92　11　6

4

C57BL/6　　3　ICR

3.4%

30

2　Monash

HES2

HES2

HES2

Embryonis stem (ES) cells are pluripotent cells which can differentiate into most lineages in the body. Recently a number of studies indicate that ES cell may be useful in the study of developmental biology and toxicology. These cells may also provide an unlimited source of cells or tissues or organs for transplantation therapy. According to the original goals, we have derived in total 4 mouse ES cell lines (one is C57BL6 and 3 are ICR strain mice). The effieciency of establishment is about 3.4% and two of the lines (one is C57BL6 and one is ICR strain mice) have been passaged for more than 30 generations. After injection of these ES-like cells into the SCID mice, termatoma formed which contained cells originated from all three germ layers. Recently we are also characterizing these cells by examining the stem cell markers (SSEA-1, SSEA-3, SSEA-4 and alkaline phosphatase) of these ES cell lines. We also are working on establishing human ES cell line from spare human embryo obtained during IVF. However as yet we can only obtain ES-like colony and grow to 3rd passage. In addition, we are also mainiaining the human ES cell line (HES2) obtained from Monash University in Australia, the condition of these cells are also fair and in many circumstances the cell will differentiate. Therefore further refinement of the culture condition is needed. However during the differentiation of the HES2 cells, we observe some morphological changes of the cells which suggested the potential of germ cell differentiation. Therefore further study will hopefully identify the potential of these cells.

Stem cells are unique cells with the ability of self-renewal and are capable of developing into one or sometimes many specialized cell types. Stem cells can roughly be divided into mono- (or multi-) potent and pluripotent ones. As yet, three pluripotent stem cell lines have been isolated – embryonic carcinoma (EC) cells, embryonic stem (ES) cells, and embryonic germ (EG) cells. Among them, ES cell is on the earliest up-stream cell type that theoretically is able to form all the cell lineages in the body. Recent studies suggest that under certain culture condition, ES cell can differentiate into trophoblast cell and even oocytes. These indicate that ES cell is very likely a totipotent cell type.

Human ES cell lines were first isolated by Thomson et al. in 1998 and subsequently be by Reubinoff in 2000. Many other informal reports also claimed the establishment of more than 70 human ES cell lines. These cell lines retain the ability to differentiate into all three primary embryonic germ layers. The study of human ES cells is scientifically very important due at least to the following reasons. First through the observation of ES cell differentiation into a number of cell lineages, the key factors and environmental influences that direct earliest decisions toward specific cell fate may be delineated. Secondly, from clinical and pharmaceutical point of view, the use of ES cells also provides an extremely efficient *in vitro* model for studying a number of chemicals and toxins. Last, the differentiated product of ES cells will likely provide an unlimited source of tissues or organs for human transplantation therapy.

Although there have been approximately 79 cell lines registered in NIH, there is as yet no domestic Taiwanese cell line. From the practical point of view, we in Taiwan need to have our own cell lines in order to make more researchers assessable to the cells. In addition, each ES cell line is not the same. It is therefore important to establish a practically workable protocol for researchers in Taiwan to isolate our own ES cell lines. Using these cell lines as a starting point, further study on the *in vivo* and *in vitro* differentiation of ES cells can be performed to expand and fulfill the potentiality of these pluripotent stem cells. However isolation of ES cell lines is not easy. Therefore, the original design of this project was first to establish murine ES cell line as a starting point. Using the experience from the animal works and also by learning ES cell culture from foreign lab (e.g. Dr. Pera's lab in Monash University), we hoped to establish Taiwanese human ES cell line. During the period, we also tried to contact with Monash University about obtaining the established human ES cell line, so that culture and differentiation study can be performed in our lab as soon as possible even before establishment of our own cell line.

[1] Modify the protocol of mouse ES cell isolation to increase the efficiency of cell line establishment
[2] Characterize the newly derived mouse ES cell lines
[3] To isolate and establish human ES cell line
[4] Culture and maintain the human ES cell line

Isolation Of ES Cell Lines

The isolation of murine ES cells has been reported as early as 1981. Recently, successful derivation of primate ES cell has been reported in several groups. It has been found out that morphologically there are some subtle but important differences between primate and murine ES cells and there are also a number of differences in the cell surface markers between the two cell lines.

There have been a number of well established reports on the derivation of ES cell lines from human embryos (Thomson 1998; Reubinoff 2000). Spare embryos donated by patients participating in the in vitro fertilization (IVF) and embryos obtained by fertilization from donated gametes have both been used. According to those reports, the efficiency of isolation is not always satisfactory (a ES cell line production rate from 3/18 to 5/14 cell lines/inner cell mass have been achieved). It is thought that there is still room for improvement of isolation and establishment of human ES cell line.

Defining the pluripotency of a ES cell-like cell line

There are three independent methods [1;2] to test the differentiation potential of ES-like cells once it is established – including in vitro differentiation in a culture dish, injection of ES cell into an immune-compromised mice to produce a teratoma, and chimera formation after injection into a blastocyst. Only the first two methods are practically applicable in human ES cells and both have been used successfully.

Development and Differentiation of ES cells

The differentiation of ES cells can be achieved by growing the cells in high density, on different feeder layers, by supplement of certain growth factors or introduction of transcription factors, or by culture on extracellular matrix substrates. The developmental and differential potential of murine ES cells have been tested in a big number of studies through trial and error and most denoted to the potential of ES cells to differentiate to many cell lineages when the culture environments are appropriately altered. Accumulating evidences also suggest that human ES cells can behave in a similar fashion.

(Methods of Differentiation Induction)

In vitro suspension culture leads to the formation of embryoid bodies that contains a number of differentiated cell types [1]. However these differentiations are generally undirected and uncontrollable. Addition of growth factors into the culture media is another effective way to induce the differentiation of ES cells [3]. However, the factors for the differentiation of a specific cell type have not been well established and no known single growth factor can induce ES cells to develop into a single cell lineage [3]. Finally coculture with a specific stroma cell lines may be proved to be an useful option since the feeder cells can provide the inherent dynamics of growth factor production that are suitable for the development of ES cells into specific lineage [4;5]. Different stroma types have been employed to promote hematopoietic commitment [4-6] and to direct production of dopaminergic neurons [7]. ES cells are induced to differentiate to hematopoietic cells by coculture with stroma

cell line RP010 and cytokine IL-3 and IL-6 [4]. Nakano et al. showed that on a macrophage colony-stimulating factor-deficient stroma cell line OP9, mouse embryonic stem cells were shown to give rise to primitive and definite erythrocytes sequentially [6]. The group in the Kyoto University furthermore identified a stromal cell (PA6 stroma cell) derived inducing activity (SDIA) that promotes the neural differentiation of mouse ES cells [7]. The SDIA accumulates on the surface of the stroma cells and induce efficient differentiation of cocultured ES cells in a serum-free medium without addition of other growth factors. These observations indicate that specific stromal cell lines may provide the unknown but necessary growth factors to direct ES cell to develop to a certain cell lineage. Theoretically the stroma cells should arise from the environment where the target cell of ES cell differentiation is located.

(Differentiation to germ cell)

Mouse ES cells have been repeatedly proved to be capable of differentiating to every cell lineage including germ line. Evidences from differentiation of human ES cells into teratoma also suggested that they are pluripotent, and specifically evidences are also accumulating that they can differentiate into germ cells *in vivo* [1;3;8;9]. However as far as our knowledge is concerned, *in vitro* development of human ES cells into germ cells or its related tissues have not been tested or, at least not reported previously. Irrespective of the potential ethical concerns that might arise, the study to examine this potential of ES cell has evident merits both in basic science and clinically.

In the search of the factors to direct ES differentiation into germ cells, there are several candidates that need to be noted. The functional gametes first arise as a migratory population of cells, called the primordial germ cells. These cells migrate to and colonize on the genital ridges during early organogenesis period, and finally give rise to a completely differentiated gonad. During this migration they proliferate and the number of primordial germ cells in mouse embryo increase from less than 100 to approximately 4000. It has been shown that extraembryonic ectoderm-derived factors instruct the pluripotent epiblast cell (a part of inner cell mass of blastocyst) towards restricted primordial germ cell fate during murine gastrulation [10]. BMP4 and BMP8b proteins (which are expressed by extraembryonic ectoderm) produced by COS cells form heterodimer to direct murine primordial germ cell formation [10]. Subsequently, there were studies identifying that the proliferation and migration of primordial germ cells in culture were influenced by the soluble factors released from the genital ridge. It was shown that genital ridges exert long-range effects on murine primordial germ cells, including their cell number and direction of migration in culture [11]. Furthermore, the chemotropic effect is at least partly mediated by TGF- 1 [12]. It is therefore suggested that culture media conditioned by genital ridges might contain some factors that are closely related to this effect. Other feeder layers have been used for germ cells proliferation, including STO cell line, sertoli cells and granulosa cells [13]. Messenger RNA encoding primordial germ cell marker, vasa, could be present for more than 30 days when Zebrafish embryonic cells were co cultured with rainbow trout spleen

cell line RTS34st [14]. After this short-term culture, these embryonic cells were able to produce germ line chimera when injected into a host cell. Therefore, co-cultures with these cell lines may be able to direct ES cells toward germ line lineage. Since germ cells survive well on monolayer derived from the cells of embryonic gonads [15], the use of gonad as a feeder of culture is another alternative.

Growth differentiation factor 9 (GDF-9) is a cystine knot-containing hormone of the TGF- superfamily. In GDF-9 null mice, follicle development is arrested at the primary stage and GDF-9 treatment *in vitro* enhances preantral follicle growth. Presently however, there is no data to indicate the role of GDF-9 in stages during and before formation of primordial follicle.

Establishment of murine ES cell line

In this study, inbred mouse (C57BL/6 mice strain) and outbred ICR strain mouse embryos are used.

Culture medium and LIF

The culture medium for mouse ES cells isolation is DMEM supplemented with 0.1 mM 2-mercaptoethanol, 2 mM glutamine, and 15% FBS (Hyclone), 1% non-essential amino acid, 50-100 U/mL penicillin, and 50-100 g/mL streptomycin. LIF is also used at this stage.

Isolation of mouse ES cells from blastocyst

The steps for isolation follow the original report of Ledermann et al. and Wasserman et al. The blastocyst can be plated on to feeder layer directly and after 4 days of culture, the ICM colonies are collected by a Pasteur pipette and replated onto a new feeder.

After further 7-14 days, ES-like colony is collected and passaged. Blastocysts can also go through a procedure called immunosurgery as described below and the resulting ICM plated onto a feeder.

Establishment of human ES cell line

The protocols of isolation of human ES cell line is basically modified from some previous reports in primates [16](Suemori, Developmental Dynamics, 2001) and human [1;2;17;18].

Obtaining human embryos and in vitro culture to blastocyst

Fresh or frozen human embryos are donated from patients undergoing IVF. 4-8 cell stage embryos are first cultured using G1.2 and G2.2 sequential media until expanded blastocyst stage.

Immunosurgery

Blastocysts are first subjected to proteinase to remove the zona pellucida. Then, the blastocysts are exposed to 1:10 dilution of rabbit antiserum for 30 min, and then exposed to heat-inactivated guinea pig complement for 30 min (1:5 dilution) to remove the trophectoderm.

Culture of ICM and subsequent propagation of ES cells

ICM is cultured on the mitomycin C-treated PMEF feeder using the ES medium. After 3-7 days, expanded ICM is mechanically removed from differentiated cell outgrowth, treated with 0.25% trypsin/1 mM EDTA, and replaced in a new PMEF feeder. After 7-14 days, the resulting ES-like colonies are propagated in clumps of cells (about 50-100 cells/clump) every 3-7 days, using type IV collagenase (1 mg/mL; Gibco/BRL).

Characterization of ES-like cell line

The resulting cell lines need to be

characterized by a number of experiments. Four studies will be done – (1) examination of the ES cell marker expressions (2) examination of teratoma formation after injection into SCID mice (3) examination of length of telomere and the expression of telomerase (4) examination of the karyotype.

Maintenance and culture of HES2 cell line

After thawing of the HES2 cell line, the colony pieces are cultured using the protocol provided by ESI. Differentiation of these cell colonies are also observed and characterized.

(Results)

Establishment of murine ES cell line

After serial experiments right from scratch, we finally establish 4 murine ES cell lines after many experiments (Table 1 and figure 1). One is from the inbred C57BL/6 mouse and the others from the outbred ICR strain mouses. The C57BL/6 line has been propagated to the 32th passage and was terminated due to contamination. At the 32th passage, the growth and proliferation of this cell line was still very vivid and the gross morphology was normal. Similarly one of the ICR cell line has been propagated to 31th passage and the passage was terminated due to the New Year. Again, the growth and proliferation speed remained perfect at that passage. Now we have ample stocks of these two cell lines in liquid nitrogen. Recently, we have used immuosurgery for mouse ICM harvet and the ICM morphology seems to be improved. At passage 10, both cell lines (one C57BL/6 mouse and one ICR strain) have been injected into SCID mice and both developed teratoma. After removal and stain, these tumors showed cell types from all three germ layers (including neural tissues, bone,

cartilage, GI tissues and renal tissues) (histological slides are available). We are now characterizing these cell lines (two of them) by examining SSEA-1,3, 4 and AP.

Obtain human ES cell line (HES2)

We have obtained a human ES cell line (HES2) from ESI (ES cell International (Figure 2). This cell line was shipped here in late February 2003 and we started to culture this line since June. At present the culture is not smooth yet and further refinement of the culture condition is needed to maintain its undifferentiated state. However in the observation of the in vitro differentiation of HES2, we find out some morphological change which may suggest the differentiation to germ cell lineage. Now we are using immunohistochemical staining and PCR to identify the potential germ cell marker (c-kit, vasa etc.), which hopeful may appear in these cells.

Human ES cell line establishment

Due to the lack of donated human embryos, we have as yet tested on 20 human embryos to establish the ES cell line. However, most of them are poor embryos (1P or 3P embryos, i.e. very likely chromosomally abnormal embryos). The results of immunosurgery on these embryos are basically favorable (Table 2, Figure 3) and one of the tested embryo resulted in a ES cell–like colony which survived and growed for three passages. However as yet no ES line has been established.

The efficiency of establishment of mouse ES cell line at this early stage was approximately 3.3~3.4%. This is comparatively lower than those reported in

literature (which is approximately at the level of 5-20%). Therefore there is still room for improvement. However since the result was obtained at an early stage when we have just learned to establish cell line and we did not use immunosurgery for ICM collection. Therefore, future modification of the protocols and the use of immunosurgery will probably improve the efficiency. Actually we have done immunosurgery on mouse embyos and it was found that the resulting ICM has a better morphology than those without immunosurgery. In addition, the formation of all three germ layer cells after injection into SCID mice indicates that these cell lines have the potential to develop to many cell types in the body. This result (accompanied with prolonged ability to proliferate up to 32 passages) basically fulfills the criteria of embryonic stem cells. However we still need to test other stem cell parameters to confirm the characteristics.

We have tested the viability and proliferation efficiency of HES2 in our lab. Preliminary experience indicate that it is quite difficult to maintain its long-term undifferentiated state. Similar experience actually have been met in   . Therefore we need more time and experience to obtain a smooth culture of this cell line. However the observation of the differentiated cell morphology, which reminds us of the germ cell lineage, suggested that our initial goal to direct human ES cell differentiation to germ cell lineage may be feasible.

The process of human ES cell line establishment is delayed recently basically due to the lack of donated human embryos. However since the feeder layer maintenance and the immunosurgery is basically smooth recently, we will more actively contact with IVF patients for the possibility of embryo donation. We have come to the idea that the success of ES establishment will depend mostly on the availability of good embryos.

The goal of this study was: [1] to isolate and establish mouse ES cell line; [2] to establish Taiwanese human ES cell line; [3] to culture and maintain HES2 cell line; [4] to direct the differentiation of human ES cell to germ cell lineage. The experiments going on in this year are basically agreeable with the original design. Up to now, we have established the mouse ES cell lines, have obtained human ES cell line from Monash and culture, and also have record some preliminary observation of in vitro HES2 cell differentiation. However, the establishment of our own human ES cell line is still going on and not yet successful (which in the original design, we actually did not think it definitely possible to establish in a year). The culture of the HES2 cell cannot be regarded as smooth, neither.

In conclusion, from a practical point of view, the present study basically follows the pre-designed way and the results can be rated as O.K. In addition, the results available up to now are not yet sufficient for publication and one more year of study will be needed for a more thorough analysis of the data. However it is likely that these data would provide some or enormous contribution to the future academic and clinical science.

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Table 1: Successfully established mouse ES cell lines

| Mouse strain	Number of 3.5 d.p.c. embryo (blastocyst) plated	Number of ICM colonies formation	Number of ES cell line established
ICR	132	116	4 (4/116; 3.3%)
C57BL/6	40	30	1 (1/30; 3.4%)

Table 2: Result of ES cell Establishment on Human Embryos

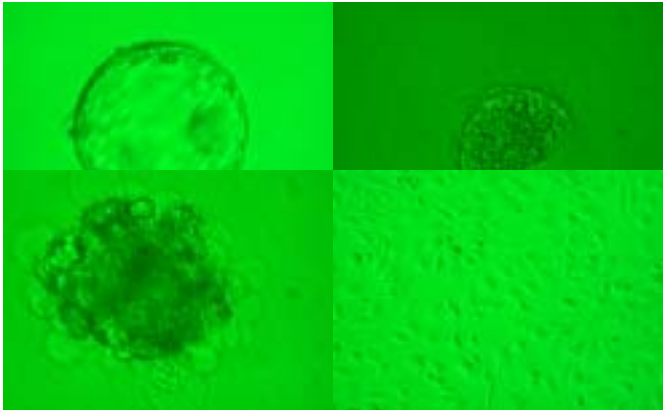| Embryo	Initial condition	Successful trophectoderm destruction by immunosurgery	Successful implantation of ICM on feeder	ES cell line formation
Fresh embryos	12 (mostly 1P or 3P)	8	6	0
Frozen embryo	3 (morphologically good in 1)	3	3	0

Figure 1: mouse ES cell line (ICR strain, left; C57BL6, right)
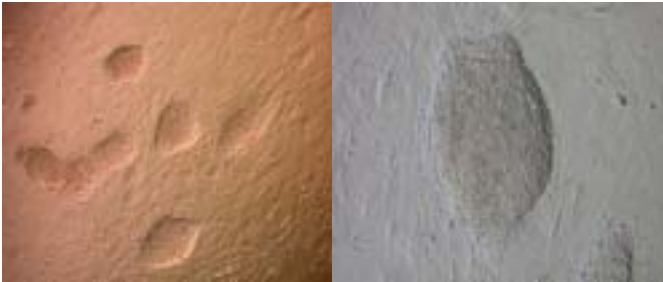
Figure 2: human ES cell line (HES2)
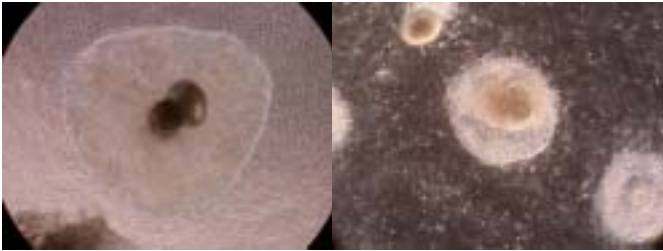
Figure 3: Immunosurgery

Figure 4: human ES-like colony at 3rd passage (beginning to differentiate)