Passaging Method for Expansion of Undifferentiated Human Embryonic Stem Cells by Pipetting Technique

Sung-Geum Lee[a], Sung-Hwan Moon[a], Soo-Hong Lee, Hey-Jin Lee, Jae-Hwan Kim and Hyung-Min Chung[†]

Stem Cell Research Laboratory, CHA Stem Cell Institute, Pochon CHA University and CHA Biotech Co., Ltd., Seoul 135-081, Korea

ABSTRACT

We have developed a new passaging technique for the expansion of human embryonic stem cells (hESCs) that involves simply pipetting portions of hESCs acquired from colonies, reducing the laborious and time-consuming steps in the expansion of hESCs. Compared to general mechanical methods of passaging, our pipetting method allowed hESCs colonies to be broken into small fragments, which showed significantly higher attachment rates onto feeder cell layers. This technique produced three times the number of hESCs colonies than conventional mechanical methods. In addition, this pipetting method allowed us to distinguish differentiated hESCs from undifferentiated hESCs during hESCs colony pipetting. The hESCs cultured by pipetting method displayed normal human chromosomes for over 60 passages. According to RT-PCR and immunohistochemical analysis, the hESCs successfully maintained their undifferentiated state and pluripotency which was also confirmed by teratoma formation *in vivo*. Therefore, the pipetting method described in this study is a useful tool to efficiently and quickly expand hESCs on a large scale without enzyme treatment.

(Key words : Human embryonic stem cells, Cell expansion, Pipetting technique)

INTRODUCTION

Human embryonic stem cells (hESCs) exhibit pluripotency, the ability to differentiate into any type of cell through three germ layers (ectoderm, endoderm and mesoderm). Since Thomson *et al.*, successfully achieved hESCs culture on mouse embryonic fibroblasts in 1998 (Thomson *et al.*, 1998), the possibility of cell therapy using hESCs has stimulated world-wide interest. Not only can hESCs differentiate into specific cell types depending on their external environment, they also proliferate with high self-renewal activity. Recently, many studies have focused on the differentiation and proliferation of hESCs stimulated by environmental conditions such as the presence of cytokines and three-dimensional cultures (Gerecht-Nir *et al.*, 2004a,b; Ramirez-Bergeron *et al.*, 2004; Xu *et al.*, 2005; Stewart *et al.*, 2006).

Even though hESCs culture can be accomplished, cell expansion on a large scale is required to apply hESCs to clinical applications. In previous studies, hESCs have been transferred in the presence of enzymes such as collagenase, trypsin, and dispase (Xu *et al.*, 2001; Richards *et al.*, 2002; Hovatta *et al.*, 2003). This enzyme treatment allows effective expansion of hESCs, however, there is a possibility that hESCs will develop abnormal chromosomes due to consecutive exposures to enzyme after several passages. Therefore, a novel culture method is needed to acquire sufficient hESCs with high purity for clinical applications.

Recently, a mechanical isolation technique has been developed to culture hESCs without enzyme treatment, in which hESCs colonies were scratched by glass pipettes and then separated from feeder layers (Heins *et al.*, 2004; Oh *et al.*, 2005a,b). Subsequently, mechanically isolated cells were effectively sub-cultured on the feeder cells for successive passages. This technique has an advantage in not using enzymes during passaging but it is a lengthy process involving precise mechanical cutting done step by step under a microscope (Oh *et al.*, 2005b). Alexis *et al.*, developed an automated technique that utilizes a tissue chopper with a razor blade (Joannides *et al.*, 2006). However, the automated technique still required extended amounts of time as well as expensive machinery that may produce contaminations. In this study, pipetting technique allowed us to expand hESCs effectively without using enzyme treatment compared to general mechanical technique. Furthermore, simply pipetting hESCs facilitated passaging step so that we were able to reduce laborious and time-consuming

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[a] These authors equally contributed for this work.

[†] Corresponding author : Phone: +82-2-3468-3391, E-mail: stem_chung@naver.com

process. Therefore, this pipetting method will be a useful tool to expand hESCs on a large scale in relatively short period.

MATERIALS AND METHODS

hESCs Culture

The hESCs line CHA-3 were cultured as described in a previous study (Ahn *et al.*, 2006). Mitomycin C- (Sigma, St. Louis, MO) treated STO feeder cells (mouse embryonic fibroblasts; ATCC, Manassas, VA, USA,) were seeded on 0.1% gelatin-coated 35mm tissue-culture dishes. The following day, the STO cell medium was replaced with 2.5 ml of Dulbecco's Eagle's medium DMEM /F12 supplemented with 20% knockout serum replacement, 4 ng/ml human basic fibroblast growth factor, 1 mM L-glutamine, 1% nonessential amino acid, 1% penicillin-streptomycin, 0.1 mM β-mercaptoethanol (Gibco BRL, Gaithersburg, MD). For expansion of the STO cells, DMEM (high glucose with L-glutamine; Gibco) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 110 mg/l sodium pyruvate (Gibco), 1% nonessential amino acid, 1% penicillin-streptomycin, and 0.1 mM β-mercaptoethanol was used. hESCs cultured for 7 days were seeded on top of the prepared STO feeder layer, allowed to attach, and further cultured to allow colony formation.

Expansion of Human Embryonic Stem Cells by Pipetting Method

For hESCs transfer, the surrounding STO feeder layers were carefully scrubbed away from the hESCs colonies with a dissecting pipette (Fig. 1A). Then each isolated hESCs colony was left to spontaneously assemble (Fig. 1B). Several colonies were collected (Fig. 1C) and then fragmented by mixing gently using a micropipette 8~10 times. Small fragments produced by the procedure were seeded on fresh feeder layers for expansion (Fig. 1D). The hESCs colony fragments successfully attached and grew on the feeder layer after 48 hr (Fig. 1E and 1F), and the resulting hESCs were cultivated for 7 days and transferred onto a new feeder layer. The culture media was refreshed each day during hESCs culture.

Reverse Transcription-Polymerase Chain Reaction

The hESCs colonies were harvested by mechanical isolation and then washed twice with PBS (Gibco). Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. cDNA was synthesized from 1 μ g of RNA using Maxime RT PreMix Kit (iNtRON BIOTECHNOLOGY, Sungnam, Kyungki-Do, Korea). PCR products were separated on a 1.5% agarose gel. The following primer

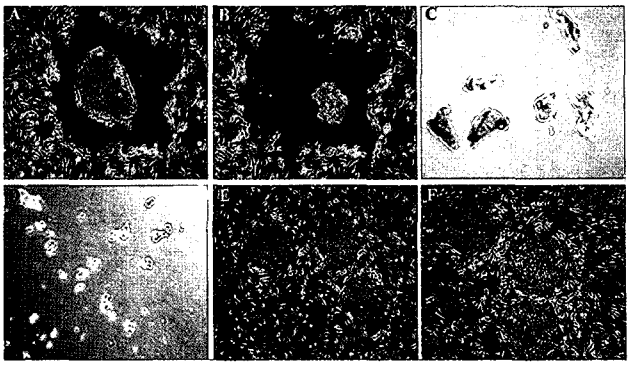

Fig. 1. The maintenance and passage of human embryonic stem cells (hESCs) by pipetting method. (A) hESCs colony surrounded by the feeder layers, which was pushed away from hESCs colonies using the dissecting pipette. (B) hESCs clump formed from the hESCs colony. hESCs were isolated from the surrounding STO feeder layer. (C) Several hESCs clumps transferred into a petri dish. (D) hESCs fragments produced from the hESCs clumps by pipetting method. (E) hESCs attached on the new feeder layer 2 days after pipetting method. (F) New hESCs colonies grown on the STO feeder for 4 days (×40).

sets were used: GAPDH (forward: 5'-AGCCACATCGCTCAGACACC-3', reverse: 5'-GTACTCAGCGGCCAGCATCG-3'), 55℃, 32 cycles; Oct-4 (forward: 5'-CGTGAAGCTGGAGAAGGAGAAGCTG-3', reverse: 5'-CAAGGGCCGCAGCTTACACATGTTC-3'), 55℃, 30 cycles; The procedure included denaturing, which was completed at 95℃ for 30 sec, an annealing step (at 55℃) for 45 sec, and an amplification at 72℃ for 60 sec (Kaufman *et al.*, 2001; Oh *et al.*, 2005a).

Immunocytochemistry Analysis

hESCs colonies were fixed with 4% fresh para-formaldehyde for 15 min at room temperature then washed three times with PBS. Fixed hESCs was blocked for 1 hr with 10% goat serum solution dissolved in 0.1% Triton X-100 and then incubated overnight at 4℃ with primary antibodies to markers such as Oct-4, Tra-1-60 (Chmicon, Temecula, CA) in a 2% goat serum.. Cells were washed with PBS three times, secondary antibodies were applied for 1 hr at 37℃. After washing with PBS three times, the slides were mounted with a glycerol-based mounting solution containing 4-diazabicyclo octane with 4,6-diamidino-2-phenylindole (DAPI; Sigma).

Karyotype Analysis

Chromosome analysis was performed according to standard methods with minor modifications (Dutrillaux and Viegas-Pequignot, 1981; Buzzard *et al.*, 2004). After 3 days of culture, hESCs was incubated with 100 μ l colcemide (Gibco) for 3 hr at 37℃ in a 5% CO_2 environment and then trypsinized. After hypotonic solution (1 % citrate buffer) treatment, the lysed cells were fixed in a mixture of methanol and glacial acetic acid (3:1). G-

banding was performed for identification of chromosomes.

Statistical Analysis

Ten hESCs colonies were used to compare the mechanical cutting and pipetting methods. After 2 days of hESCs post-seeding, the new colonies produced from the original 10 colonies were counted. Statistical analysis was carried out using GraphPad Prism 3.03 (Graphpad Software, San Diego, CA, http://www/graphpad.com) (Joannides *et al*, 2006).

Teratoma Formation in SCID Mice

The hESCs were maintained and harvested as described above. A suspension of 4×10^6 cells were injected into the gastrocnemius muscle of the hind limb of 6 week-old male SCID mice using a 28-gauge needle. After 12 weeks, palpable tumors were found in all mice. Dissected tumors were fixed with 10% NBF (Neutral buffer formalin), dehydrated and then embedded in paraffin. Sections were cut with an interval of 5 μ m and examined by histological staining, including hematoxyline and eosin (H&E, Dako, Glostrup, Denmark), DAPI (Sigma), Masson's trichrome (Dako), alcian blue (Dako) PAS (Periodic acid Schiff, Dako).

RESULTS

The pipetting method for hESCs passaging developed in this study not only excludes the usage of enzyme in enzymatic method but also reduces time-consuming steps in currently used mechanical methods. In addition, this method is a simple technique for mechanically isolating hESCs colonies and for efficiently and quickly expanding hESCs on a large scale. Fig. 1 shows the hESCs colonies formed by novel pipetting method. First, based on the general mechanical method, hESCs were isolated by scrubbing away the feeder layer using a dissecting pipette (Fig. 1A). The isolated hESCs colonies aggregated spontaneously, (Fig. 1B) and the hESCs clumps were transferred into a new culture dish and then pipetted gently about 15 times. At this step, differentiated hESCs clumps are hardly broken into fragments, whereas undifferentiated hESCs colonies are readily broken. This technique might be used as a simple procedure to distinguish undifferentiated hESCs from differentiated ones. Undifferentiated hESCs were exclusively collected in this experiment. Next, broken fragments were seeded on prepared feeder layers for extended culture (Fig. 1D), and they proved able to successfully generate new colonies (Fig. 1E and 1F). Attachment rates of isolated fragments were highest when the temperature of the culture dish was 40℃ (data not shown). When 20 colonies were seeded on a 6 cm diameter dish, 180~200 colonies were obtained after just one passage step.

To explore whether this pipetting method could be useful for expansion of hESCs, the number of colonies obtained by general mechanical methods and our pipetting method were counted and compared. The hESCs fragments isolated by the pipetting method showed strikingly higher attachment rates on the feeder layer compared to those separated mechanically. When 10 colonies were used for transfer, 29.6±7.0 colonies were obtained by the mechanical method. On the other hand, our pipetting method produced 101.9±10.2 colonies under the same conditions (Fig. 2A). This demonstrates that the pipetting method can produce higher numbers of hESCs colonies every passaging step compared to general mechanical methods. However, there might be slight deviations in acquiring comparable numbers of colonies, depending upon variations in pipetting times and pipetting power. When comparing the number of cells per each colony produced by both methods, there was no significant difference in net cell number between the two methods (Fig. 2).

Cytogenic analysis revealed that hESCs passaged by the pipetting method showed normal chromosomes (Fig. 3A), demonstrating that this method is also a useful tool to maintain stable karyotype of hESCs. The pipetting method allowed hESCs to be cultured continuously without any change in morphology and karyotype so that hESCs after 60 passages also showed undifferentiated morphology and normal chromosomes over extended periods (Fig. 3A).

The undifferentiated state of hESCs was also investigated by RT-PCR and immnocytochemical staining with specific markers. According to RT-PCR analysis, hESCs cultured for 40 and 60 passages by the pipetting method strongly expressed the undifferentiated marker Oct-4 with the same intensity (Fig. 3B). The immunocytochemical analysis revealed that the hESCs showed positive expression for Oct-4, Tra-1-60, confirming the hESCs also maintain pluripotency (Fig. 3C). Furthermore, we also sought to observe teratoma formation *in vivo* by hESCs. The hESCs passaged by pittpeting method were

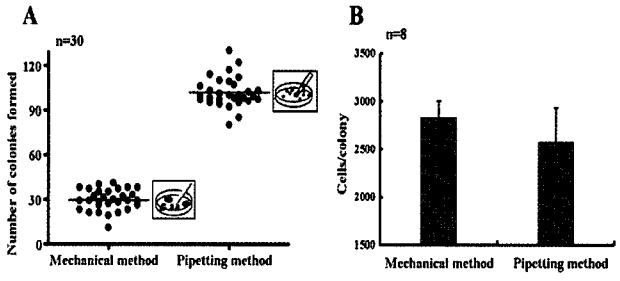

Fig. 2. Comparison of mechanical method and pipetting method. (A) The number of hESCs colonies produced after one passaging step. The greatest difference in the colony numbers between the two methods was due to attachment rates. (B) Cell numbers of hESCs per colony cultured for 7 days after passaging (p= 0.073).

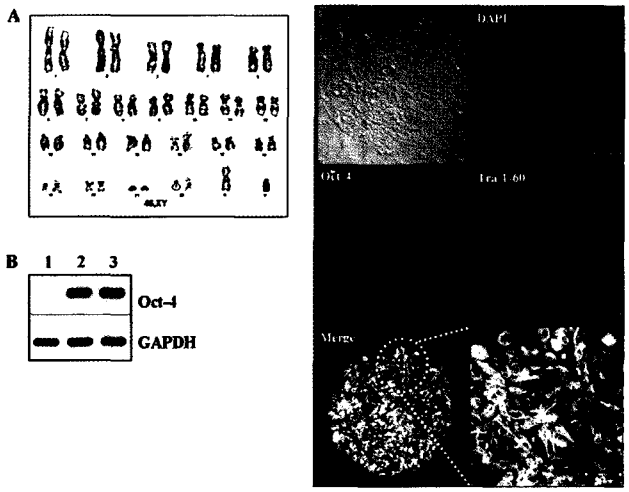

Fig. 3. Characterization of hESCs passaged by pipetting method. (A) Karyotypes of hESCs cultured for 60 passages showing normal human chromosomes. (B) The strong Oct-4 expression of the hESCs by RT-PCR (lane 1 represents STO feeder cells as a negative control, lanes 2 and 3 represent hESCs after 40 passages and 60 passages, respectively). (C) Immunocytochemical staining of the hESCs passaged by pipetting method (hESC showed undifferentiated markers of Oct-4 and Tra-1-60 in nuclei and cytoplasm, respectively. Merge image confirmed each of undifferentiated markers were expressed at their specific location)

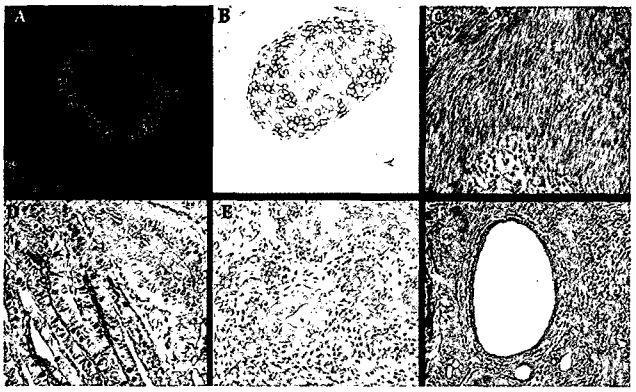

Fig. 4. Teratoma formation after injection of hESCs into SCID mice. The teratoma were confirmed by staining with several stains such as hematoxylin and eosin, masson's, alcian blue, and PAS. (A) Human nuclei (DAPI and mouse anti-human nuclei stain; ×200) (B) Cartilage tissue (mesoderm, alcian blue stain; ×100). (C) Muscle fibers tissue (mesoderm, masson's stain; ×200). (D) Epithelium tissue (ectoderm, PAS stain; ×200). (E) Neural rosette structures (ectoderm, H&E stain; ×200). (F) Gut epithelium tissue (endoderm, masson's stain; ×100).

injected into SCID mouse that were sacrificed after 12 weeks. Teratoma were found where hESCs were injected and they successfully developed into three germ layers, namely, endoderm (gut epithelium), mesoderm (cartilage, muscle fiber) and ectoderm (epithelium, neuronal rosette) (Fig. 4).

DISCUSSION

It is necessary to expand hESCs by passaging steps for cell studies and therapeutic cell applications. In spite of this requirement, only two techniques (enzyme treatment and mechanical cutting) have been widely used. Enzyme treatment uses enzymes such as collagenase and trypsin to detach hESCs from feeder cells. This technique has the great advantage of bulk expansion in relatively short time. However, there are some drawbacks on this technique, which not only include differentiated hESCs colonies, but also increase incidences of chromosomal alterations after repeated enzyme treatments (Buzzard *et al.*, 2004; Draper *et al.*, 2004; Inzunza *et al.*, 2004). On the other hand, the mechanical cutting technique isolates hESCs mechanically from feeder layers by dissecting pipette without enzyme treatment. This technique has the advantage of not using enzymes so that hESCs are able to maintain their undifferentiated state and stable karyotype even after many repeated passage steps. However, this method requires laborious and time-consuming steps to expand hESCs. Joannides *et. al* reported automated mechanical passaging to reduce labor and time spent on mechanical cutting. They utilized a McIlwain tissue chopper that automatically cuts hESCs colonies into relatively uniform sized clumps. However, this technique requires automatic equipment that can be expensive and readily contaminated.

The pipetting technique developed in this study is very simple and easy to implement for the transfer and expansion of hESCs without enzyme treatment or expensive machinery. Even though the pipetting technique failed to make colony sizes more uniform compared to the mechanical technique, it allows hESCs segments to attach to feeder layers effectively, resulting in more colonies (see Fig. 2). According to our results, the pipetting technique produced more than three times as many colonies as the mechanical technique every passage step. This demonstrates that our pipetting technique can be of great use in the simple expansion of hESCs on a large scale without enzyme treatment.

This pipetting technique can also be used to distinguish between differentiated and undifferentiated hESCs. Most already differentiated hESCs were intensively aggregated and were not able to be separated by even repeated and strong pipetting. Undifferentiated hESCs secrete very small amounts of extracellular matrix (ECM) proteins, which provide anchorage and support for cells. However, hESCs secrete more amount of ECM as they are differentiated (Kuri-Harcuch *et al.*, 1984; El-Sabban *et al.*, 2003).

In addition, the pipetting technique is safer and more stable because it might expose cells to fewer contaminants that might be contacted during additional steps necessary for enzyme treatment, mechanical cutting and

automatic machinery. According to our results, hESCs passaged by our pipetting technique maintained normal chromosomes and morphologies for over 60 passages and formed teratomas consisting of three germ layers *in vivo*, confirming pluripotency. Thus, we strongly believe that this pipetting technique will allow us to easily expand hESCs on a large scale for clinical application.

REFERENCES

1. Ahn SE (2006): Primary bone-derived cells induce osteogenic differentiation without exogenous factors in human embryonic stem cells. Biochem Biophys Res Commun 340:403-408.
2. Buzzard JJ, Gough NM, Crook JM, Colman A (2004): Karyotype of human ES cells during extended culture. Nat Biotechnol 22:381-382; author reply 382.
3. Draper JS (2004): Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. Nat Biotechnol 22:53-54.
4. Dutrillaux B, Viegas-Pequignot E (1981): High resolution R- and G-banding on the same preparation. Hum Genet 57:93-95.
5. El-Sabban ME (2003): ECM-induced gap junctional communication enhances mammary epithelial cell differentiation. J Cell Sci 116:3531-3541.
6. Gerecht-Nir S, Cohen S, Itskovitz-Eldor J (2004a): Bioreactor cultivation enhances the efficiency of human embryoid body (hEB) formation and differentiation. Biotechnol Bioeng 86:493-502.
7. Gerecht-Nir S, Cohen S, Ziskind A, Itskovitz-Eldor J (2004b): Three-dimensional porous alginate scaffolds provide a conducive environment for generation of well-vascularized embryoid bodies from human embryonic stem cells. Biotechnol Bioeng 88:313-320.
8. Heins N (2004): Derivation, characterization, and differentiation of human embryonic stem cells. Stem Cells 22:367-376.
9. Hovatta O (2003): A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells. Hum Reprod 18:1404-1409.
10. Inzunza J (2004): Comparative genomic hybridization and karyotyping of human embryonic stem cells reveals the occurrence of an isodicentric X chromosome after long-term cultivation. Mol Hum Reprod 10:461-466.
11. Joannides A (2006): Automated mechanical passaging: a novel and efficient method for human embryonic stem cell expansion. Stem Cells 24:230-235.
12. Kaufman DS, Hanson ET, Lewis RL, Auerbach R, Thomson JA (2001): Hematopoietic colony-forming cells derived from human embryonic stem cells. Proc Natl Acad Sci USA 98:10716-10721.
13. Kuri-Harcuch W, Arguello C, Marsch-Moreno M (1984): Extracellular matrix production by mouse 3T3-F442A cells during adipose differentiation in culture. Differentiation 28:173-178.
14. Oh SK (2005a): Derivation and characterization of new human embryonic stem cell lines: SNUhES1, SNUhES2, and SNUhES3. Stem Cells 23:211-219.
15. Oh SK (2005b): Methods for expansion of human embryonic stem cells. Stem Cells 23:605-609.
16. Ramirez-Bergeron DL (2004): Hypoxia affects mesoderm and enhances hemangioblast specification during early development. Development 131:4623-4634.
17. Richards M, Fong CY, Chan WK, Wong PC, Bongso A (2002): Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. Nat Biotechnol 20:933-936.
18. Stewart R, Stojkovic M, Lako M (2006): Mechanisms of self-renewal in human embryonic stem cells. Eur J Cancer 42:1257-1272.
19. Thomson JA (1998): Embryonic stem cell lines derived from human blastocysts. Science 282:1145-1147.
20. Xu C (2001): Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol 19:971-974.
21. Xu RH (2005): Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nat Methods 2:185-190.

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