b</sup> meant that there was statistical difference between them ( $P < 0.05$ ).  $n$ : number of repetition.

### 3.2 Comparison of attachment ratio of morula and blastula, ICM growing ratio and ratio of growth ICM after passage (Table 2)

**Table 2.** Comparison of attachment ratio of morula and blastula, ICM growing ratio and ratio of growth ICM after passage ( $n = 15$ )

Categories of embryo	Number of embryo	Ratio of attaching wall cultured 72 h (%)	Ratio of ICM growing cultured 96 h (%)	Ratio of growth ICM after passage cultured 96 h (%)
Morula	164	38.49 ± 2.46 <sup>a</sup>	37.16 ± 2.57 <sup>a</sup>	27.40 ± 2.01 <sup>a</sup>
Blastula	149	80.34 ± 3.11 <sup>b</sup>	73.45 ± 3.92 <sup>b</sup>	40.50 ± 3.02 <sup>b</sup>

<sup>a,b</sup> meant that there was statistical difference between them ( $P < 0.05$ ).  $n$ : number of repetition.

### 3.3 Effect of different culture medium on embryo culture (Table 3)

**Table 3.** Result of embryo culture in different culture medium ( $n = 6$ )

Culture medium	Numbers of blastula	Ratio of attaching embryo cultured 72 h (%)	Ratio of ICM growing cultured 96 h (%)	Ratio of growth ICM after passage cultured 96 h (%)
I	89	80.34 ± 3.11 <sup>a</sup>	73.45 ± 3.92 <sup>a</sup>	40.50 ± 3.02 <sup>a</sup>
II	71	79.50 ± 5.61 <sup>a</sup>	71.79 ± 5.0 <sup>a</sup>	44.33 ± 5.60 <sup>a</sup>
III	75	60.13 ± 4.03 <sup>b</sup>	48.44 ± 4.93 <sup>b</sup>	26.54 ± 6.24 <sup>b</sup>
IV	69	55.32 ± 5.98 <sup>b</sup>	43.65 ± 6.05 <sup>b</sup>	39.55 ± 6.81 <sup>a</sup>

<sup>a,b</sup> meant that there was statistical difference between them ( $P < 0.05$ ).  $n$ : number of repetition.

### 3.4 Effect of ES cell culture with different methods (Table 4)

### 3.5 Growth and identification of ES cells

24 hours after being cultured in MEF dish, small ICM mass attached to the wall and proliferated successfully, and various differentiated cells were observed 2 – 3 days later. It was generally about 4 – 6 days that typical ES cell colonies began to appear (Figure 1). Meanwhile, typical ES cell colonies were dispersed and passaged. ICM, ES colony cells and differentiated cells were identified by staining with AKP. The result showed that well growing ICM were strong AKP positive with black color colonies. Trophoblast-like cells and epithelium were AKP negative and light yellow or achromatic color (Figure 2), the fourth generation cells were strong positive and the colonies were black (Figure 3), and fully differentiated ICM formed into trophoblast-like cells, epithelium-like cells and fibroblasts, which were AKP

**Table 4.** Result of ES cell culture in different methods ( $n = 6$ )

Culture medium	Number	Generation (%)				
		1st	2nd	3rd	4th	5th
I	30	20 (49.26 ± 2.77 <sup>a</sup> )	6 (20.0%)	2 (6.67%)	1 (3.33%)	1 (3.33%)
II	36	13 (35.75 ± 2.67 <sup>b</sup> )	4 (11.11%)	1 (2.78%)		
III	40	14 (34.48 ± 2.16 <sup>b</sup> )	4 (10.0%)			
IV	27	15 (48.19 ± 5.14 <sup>a</sup> )	6 (22.22%)	2 (7.41%)	2 (7.41%)	1 (3.70%)

<sup>a,b</sup> meant that there was statistical difference between them ( $P < 0.05$ );  $n$ : number of repetition; ES cell ratio: (number of ICM forming ES cell colony/number of incipient) × 100%.

negative and light yellow or achromatic color (Figure 4).

## 4 Analysis and Discussion

### 4.1 Nature mating and superovulation

There were no significant difference in wall-attaching ratio of 72 h culture and ICM forming ratio between natural mating and superovulation, but there was significant difference in ratio of ICM which could be passaged. It was concluded that embryos of nature mating was better for isolation and clone of mouse ES cells than superovulation. Maybe it was due to high concentration hormone in blood stream affecting uterine environment.

### 4.2 Morulae and blastulae

ES cells could be isolated from morula and blastula. The study result were different in the establishment of ES cell lines (Bryja *et al*, 2006). Comparing the effects of morulae and blastulae on establishment of ES cell lines during dispersing morulae into single blastomeres which were cultured in MEF to forme cell colonies. Eistetter *et al* (1989) found that morulae were better than blastulae on the establishment of ES cell lines. In this study which routine culture method was used, the attachment ratio of morulae in 72 hours culture was low (38.49 ± 2.46), but most of blastulae attached to the wall in 72 cultured (80.34 ± 3.11) and others degenerated gradually. Morulae developed into blastula and hatched blastocysts which attached to the wall later. If culture condition and other factors were not good for the development of morula, the number of hatched blastocysts and expanded blastocysts decreased, the hatching ratio of embryos and ratio of ICM formation were low and the number of isolated ES cell was poor. The results showed that blastulae were better than morulae in the isolation of ES cell.

### 4.3 Culture medium

Compared with the high sugar culture medium, the low

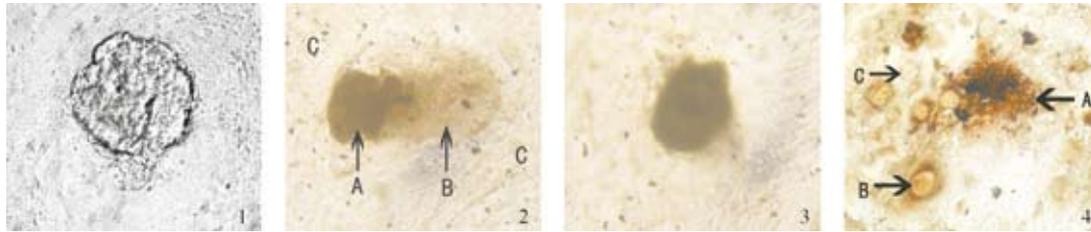
sugar culture medium produced the low speed of embryo development and the less number of ICM growth. The low speed of ICM differentiation was advantageous to passage and digestion in time, therefor number of growth ICM after passage was not very less (44.33 ± 5.60 vs. 39.55 ± 6.81). Glutamate was important energy substance for embryo developmen.  $\beta$ -mercaptoethano promoted embryonic cells division growth and protected intracellular enzyme and sulfhydryl groups of protein from oxygenization. The results showed that culture medium with 0.1 mmol/ $\beta$ -mercaptoethano have no significant influencing on embryo devepoment. Low sucrose DMEM was suitable to culture observation of ES cells, and the high sugar culture medium was suitable to acquiring ES cells.

DMEM containing serum was a basic culture medium, but there was difference in culture medium with different serum. The serum was natural medium which provided nutritional ingredient to cells, promoted DNA synthesis and contained some growth regulatory factors for cells to grow and propagate. Also serum provided proteinase inhibitors to cells to protect itself from the damage of proteinase of dead cell. When the concentration of serum was increased in culture medium, the speed of cell growth increased, but high concentration of serum was not always suitable to ES cell culture. The medium supplemented with 15% serum was better for culture of ES cell than 10% and 20% group.

In the culture process of ES cell, there was no significant difference between low sucrose and high sucrose DMEM, but comparing to high sucrose DMEM, ES cell colonies grew slowly in low sucrose DMEM with the small size of ES clones and later differentiation ES.

### 4.4 Identification of ES cells

The activity of alkaline phosphatase in early ES cells was high. Undifferentiated ES cells had typical cloned configuration which were strong AKP positive. Once ES cells began to differentiated, their AKP became negative (Thomson *et al*, 1998). In this study, ES cell colony was



**Figure 1.** Identification of ES cells. Figure 1.1: ES cell clone,  $\times 100$ ; Figure 1.2: staining of ICM, arrow A shows ICM in black, arrow B points to trophoblast-like cells in light yellow and MEF was achromatic as marked in c area,  $\times 100$ ; Figure 1.3: Clone at passage 4 shows strong AKP positive,  $\times 100$ ; Figure 1.4: Staining of differentiated ICM, arrow A points to trophoblast that was weakly positive and light black, arrow B points to giant cell (epithelioid-like cells), both of fibroblast (c) and giant cell were weakly positive and light yellow,  $\times 100$ .

strong AKP positive, which meant the ES cells were not differentiated.

## 5 Conclusion

Development ability of embryos of natural mating was better than superovulation, blastulae had more developmental potential than morulae in the establishment of ES cell lines. Both low sucrose and high sucrose DMEM could be used to culture ES cells. The medium supplemented with 15% serum was better for culture of ES cell than 10% and 20% group.

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