

양수 세포를 이용한 인간배아줄기세포의 배양

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Human Amniotic Fluid Cells Support Expansion Culture of Human Embryonic Stem Cells

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Objective: This study was performed to evaluate the possibility of prolonged culture of human embryonic stem cells (hESC; SNUhES2) on human amniotic fluid cells (hAFC), which had been stored after karyotyping.

Method: The hAFC was prepared for feeder layer in the presence of Chang's medium and STO medium (90% DMEM, 10% FBS) at 37 in a 5% CO₂ in air atmosphere. Prior to use as a feeder layer, hAFC was mitotically inactivated by mitomycin C. The hESCs on hAFC were passaged mechanically every seven days with ES culture medium (80% DMEM/F12, 20% SR, bFGF).

Results: The hAFC feeder layer support the growth of undifferentiated state of SNUhES2 for at least 59 passages thus far. SNUhES2 colonies on hAFC feeder appeared slightly angular and flatter shape as compared with circular and thicker colonies observed with STO feeder layer and showed higher level with complete undifferentiation in seven days. Like hESC cultured on STO feeders, SNUhES2 grown on hAFC expressed normal karyotype, positive for alkaline phosphatase activity, high telomerase activity, Oct-4, SSEA-3, SSEA-4, Tra-1-60 and Tra-1-81 and formed embryoid bodies (EBs).

Conclusion: The hAFC supports undifferentiated growth of hESC. Therefore, these results may help to provide a clinically practicable method for expansion of hESC for cell therapies.

Key Words: Human embryonic stem cells (hESC), Human amniotic fluid cells (hAFC), Feeder layer, Undifferentiation, Expansion of hESC

(human embryonic stem cells, ,
hESC) (inner cell (karyotype)
mass, ICM) 가 , ,

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(cell therapy)¹⁻³ (fetal abnormalities)
 (prenatal genetic diagnosis) 가
 11,12

(mouse embryonic fibroblast, MEF)
 (feeder layer) factor-4 (Oct-4)가 octamer-binding transcription
 가¹² 가¹³

(coculture) 가
 RNA (retrovirus)
 가
 4 (mouse embryonic stem cell, mESC)

gelatin leukemia 1.
 mia inhibitory factor (LIF) 가
 5 LIF가 (IVF-ET)
 10

6 (SNUhES2)
 Xu⁷ extracellular matrix Matrigel
 (conditioned media) 가

2.
 1)
 10 ml (1000 rpm, 8)
 가 Chang's (Irvine scientific,
 Santa Anna, CA, USA) 1 ml 가
 muscle) (fetal skin) (fetal 35 mm
 (adult fallopian tuba) (epithelial cell) 0.5 ml
 37 , 5% CO₂

, Amit⁴ Hovatta⁹ (foreskin) 2 ml 가
 , Cheng¹⁰ (adult bone ma-
 row) 4~5
 0.25% trypsin-EDTA
 (human amniotic fluid cells, hAFC)
 (genetic alteration)

가 70~80% bFGF

0.2

Ma-

150 μ m 35 mm

mitomycin C (0.01 mg/ml)

Chang's 가

10% Fetal Bovine Serum 3)

(HyClone, Utah, USA), penicillin streptomycin

Dulbecco's Modified Eagle Medium (DMEM, GIBCO) 가

2) (cell surface marker)

AP, SSEA-1, 3 4 Tra 1-60, 1-81

STO (ATCC, USA) Oct-4 telomerase PCR

71 Oh

(SNUhES2) 14

alkaline phosphatase (AP) 4)

Oct-4, stage-specific embryonic antigen-3, 4 (SSEA-3, 4) 500 4~

(46,XX) 5 ml (DMEM/F12, 20% SR, 1% non-essential amino acid, 0.1 M β -mercaptoethanol, 0.5% penicilline/streptomycin)가

14

(1) 0.1% gelatin

mitomycin C (three germ layer) (derivatives)

mitomycin C

(colony) glass

knife 100~200 가

7

DMEM/F12 (GIBCO)

, 20% serum replacement (SR, GIBCO),

0.4 ng/ml basic fibroblast growth factor (bFGF, Invitrogen), 1% non-essential amino acid, 0.1 mM β -mercaptoethanol, 0.5% penicilline/streptomycin 가

(2)

Mitomycin C (2 \times 10⁶ cells) 25

cm² flask

FBS가 DMEM

(endoderm) amylase albumin,

(mesoderm) cartilage matrix protein

(CMP) enolase (ectoderm)

neurofilament heavy chain (NFH) keratin

RT-PCR primer

sequences PCR Amylase (forward: 5'-GCTGGGCTCAGTATTCCCAAATAC-3', reverse: 5'-GACGACAATCTCTG-3'), albumin (forward: 5'-CCTTTGGCACAATGAAGTGGGTAACC-3', reverse: 5'-GACGACAATCTCTGACCTGAGTAGC-3'), CMP (forward: 5'-ATGACTGTGAGCAGGTGTGTCATCAG-3', reverse: 5'-CTGGTTGATGGTCTTGAAGTCAGCC-3'), enolase (forward: 5'-TGA CTTCAGTCGCCTGATG-ATCCC-3', reverse: 5'-TGCGTCCAGCAAAGATTGC-

CTTGTC-3'), NFH (forward: 5'-TGAACACAGACGC-TATGCGCTCAG-3', reverse: 5'-CACCTTTATGTGA-GTGGACACAGAG-3'), keratin (forward: 5'-AGGAA-ATCATCTCAGGAGGAAGGGC-3', reverse: 5'ATCT-CAGGAGGAAGGGC-3', reverse: 5'-AAAGCACAGA-TCTTCGGGAGCTACC-3'). PCR primer 94
 30 , 68 30 primer
 72 30
 30 PCR 2% agarose gel

12 ,
 (over-
 growth) mitomycin C
 mitomycin C
 mitomycin C
 가 , 가 가
 mitomycin C
 mitomycin C
 (prolife-
 ration) 가

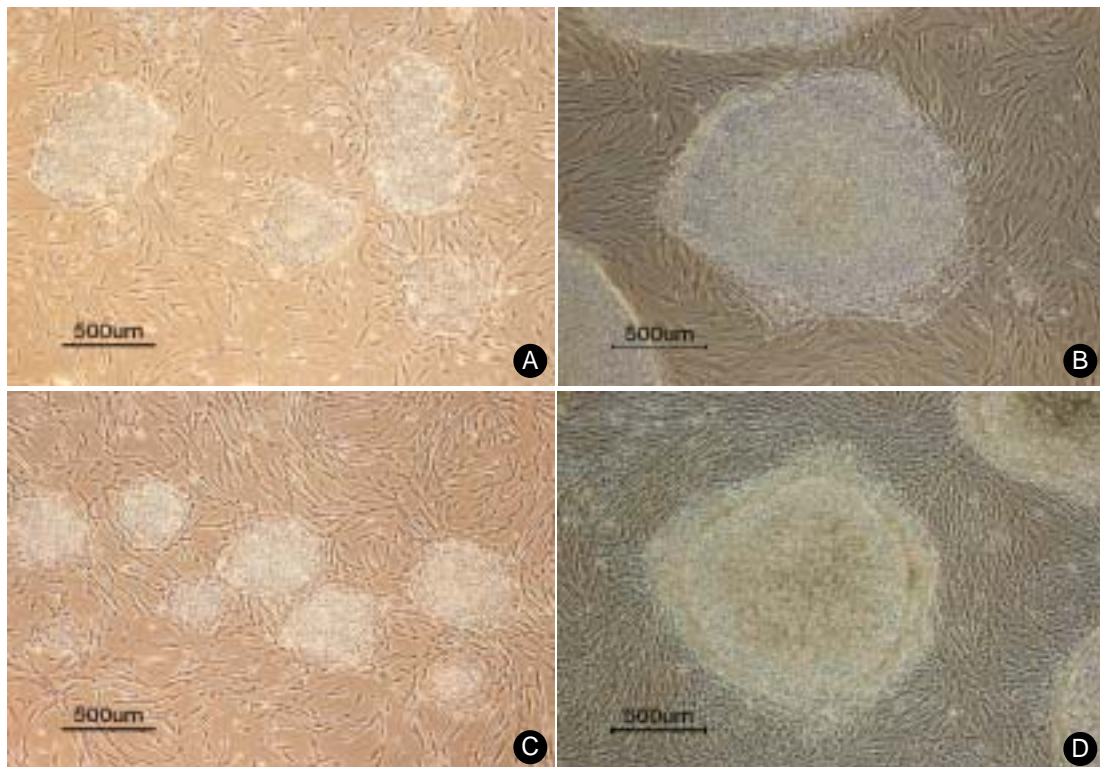


Figure 1. Morphology of SNUhES2 cell line grown on hAF feeder layer. (A) SNUhES2 P71-5 cell colony at day 2 on hAFC treated with mitomycin C (B) SNUhES2 P71-5 cell colony at day 7 on hAFC treated with mitomycin C (C) SNUhES2 P71-5 cell colony at day 2 on hAFC non-treated with mitomycin C (D) SNUhES2 P71-5 cell colony at day 7 on hAFC non-treated with mitomycin C

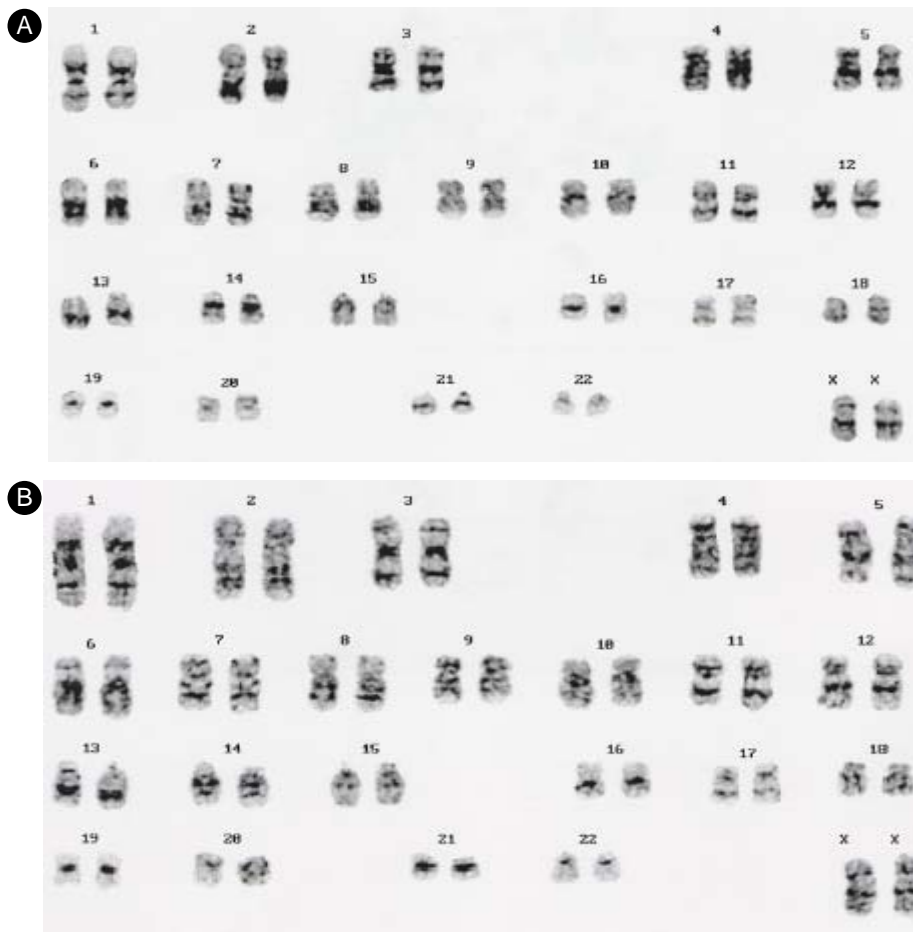


Figure 2. Results of karyotyping. (A) SNUhES2 P71-25 on hAFC treated with mitomycin C: 46,XX (B) SNUhES2 P71-25 on hAFC non-treated with mitomycin C: 46,XX

STO (Figure 2A) (46,XX) (Figure 2B)

mitomycin C (Figure 1A, B) mitomycin C (Figure 1C, D)

AP (Figure 3A, G) SSEA-4 (Figure 3F, L), Tra-1-60 (Figure 3B, H), Tra-1-81 (Figure 3C, I)가 SSEA-1 (Figure 3D, J) SSEA-3 (Figure 3E, K)

Oct-4 (Figure 4A) Oct-4 (Figure 4B) telomerase (Figure 5A, B) (Figure 5C)

가 가 가 가

Mitomycin C

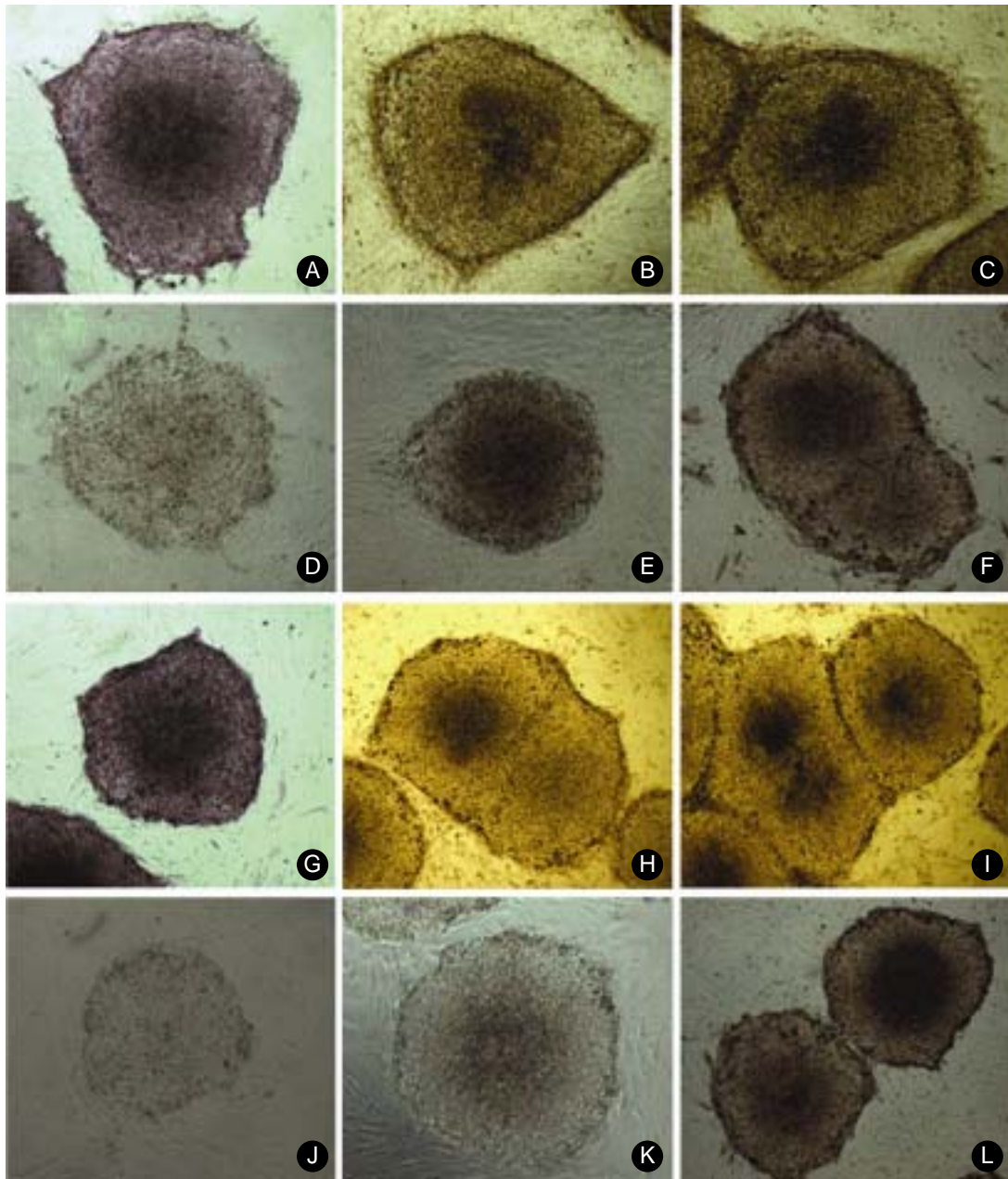


Figure 3. Immunocytochemical staining results of cell surface markers for detecting undifferentiation state of hESC grown on hAFC; (A)~(F) SNUhES2 P71-25 on hAFC treated with mitomycin C (G)~(L) SNUhES2 P71-25 on hAFC non-treated with mitomycin C (A, G) AP (+) (B, H) Tra-1-60 (+) (C, I) Tra-1-81 (+) (D, J) SSEA-1 (-) (E, K) SSEA-3 (+) (F, L) SSEA-4 (+).

mitomycin C
Matrigel

4~5

(Figure 6).

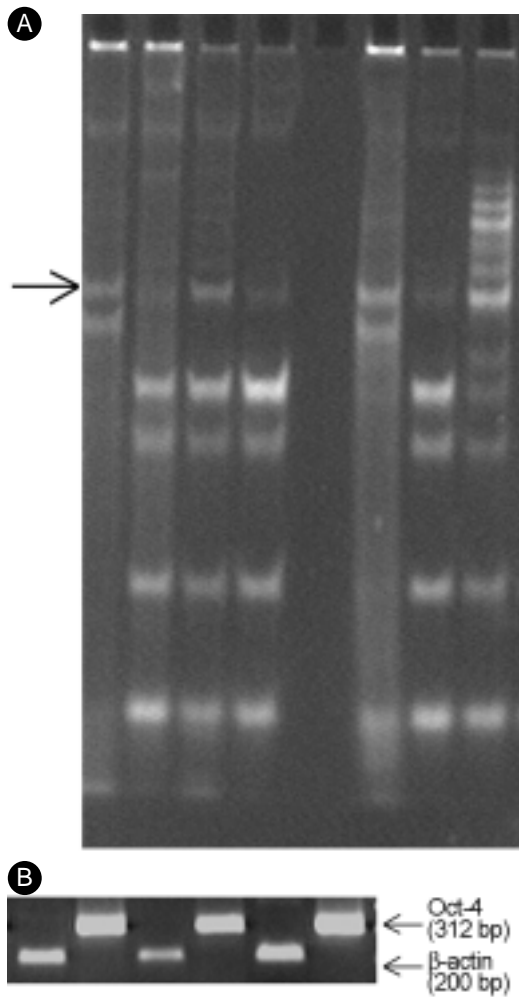


Figure 4. Detection of telomerase activity and Oct-4.
(A) telomerase activity.
 Lane 1: SNUhES2 cells grown on hAFC treated with mitomycin C, Lane 2: Heat inactivated control of lane 1 sample, Lane 3: SNUhES2 cells on hAFC non-treated with mitomycin C, Lane 4: Heat inactivated control of lane 3 sample, Lane 6: hAFC, Lane 7: Heat inactivated control of lane 6, Lane 8: Positive control
(B) Oct-4 expression of SNUhES2 grown on hAFC.
 Lane 1, 3, 5: beta- actin of lane 2, 4 and 6, respectively, Lane 2: SNUhES2 cells grown on hAFC treated with mitomycin C, Lane 4: SNUhES2 cells on hAFC non-

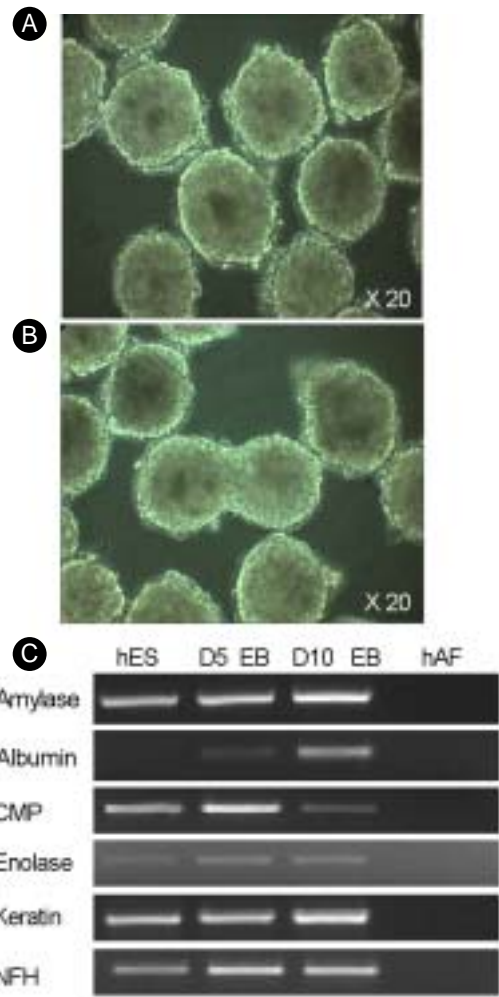


Figure 5. Morphology and gene expression in embryoid bodies. **(A)** EBs formed with SNUhES2 P71-5 cells at day 7 on hAFC treated with mitomycin C **(B)** EBs formed with SNUhES2 P71-5 cells at day 7 on hAFC non-treated with mitomycin C **(C)** RT-PCR results for detection of three germ layer markers: Endoderm (amylase and albumin), mesoderm (CMP and enolase) and ectoderm (keratin and NFH). Lane 1 is undifferentiated hESC grown on hAFC treated with mitomycin C, lane 2 is EB for day 5, lane 3 is EB for day 10 and lane 4 is hAFC as negative control.

가
 가
 가
 가 (fetal bovine serum, FBS) 가

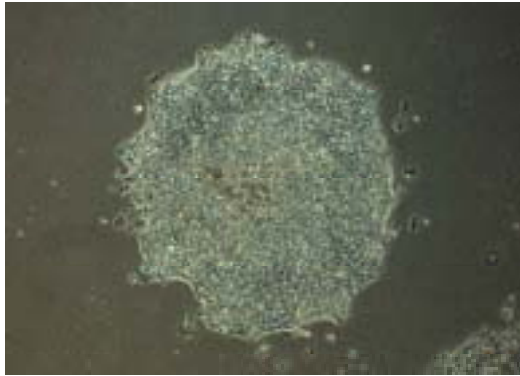


Figure 6. Morphology of colony of SNUhES2 cell on day 7 grown on feeder free condition (magnification:

12
RNA
가
4
59
Mitomycin C
(primate)
16,17
tomycin C
AP, SSEA-4, TRA-1-60, TRA-1-81
, SSEA-3가
, SSEA-1
(suspension)
가
mitomycin C

30
10
가
가
serum-free
bFGF가 가 serum replacement
(SR)
SR 가
1998 Thomson 1
가 2000 Xu 7
extracellular matrix
(soluble factor)가
onectin matrigel laminin, collagen , fibr-
trix extracellular ma-
가
가
Cheng 10
mitomycin
C
, mitomycin C
가
mitomycin C
mitom-

ycin C 가
 Cheng
 mitomycin C SSEA-4 TRA-1-60
 TRA-1-81
 flow cytometer , early passage
 late passage (gene expression)
 mitomycin C microarray 가
 가 mitomycin C
 (cytotoxicity)가 가
 (carcinogen) 가
 가 Amit ²² fibronectin
 mito- matirx , SR, transforming growth factor β1
 mycin C (TGFβ1), LIF bFGF
 mitomycin C 가
 가 Amit ⁴ 가
 가 2 SR 가
 가 3
 가 3 (embryonic) (fe-
 tal)
 가 Hovatta ⁹ ^{23,24}
 Richards ¹⁹ 가 11 ¹³
 Richards ⁸
 가 mesenchymal
 (transition)
 가 50 가
 , 90% (embryonic stem cell, ESC),
 80% (embryonic germ cell, EGC)
 , Carpenter ²⁰ Rosler ²¹ (embryonic carcinoma cell, ECC)
 Xu ⁷ Oct-4가
 Thomson ¹ , ^{25,26} telom-

erase 가 27 AP, SSEA-4, TRA-1-60 TRA-1-81
, telomerase 가
merase (Figure 4A). Oct-4 telo- 13
가 (in vivo)
가 , , 가 가
, , 가
extracellular matrix 가 proteomics
(factor) 6 proteomics
2000 cytokine (growth fa-
ctor) (factor)
가
bFGF bone morphogenic protein 4 (BMP4)

1. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. *Science* 1998; 282: 1145-7.
2. Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: Somatic differentiation in vitro. *Nat Biotechnol* 2000; 18: 399-404.
3. Pera MF, Reubinoff B, Trounson A. Human embryonic stem cells. *J Cell Sci* 2000; 13: 5-10.
4. Amit M, Margulets V, Segev H, Shariki K, Laevsky I, Coleman R, et al. Human feeder layers for human embryonic stem cells. *Biol Reprod* 2003; 68: 2150-6.
5. Williams R, Hilton D, Pease S, Wilson T, Stewart C, Gearing D, et al. Myeloid leukemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* 1988; 336: 684-7.
6. Lim KWE, Bodnar A. Proteome analysis of conditioned medium from mouse embryonic fibroblast feeder layers which support the growth of human embryonic stem cell. *Proteomics* 2002; 2: 1187-203.

7. Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, et al. Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol* 2001; 19: 971-4.
8. Richards M, Fong CY, Chan WK, Wong PC, Bongso A. Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. *Nat Biotechnol* 2002; 20: 933-6.
9. Hovatta O, Mikkola M, Gertow K, Stromberg AM, Inzunza J, Hreinsson J, et al. A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells. *Hum Reprod* 2003; 18: 1404-9.
10. Cheng L, Hammond H, Ye Z, Zhan X, Dravid G. Human adult marrow cells support prolonged expansion of human embryonic stem cells in culture. *Stem Cells* 2003; 21: 131-42.
11. Milunsky A. Amniotic fluid cell culture. In Milunsky, A. (ed). *Genetic Disorder and the fetus*. Plenum Press; New York, 1979. pp. 75-84.
12. Hoehn H, Salk D. Morphological and biochemical heterogeneity of amniotic fluid cells in culture. *Methods Cell Biol* 1982; 26: 11-34.
13. Prusa AR, Marton E, Rosner M, Bernaschek G, Hengstschlager M. Oct-4 expressing cells in human amniotic fluid: A new source for stem cell research? *Hum Reprod* 2003; 18: 1489-93.
14. Oh SK, Kim HS, Ahn HJ, Seol HW, Kim YY, Park YB, et al. Derivation and characterization of new human embryonic stem cell lines, SNUhES1, SNUhES2 and SNUhES3. *Stem Cells* In press 2005.
15. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; 162: 156-9.
16. Thomson JA, Kalishman J, Golos TG, Durning M, Harris CP, Becker RA, et al. Isolation of a primate embryonic stem cell line. *Proc Natl Acad Sci USA* 1995; 92: 7844-8.
17. Thomson JA, Kalishman J, Golos TG, Durning M, Harris CP, Hearn JP. Pluripotent cell lines derived from common marmoset (*Callithrix jacchus*) blastocysts. *Biol Reprod* 1996; 55: 254-9.
18. Amit M, Carpenter MK, Inokuma MS, Chiu CP, Harris CP, Waknitz MA, et al. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev Biol* 2000; 227: 271-8.
19. Richards M, Tan S, Fong CY, Biswas A, Chan WK, Bongso A. Comparative evaluation of various human feeders for prolonged undifferentiated growth of human embryonic stem cells. *Stem Cells* 2003; 21: 546-56.
20. Carpenter MK, Rosler ES, Fisk GJ, Brandenberger R, Ares X, Miura T, et al. Properties of four human embryonic stem cell lines maintained in a feeder-free culture system. *Dev Dyn* 2004; 229: 243-58.
21. Rosler ES, Fisk GJ, Ares X, Irving J, Miura T, Rao MS, et al. Long-term culture of human embryonic stem cells in feeder-free conditions. *Dev Dyn* 2004; 229: 259-74.
22. Amit M, Shariki C, Margulets V, Itskovitz-Eldor J. Feeder layer- and serum-free culture of human embryonic stem cells. *Biol Reprod* 2004; 70: 837-45.
23. Gosden CM. Amniotic fluid cell types and culture. *Br Med Bull* 1983; 39: 348-54.
24. Prusa AR, Hengstschlager M. Amniotic fluid cells and human stem cell research: A new connection. *Med Sci Moni* 2002; 8: 253-7.
25. Donovan PJ. High Oct-ane fuel powers the stem cell. *Nature Genet* 2001; 29: 246-7.
26. Pesce M, Scholer HR. Oct-4: Gatekeeper in the beginnings of mammalian development. *Stem Cells* 2001; 19: 271-8.
27. Mosquera A, Fernandez JL, Campos A, Goyanes VJ, Ramiro-Diaz JR, Gosalvez J. Simultaneous decrease of telomere length and telomerase activity with ageing of human amniotic fluid cells. *J Med Genet* 1999; 36: 494-6.
28. Schuldiner M, Yanuka O, Itskovitz-Elder J, Melton DA, Benvenisty N. *Proc Natl Acad Sci USA* 2000; 97: 11307-12.