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Experimental Protocols for Embryonic Stem Cell Researh

Methods for Expansion of Human Embryonic Stem Cells

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ABSTRACT

The manipulation of human embryonic stem cells (hESCs) requires refined skills. Here we introduce both mechanical and enzymatic transfer methods for hESCs depending on experimental purpose. We use the mechanical transfer method for maintenance of hESC lines. Although the method is laborious and time-consuming, the technique permits efficient transfer of undifferentiated hESCs and results in similar clump sizes. We implement the enzymatic transfer method when we need the bulk production of cells for various experiments. The enzyme-treated expansion rapidly produces greater amounts of hESCs within a limited time frame. However, the cell clumps vary in size, and there is a probability that both the differentiated and undifferentiated cells will be transferred. In cases in which there are differentiated colonies, the combination of two methods allows mass production of hESCs by excluding differentiated colonies from passage by manual selection before enzyme treatment. STEM CELLS *2005;23:605–609*

INTRODUCTION

The first derivation of human embryonic stem cells (hESCs) from the inner cell mass of preimplanation blastocyst was reported in 1998 [1]. Since then, several groups, including ours, have established new hESC lines [2–8]. The derivation and characterization of hESCs have drawn much interest in respect to their potential use for direct cell therapy for human patients [9, 10]. However, unlike mouse ESC culture, manipulation of hESCs is a relatively delicate process and requires refined skills for expansion. Various techniques are used to expand established hESCs. Some groups mechanically transfer hESCs, whereas others use enzymes such as collagenase, trypsin, and dispase for expansion [1–8, 11–13].

We introduce here methods of efficiently expanding our hESCs on STO feeder layers by both mechanical process and enzymatic treatment using collagenase IV. The selection of transfer method is based on experimental purpose. The mechanical transfer method requires a finely drawn Pasteur pipette to physically segregate the hESC colony into clumps of 150 to 200 cells for transfer. The advantages lie in the absence of cell-dissociating enzyme and the ability to isolate undifferentiated hESCs from differentiated cells. This process is ideal for maintaining hESC lines. However, mechanical transfers are laborious and time-consuming, making it difficult to process many cells at a time. The enzymatic transfer method, a faster and simpler method than the

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previous, uses the enzyme collagenase to separate hESCs from STO feeder layer. Once the colonies are isolated from the feeder layer by enzyme treatment followed by gentle pipetting, the colonies are pipetted into small cell clumps for transfer. However, these cell clumps vary in size, and in some cases, both differentiated and undifferentiated cells are transferred. This method is used to increase cell number for experiments that require large quantities of cells. The selection of transfer technique depends ultimately on the experimental purpose.

Expansion of hESCs by Mechanical Transfer

The mechanical transfer method was used for hESC line maintenance. For mechanical transfers, the glass pipettes were thoroughly sterilized and crafted into two different tools (Figs. 1A, 1B). The 9-inch glass Pasteur pipettes were sonicated for 60 minutes, with the water bath replaced three times. The sonicated pipettes were dried at 120°C in the oven for 3 hours and then autoclaved before storage. These sterilized pipettes were crafted into two unique tools, a dissecting pipette (Fig. 1A) and a transfer pipette (Fig. 1B). To make the dissecting pipette, a sterilized pipette was finely drawn out and its tip curved over an ethanol lamp for mechanically dissecting the colonies into small clumps (Fig. 1A). For the transfer pipette, the tip of another pipette was carefully heated and rounded out to prevent the sticky cell clumps from adhering to its edges (Fig. 1B).

One day before hESC transfer, a new transfer dish was prepared. A 0.1% gelatin-coated 35-mm culture dish was seeded with 3×10^5 mitomycin C–treated (Sigma, St. Louis) STO cells (ATCC, Manassas, VA). The STO cell medium was replaced on the day of hESC culture with 2 ml of hESC medium.

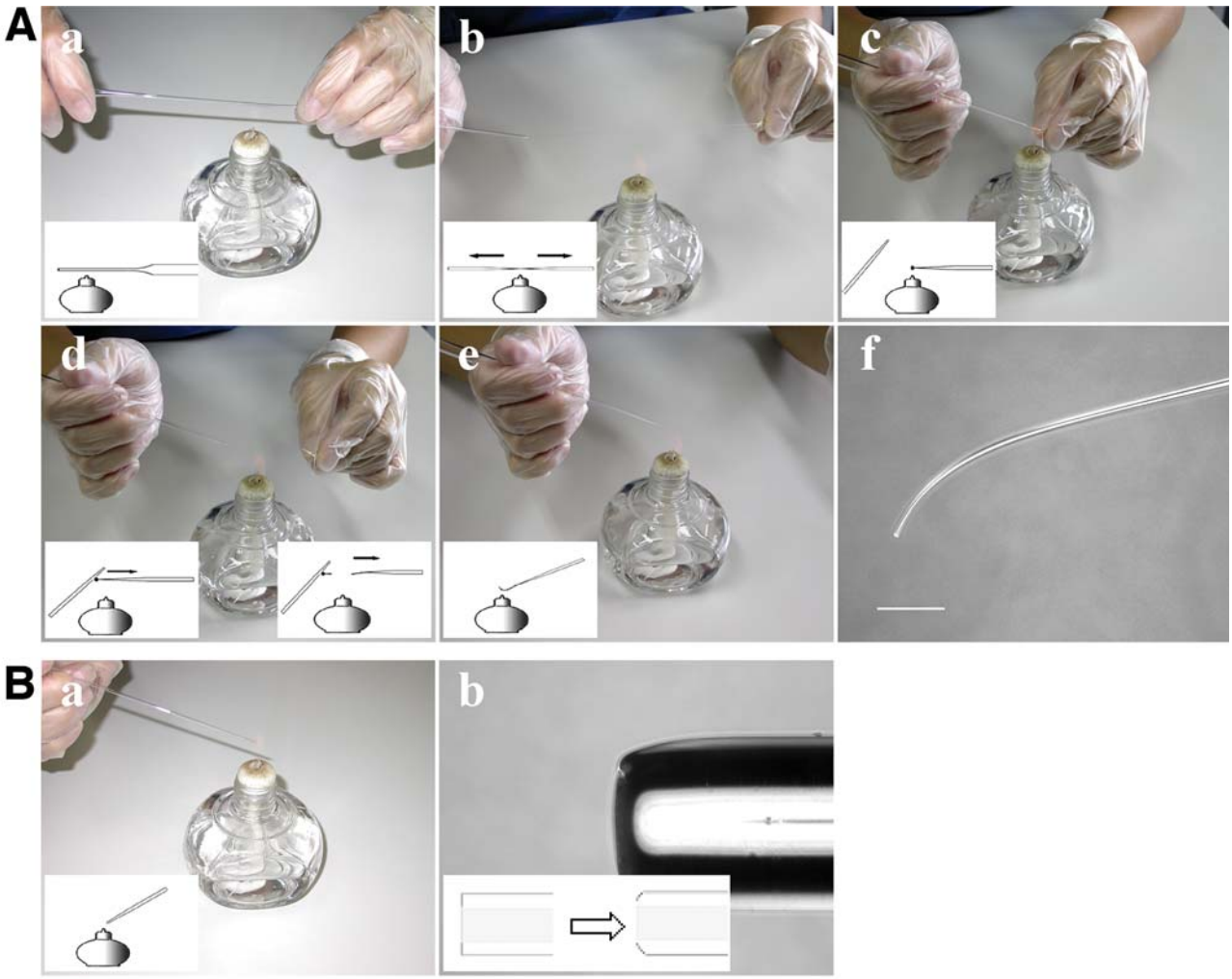

Figure 1. (A): How to make a dissecting pipette. (a): Long and thin section of glass pipette is heated over ethanol lamp. (b): The heated pipette is quickly drawn out. (c): The finely drawn-out edge is quickly heated to form a glass ball. (d): Extra-fine glass pipette is drawn out by gently reattaching the glass ball and drawing the edge out evenly. (e): The edge is slightly bent by heating over top of flame. (f): Completed glass pipette. Insets represent illustrations of the figures. **(B):** How to make a transfer pipette. (a): Glass pipette is heated over top of flame to round out the edge without completely blocking off the pipette. (b): Completed pipette. Scale bar, 500 μm.

For hESC transfer, the surrounding STO feeder layers were carefully moved aside from the colonies with a dissecting pipette (Figs. 2A, 2B). Once the hESC colonies were isolated from the surrounding STO feeder layer (Fig. 2C), they were mechanically divided into small clumps (Fig. 2D). A colony of approximately 1,000 µm in diameter was made into 10 to 15 small clumps (Fig. 2E). The small clumps were transferred to a freshly prepared culture dish using a transfer pipette, and each clump was evenly spaced out on the feeder layer (Fig. 2F). The dish was incubated for 2 days at 37°C and in 5% CO_2 for cells to attach to the culture dish. The transferred hESCs require sufficient time for complete attachment. Earlier exchange of medium could result in the dislodgement of weakly attached hESCs from the dish. Two days after transfer of cells, the attachment of cells was verified under a stereomicroscope. The unattached and dead cells were removed with a micropipette when 1 ml of old medium was replaced with 1 ml of new medium to avoid sudden changes in hESC culture conditions by replacing all the media. The hESC morphology was inspected daily under phase-contrast microscope. On average, the cells were cultured for approximately 5–7 days before passage.

In cases in which there are differentiated cells within hESC colonies (Fig. 3A), a finely drawn-out dissecting pipette was used to remove the STO feeder layer (Figs. 3B, 3C). Then the undifferentiated cells were cut away from differentiated cells as small clumps (Figs. 3D, 3E). The small clumps were gathered and transferred using a transfer pipette to a freshly prepared culture dish and evenly spaced out on the feeder layer. The dish was incubated at 37°C in 5% CO_2 for 2 days to allow cells to adhere to the bottom. The cell morphology was inspected daily and passaged every 5–7 days. However, if many differentiated colonies appeared in the dish, the undifferentiated cells were selected and transferred to a new feeder layer before regular transfer periods.

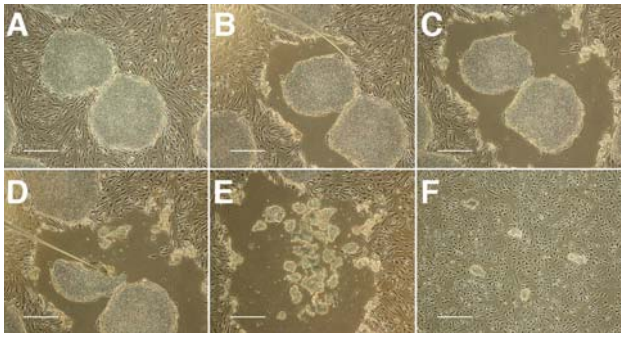

Figure 2. Mechanical transfer of human embryonic stem cells (hESCs) for maintenance. **(A):** At day 6, undifferentiated colonies shown on STO feeder layer. **(B):** The feeder layers pushed away from hESC colonies using the dissecting pipette. **(C):** Complete separation between feeder layer and hESC colonies. **(D):** Dissecting with pipette into small clumps. **(E):** Completely dissected clumps. **(F):** Transfer to new culture dish using the transfer pipette. Scale bar, 500 µm.

Expansion of hESCs by Enzyme Treatment

We used this method for experiments requiring large quantities of cells. A collagenase IV (Gibco-BRL/Invitrogen, Carlsbad, CA) solution was stored in 1-ml (2-mg/ml) aliquots at –70°C. The enzyme was thawed out at 37°C for 30–60 minutes before use.

The hESCs, grown on STO feeder layer in a 0.1% gelatin-coated 35-mm culture dish, were inspected before collagenase treatment, and at times differentiated hESCs were removed with a dissecting pipette. For transfer, hESC culture dish was washed with phosphate-buffered saline once and then treated with 1 ml of prewarmed collagenase at 37°C in 5% CO_2 for approximately 30 minutes (Fig. 4A). The collagenase was removed, and 2 ml of culture medium was added as hESC colonies began to peel

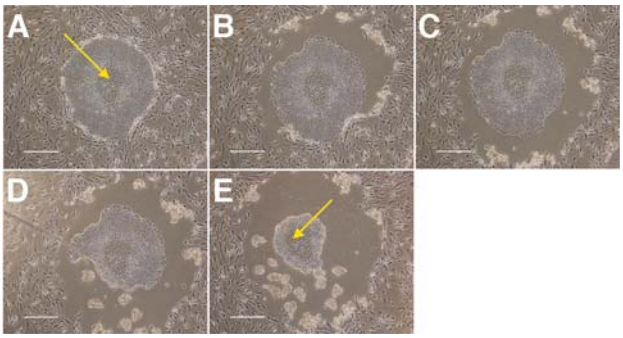

Figure 3. Mechanical separation and transfer of undifferentiated human embryonic stem cells (hESCs) from differentiated cells. Arrows indicate differentiated hESC portion. **(A):** Differentiated cells at day 6, indicated by arrow within hESC colony. **(B):** The feeder layers pushed away from hESC colonies using the dissecting pipette. **(C):** Complete separation between feeder layer and hESC colony. **(D):** Separation of undifferentiated cells from differentiated cells using the dissecting pipette. The undifferentiated cells are dissected into small clumps. **(E):** The differentiated cells remain, and all of the undifferentiated cells are dissected into small clumps. Scale bar, 500 µm.

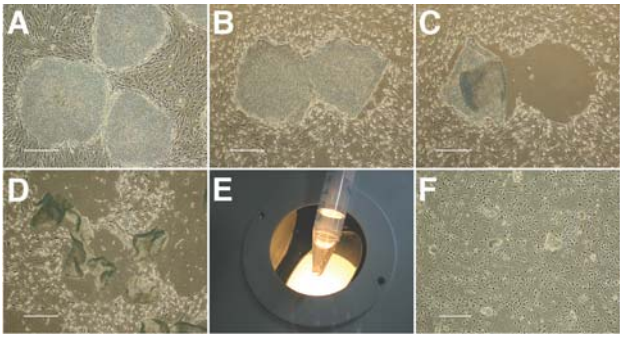

Figure 4. Enzymatic transfer of human embryonic stem cells (hESCs) using collagenase IV. **(A):** Treatment of undifferentiated hESC colonies with collagenase. **(B):** After 30 minutes of enzyme treatment, the cells began to detach around the edges. At this time point, collagenase was removed and new medium was added. **(C):** The colonies lifted off the dish by gently pipetting with a 200-µl micropipette. **(D):** Multiple colonies completely detached from dish. **(E):** The detached hESC colonies were collected in a 15-ml conical tube, allowed to settle to bottom, and pipetted multiple times to make small clumps. **(F):** Small clumps transferred to new culture dish. Scale bar, 500 µm.

away from the surrounding STO feeder layer (Fig. 4B). The hESC colonies were gently pipetted with a 200-µl micropipette to detach from STO feeder layer (Figs. 4C, 4D). The isolated colonies were collected in a 15-ml conical tube with a 200-µl pipette, and medium was added to a final volume of 2 ml (Fig. 4E). The hESC colonies were allowed to settle to the bottom of the tube (~20 seconds). Once the hESC colonies settled, the supernatant containing single cell or STO cells was removed. This process was repeated twice before the colonies were made into small clumps by pipetting approximately five times with a 200-µl micropipette in a small volume of medium. The hESC clumps in 1-ml volume were spaced out evenly on a feeder layer of a 35-mm culture dish containing 1 ml of medium to a final volume of 2 ml (Fig. 4F). The dish was incubated at 37ºC in 5% CO_2 for 2 days to allow small clumps to attach to the dish. Two days after transfer of cells, the attachment of cells was verified under a stereomicroscope. Unattached cells were removed with a micropipette when 1 ml of old medium was replaced with 1 ml of new medium. The hESC morphology was inspected using phase-contrast microscope daily. The cells were cultured for approximately 5–7 days for transfer and were processed for experiments if cell quality and counts were sufficient after several passages.

Conclusions

The proper maintenance of stem cells is an important issue in the study of hESCs. Each laboratory uses different methods for passaging hESCs [1–8, 11–13]. We use both mechanical and enzymatic transfer methods for hESCs grown on feeder layers depending on experimental purpose. In general, we use the mechanical transfer method for maintenance of hESC lines with the least amount of differentiated cells. Two glass tools, the dissecting and transfer pipettes, are used to maintain hESC subcultures by this method. In suboptimal culturing conditions, the hESC lines may differentiate. In such a case, the undifferentiated hESCs are easily dissected from the differentiated portions with the dissecting pipette and transferred to a new culture dish with the transfer pipette. During the early passages of hESCs, this mechanical transfer method is favorable because often the hESCs quite readily differentiate. Furthermore, the mechanical dissection of hESC colonies results in similar cell clump sizes. This is particularly advantageous when creating consistent sizes of embryoid bodies or getting similar sizes of hESC colonies.

The mechanical transfer method, however, is laborious and time-consuming. Because some experiments necessitate larger quantities of hESCs, this method is less than ideal. The enzyme-treated expansion rapidly produces greater amounts of hESCs. However, the cell clumps are different in size, and there is a probability that both the differentiated and undifferentiated cells will be transferred. Thus, the combination of mechanical transfer and enzyme treatment permits excluding the differentiated colonies from passage by manual selection before enzyme treatment. This allows mass production of hESCs with fewer differentiated colonies.

There have been recent reports by two groups [14, 15] regarding the appearances of chromosomal alterations in hESCs. Shortly after this report, it was reported that the use of enzymes in transferring hESCs resulted in cytogenetic aberrations, whereas the use of mechanical transfers maintained a stable karyotype [16]. Mitalipova et al. [17] reported also that hESC aneuploids were detected when using cell-dissociating buffer and/or collagenase/trypsin enzymes to transfer their cell lines.

The experimental purpose that we mention here refers to either the efficient maintenance of undifferentiated stem cells by mechanical transfer or the bulk production of stem cells for various experiments by enzymatic transfer within a limited time frame. We did not compare two methods quantitatively in other aspects such as karyotypic stability. Further investigations are needed to precisely evaluate the advantages and disadvantages of the two methods in other aspects.

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References

1 Thomson JA, Itskovitz-Eldor J, Shapiro SS et al. Embryonic stem cell lines derived from human blastocysts. Science 1998;282:1145–1147.

2 Oh SK, Kim HS, Ahn HJ et al. Derivation and characterization of new human embryonic stem cell lines, SNUhES1, SNUhES2, and SNUhES3. STEM CELLS 2005;23:211–219.

3 Reubinoff BE, Pera MF, Fong CY et al. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 2000;18:399–404.

4 Park JH, Kim SJ, Oh EJ et al. Establishment and maintenance of human embryonic stem cells on STO, a permanently growing cell line. Biol Reprod 2003;69:2007–2014.

5 Cowan CA, Klimanskaya I, McMahon J et al. Derivation of embryonic stem cell lines from human blastocysts. N Engl J Med 2004;350:1353–1356.

6 Suss-Toby E, Gerecht-Nir S, Amit M et al. Derivation of a diploid human embryonic stem cell line from a mononuclear zygote. Hum Reprod 2004;19:670–675.

7 Heins N, Englund MC, Sjoblom C et al. Derivation, characterization, and differentiation of human embryonic stem cells. STEM CELLS 2004;22:367–376.

8 Hwang WS, Ryu YJ, Park JH et al. Evidence of a pluripotent human embryonic stem cell line derived from a cloned blastocyst. Science 2004;303:1669–1674.

9 Lindvall O, Kokaia Z, Martinez-Serrano A. Stem cell therapy for human

neurodegenerative disorders: how to make it work. Nat Med 2004;10: S42–S50.

10 Stojkovic M, Lako M, Strachan T et al. Derivation, growth and applications of human embryonic stem cells. Reproduction 2004;128:159–267.

11 Xu C, Inokuma MS, Denham J et al. Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol 2001;19:971–974.

12 Richards M, Fong CY, Chan WK et al. Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. Nat Biotechnol 2002;20:933–936.

13 Hovatta O, Mikkola M, Gertow K et al. A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells Hum Reprod 2003;18:1404–1409.

14 Inzunza J, Sahlen S, Holmberg K et al. Comparative genomic hybridization and karyotyping of human embryonic stem cells reveal the occurrence of an isodicentric X chromosome after long-term cultivation. Mol Hum Reprod 2004;10:461–466.

15 Draper JS, Smith K, Gokhale P et al. Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. Nat Biotechnol 2004;22:53–54.

16 Buzzard JJ, Gough NM, Crook JM et al. Karyotype of human ES cells during extended culture. Nat Biotechnol 2004;22:381–382.

17 Mitalipova MM, Rao RR, Hoyer DM et al. Preserving the genetic integrity of human embryonic stem cells. Nat Biotechnol 2005;23:19–20.