the adrenal lineage. In support of this hypothesis, it has frequently been reported that MSCs are heterogeneous populations that have a different differentiation potential (1, 2, 10). In a future study, the same treatment of various mouse or human MSCs need to be carried out, followed by observations of whether both adrenal and gonadal phenotypes are obtained. This might also provide a tool for revealing the pathway leading to the differentiation of the cells into adrenal or gonadal steroidogenic cells.

In summary, we demonstrate here that MSCs have the capacity to differentiate into steroidogenic cells, both *in vivo* and *in vitro*. MSCs represent not only a powerful tool for studies of the differentiation of the steroidogenic lineage but may also offer a possible clinical stem cell resource for diseases of steroidogenic organs.

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# Immortalization of human myogenic progenitor cell clone retaining multipotentiality

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**Abstract**

Human myogenic cells have limited ability to proliferate in culture. Although forced expression of telomerase can immortalize som cell types, telomerase alone delays senescence of human primary cultured myogenic cells, but fails to immortalize them. In contrast, co stitutive expression of both telomerase and the E7 gene from human papillomavirus type 16 immortalizes primary human myogenic cell We have established an immortalized primary human myogenic cell line preserving multipotentiality by ectopic expression of telomera and E7. The immortalized human myogenic cells exhibit the phenotypic characteristics of their primary parent, including an ability undergo myogenic, osteogenic, and adipogenic terminal differentiation under appropriate culture conditions. The immortalized cells w be useful for both basic and applied studies aimed at human muscle disorders. Furthermore, immortalization by transduction of tel merase and E7 represents a useful method by which to expand human myogenic cells in vitro without compromising their ability differentiate.


*Keywords:* Muscle satellite cell; Stem cell; Immortalization; Myogenesis; Multipotentiality; Regeneration; Cell transfer therapy; Telomerase; E Papillomavirus

Telomere length is stably maintained through the action of telomerase in germ line and cancer cells, but not in most somatic tissues. The progressive shortening of telomeres in most human somatic cells culminates in replicative senescence in vitro [1–3]. Proliferative capacity has been correlated with telomere length in human muscle satellite cells [4], and the number of population doublings is limited when human myogenic cells are grown in vitro. Although the cells remain viable, the telomeres shorten during cu ture, and the cells cease to divide.

The fatal loss of muscle in patients with Duchenne mu cular dystrophy (DMD) is caused by a decline in the cap bility of muscle to regenerate. Because DMD myogen cells undergo an extra number of cell divisions, they unde go premature replicative senescence during childhoo DMD myogenic cells from muscle biopsies grow poor in vitro, and rapidly undergo senescence [5,6]. Whethe the premature senescence of DMD myogenic cells attributable to telomeric attrition remains controversi [6,7], but telomere shortening could be involved in the pr mature senescence of human myogenic cells from DM patients [8].

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Previous attempts have been made to extend the replicative capacity of human myogenic cells using viral oncoproteins such as simian virus 40 (SV40) large T antigen (Tag). However, while the transformation due to Tag was sufficient to delay senescence, it failed to induce cell immortality. In addition, extension of the life span often coincided with dedifferentiation and differentiation defects [9]. To circumvent these problems, a temperature-sensitive mutant Tag was expressed in human myogenic cells [10]. Because temperature-dependent inactivation of mutant Tag is achieved at 39 °C, mutant Tag does not prevent terminal differentiation of human myogenic cells at 39 °C. However, a leak of Tag activity might still be sufficient to alter gene expression and cause mutations in human myogenic cells. In addition, the effect of exposure to high temperatures on phenotypic stability in human myogenic cells remains to be determined.

Not all but some types of human cells appear to be immortalized by the expression of the reverse transcriptase component of human telomerase (hTERT) alone without transformation of cell properties [1,11]. Thus, it might be expected that human myogenic cells could be immortalized by introduction of hTERT, but previous attempts to reconstitute telomerase by ectopic expression of hTERT did not result in extension of the replicative life span in normal (the present study) and DMD human myogenic cells [12,13].

The present study tested different combinations of hTERT, Bmi1, and human papillomavirus type 16 genes E6 and E7 to immortalize primary human myogenic cells. Our results indicate that constitutive expression of hTERT and E7 immortalizes primary human myogenic cells. Furthermore, the immortalized human myogenic cells express myogenic lineage markers and conserve the multipotentiality that the parental cells possess.

## Materials and methods

*Cell culture.* The human myogenic cell clone Hu5 was isolated from normal subcutaneous muscle tissue of a 42-year-old woman [14] and maintained at 37 °C under 10% $CO_2$ in dishes coated with type I collagen (Sumilon, Tokyo, Japan) and containing primary cultured myocyte growth medium (pmGM) consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 2% Ultroser G (Biosepra, Cedex-Saint-Christophe, France), and glucose (4.5 mg/ml). Cells were plated at $2\times10^5$/35-mm dish and cultured in pmGM. For induction of myogenic differentiation the medium was changed to primary cultured myocyte differentiation medium (pmDM) after 48 h. pmDM consists of the chemically defined medium TIS [15,16] supplemented with 2% FBS. For low density culture, cells were plated at $1\times10^5$/35-mm dish in the indicated experiment.

For induction of terminal osteogenic differentiation, cells were cultured in DMEM supplemented with 10% FBS, glucose (4.5 mg/ml), and a combination of 10 mM β-glycerophosphate (βGP) (Sigma, St. Louis, MO) and recombinant human bone morphogenetic protein (BMP2) (500 ng/ml) (Strathman Biotech, Hamburg, Germany, or PeproTech EC, London, UK). The cells were stained with the calcium-staining dye alizarin red S (0.01%, Sigma) [14]. To induce adipogenic differentiation, we cultured myogenic cells in DMEM supplemented with 10% FBS, glucose (4.5 mg/ml), and 200 μM γ-linolenic acid (Sigma, St. Louis, MO) for up to 5 days. The cells were stained with oil red O (0.3%, Sigma) [14].

*Preparation and infection of recombinant retroviruses.* The full-length human *bmi1* cDNA was cloned from K562 cells by RT-PCR using the forward primer 5′-ACGCGTCGACCGCCATGCATCGAACAACGA GAAT-3′ and the reverse primer 5′-CGGATCCTCAACCAGAA GAAGTTGCTG-3′. The PCR product was cloned into pCLXSN to generate pCLXSN-bmi1. pCLXSN-16E6E7 was constructed by inserting an *Eco*RI–*Bam*HI segment containing *HPV-16 E6* and *E7* into pCLXSN. pCMSCVpuro comprises a CMV/LTR fusion promoter, a packaging signal Psi+, and the multicloning sequence from pCLXSN (Imgenex Co., San Diego, CA) followed by a PGK-puro cassette and a 3′ long terminal repeat of a murine embryonic stem cell virus from pMSCVpuro (Clontech, Palo Alto, CA). *HPV-16 E7* and *hTert* cDNA [17] segments were recombined in the retroviral vectors to generate pCMSCVpuro-hTERT and pCLXSN-16E7. Production of recombinant retroviruses has been described [18]. Briefly, a retroviral vector and packaging construct, pCL-10A1, was co-transfected into 293 T cells by using TransIT-293 (Mirus Co., Madison, WI) according to the manufacturer's instructions, and the culture fluid was harvested at 48–72 h post-transfection. The titer of the recombinant viruses was greater than $5\times10^5$ drug-resistant colony forming units/ml on HeLa cells.

*Establishment of immortalized cells and clonal cultures.* Hu5 cells were transfected with retroviral vectors encoding hTERT, Bmi1, HPV-16 E7, or HPV-16 E6 and E7 as described [11]. Briefly, 1 ml of the culture fluid was added to Hu5 cells seeded on type I collagen-coated 6-well plates in the presence of polybrene (4 μg/ml). Following inoculation with viruses, the infected cells were selected in the presence of 0.5 μg/ml puromycin or 400 μg/ml G418. For single-cell cloning, 1000 transfected Hu5 cells were plated on a 100 mm culture dish coated with collagen and then cultured in medium comprising equal volumes of pmGM and conditioned medium from high-density cultures of precloned transfected Hu5 cells. Colonies were isolated and expanded for experimentation. The immortalized human myogenic cell clone E18 will be available from RIKEN BioResorce Center (http://www.brc.riken.go.jp).

*Karyotyping of immortalized cell clone.* After incubation in pmGM supplemented with 2 μM colcemid at 37 °C for 10 h, E18 cells were trypsinized and incubated in 0.5 ml of 1% sodium citrate for 15 min. This was followed by addition of 0.5 ml of Carnoy's fixative (3:1 by vol; methanol:acetic acid). The fixed cells were then spun down and resuspended in 0.5 ml of Carnoy's fixative. Metaphase chromosomes were stained with 10% Giemsa solution (Wako Pure Chem., Osaka, Japan) for 10 min.

*Immunofluorescence and immunochemical analyses of myogenic lineage marker proteins.* Cultured cells were grown on collagen-coated coverslips (Iwaki, Tokyo, Japan) for immunofluorescence or immunocytochemical analysis. The cells were fixed with 4% paraformaldehyde for 10 min at room temperature or placed on ice, respectively, and were then incubated for 18–66 h at 4 °C with primary antibodies. Primary antibodies included those to mouse MyoD (Novocastra, Newcastle, UK), sarcomeric MHC [19], nestin [20] (kindly provided by Y. Arimatsu), and desmin (Progen, Heidelberg, Germany). Secondary antibodies were biotinylated or Cy3-labeled antibodies to mouse or rabbit immunoglobulin G (Jackson ImmunoResearch Laboratory, Bar Harbor, ME). The biotinylated antibodies were detected with streptavidin-conjugated horseradish peroxidase. The peroxidase reaction was performed with 3,3-diaminobenzidine (Sigma). Cell nuclei were stained with 2, 4-diamidino-2-phenylindole dihydrochloride *n*-hydrate (DAPI) (0.5 μg/ml, Sigma) or hematoxylin (Wako Pure Chem., Osaka, Japan). Samples were visualized using an upright microscope (model BX50; Olympus) and a CCD camera (DP50; Olympus). Images were post-processed using Adobe Photoshop (Adobe Systems, San Jose, CA).

*Telomerase activity.* Telomerase activity was detected using a non-radioisotopic method [21] with a TRAPeze telomerase detection kit (Intergen, Purchace, NY), according to the manufacturer's instructions. One microgram of cell protein lysed in CHAPS buffer was used for the assay. PCR products separated in 12.5% polyacrylamide gels were stained with SYBR Green I (Cambrex Co., NY), and visualized with the LAS3000 CCD-Imaging System (Fujifilm Co. Ltd., Tokyo, Japan) on a UV transilluminator.

Table 1
Sequences of PCR primers and amplification conditions

| Target gene | Primer | Sequence (5′ → 3′) | Annealing temperature (°C) | Amplification cycles (bp) | Product size |
|---|---|---|---|---|---|
| Telomerase reverse transcriptase | hTert-F | GGAAGCAGAGGTCAGGCAGC | 58 | 28 | 719 |
| | hTert-R | AGAGCAGCGTGGAGAGGATG | | | |
| Human papilloma virus-16 E6 | HPV16 E6-F | GCAACAGTTACTGCGACGTG | 53 | 28 | 234 |
| | HPV16 E6-R | GGACACAGTGGCTTTTGACA | | | |
| Human papilloma virus-16·E7 | HPV16 E7-F | GATGGTCCAGCTGGACAAGC | 53 | 24 | 143 |
| | HPV16 E7-R | GTGCCCATTAACAGGTCTTC | | | |
| Glyceraldehyde-3-phosphate dehydrogenase | hGAPDH-F | GGGCTGCTTTTAACTCTGGT | 56 | 20 | 702 |
| | hGAPDH-R | TGGCAGGTTTTTCTAGACGG | | | |

*Reverse transcription and polymerase chain reaction (RT-PCR).* Total RNA was extracted from cultured cells with TRIzol-LS (Life Technologies, Rockville, MD), treated with RNase-free DNase (RQ-1; Promega, Madison, WI), and then reverse transcribed with the use of a Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech, Buckinghamshire, UK) and random hexamers as primers. To determine ectopic expression of the transduced genes hTERT, E6, and E7, targeted genes were amplified by PCR with the primers listed in Table 1.

*Detection of ossification.* Paraformaldehyde-fixed cultured cells in 35-mm dishes were stained with the calcium-specific dye Alizarin Red S (0.01%, Sigma) for 30 min. Images of stained dishes were obtained with a digital scanner (Scanjet 5p; Hewlett-Packard, Palo Alto, CA) and then post-processed using Adobe Photoshop.

## Results and discussion

### *Immortalization of primary human myogenic progenitor cells*

We previously isolated a primary human myogenic progenitor cell clone, Hu5, from the healthy subcutaneous muscle of a nondystrophic woman [14]. In contrast to primary cultured mouse myogenic cells [22], Hu5 cells cease proliferation and undergo replicative senescence after 10–12 passages after cloning. They also exhibit compromised myogenic differentiation potential prior to replicative senescence. To circumvent alterations of their characteristics during in vitro culture, we set out to immortalize the cells in vitro.

Some types of human cells appear to be immortalized by introduction of hTERT alone, but others require inactivation of tumor suppressors in addition [1,11]. Although expression of hTERT in Hu5 cells by retrovirus-mediated gene transfer provided telomerase activity (Figs. 1A and B), it was insufficient to overcome replicative senescence (data not shown) [13,23]. We therefore transfected the cells with retroviruses encoding hTERT and either HPV-16 E7 alone or both E6 and E7 because E6 targets p53 for degradation and E7 inactivates the retinoblastoma protein Rb [11]. The resulting cells, designated Hu5/E and Hu5/EE, respectively, proliferated continuously and did not undergo replicative senescence. They were maintained as multiple clones, for which the chromosomal positions and copy numbers of the transfected genes varied. Ectopic expression of both hTERT and Bmi1, a member of the polycomb group family of proteins that suppresses transcription of the cyclin-dependent kinase inhibitor (*p16Ink4a*) gene, also

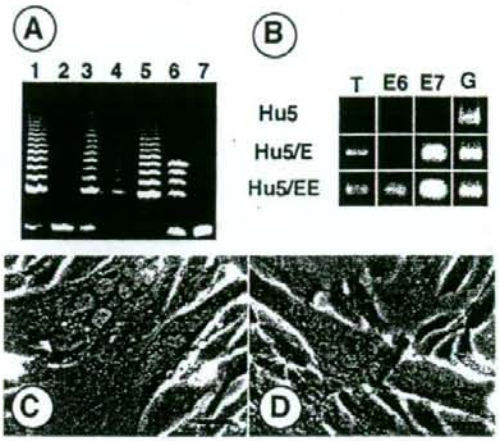


Fig. 1. Immortalization of human myogenic progenitor cells. (A) TRAP assay of parental and immortalized Hu5 cells for telomerase activity. Lanes: (1) HeLa cells (positive control), (2) Hu5 cells (passage 9), (3) Hu5 cells transduced with a retrovirus encoding hTERT alone, (4) Hu5 cells transduced with a retrovirus encoding hTERT and HPV-16 E7 (Hu5/E cells, passage 15), (5) Hu5 cells transduced with a retrovirus encoding hTERT and HPV-16 E 6/E7 (Hu5/EE cells, passage 12), (6) TSR-8 cells (positive control), (7) negative control (no cell lysate). (B) RT-PCR analysis of *hTert* (T) and *HPV-16 E6* and *E7* gene expression in parental and immortalized Hu5 cells. The human glyceraldehyde-3-phosphate dehydrogenase gene (G) served as an internal control. (C and D) Immortalized Hu5 cell derivatives Hu5/E (C) and Hu5/EE (D) were cultured in pmDM for up to 6 days and examined by phase-contrast microscopy. Scale bars, 20 μm.

resulted in a substantial extension of the life span of Hu5 cells, however, these Hu5/B cells retained little capacity for myogenic terminal differentiation in vitro and appeared to senesce within 20 passages. In a previous study, the constitutive expression of hTERT and Bmi1 in primary myogenic cells from a nondystrophic individual yielded immortalized clones, although these clones exhibited a differentiation block in vitro [12]. The life span of the primary human myogenic cells expressing hTERT and Bmi1 could be markedly extended, but immortalization is likely to occur at low frequency.

In contrast to the parental Hu5 cells, both Hu5/E and Hu5/EE cells exhibited telomerase activity (Fig. 1A).

RT-PCR analysis revealed the expression of both *hTert* and *HPV-16 E7* genes in Hu5/E cells and the expression of *hTert*, *E6*, and *E7* genes in Hu5/EE cells (Fig. 1B). To examine the myogenic differentiation potential of Hu5/E and Hu5/EE cells, we cultured them for up to 6 days in pmDM. Most Hu5/E cells differentiated into prominent myotubes (Fig. 1C), whereas only a small proportion of Hu5/EE cells underwent myogenic differentiation (Fig. 1D).

We next examined the expression of myogenic lineage marker proteins in the immortalized Hu5 cells by immunofluorescence analysis. First, the expression of a master gene for myogenesis, *MyoD*, was examined. MyoD was present in the nuclei of parental Hu5 cells (Figs. 2A and D) and a major population (approximately 80%) of Hu5/E cells (Figs. 2B and E); however, their expression was detected in only a minor population (10–20%) of Hu5/EE cells (Figs. 2C and F). Desmin and nestin, which are present primarily in proliferating parental Hu5 cells (Figs. 2G and J), were each expressed in Hu5/E cells (Figs. 2H and K); however, their expression was restricted to only a minor population (10–20%) of Hu5/EE cells (Figs. 2I and L). The immortalized line Hu5/E thus largely retains the original phenotype of the parental Hu5 cells. To date, Hu5/E and Hu5/EE cells have been cultured for >80 passages corresponding to more than 200 population doublings. We therefore conclude that they represent immortalized human myogenic progenitor cells that have retained their myogenic differentiation potential.

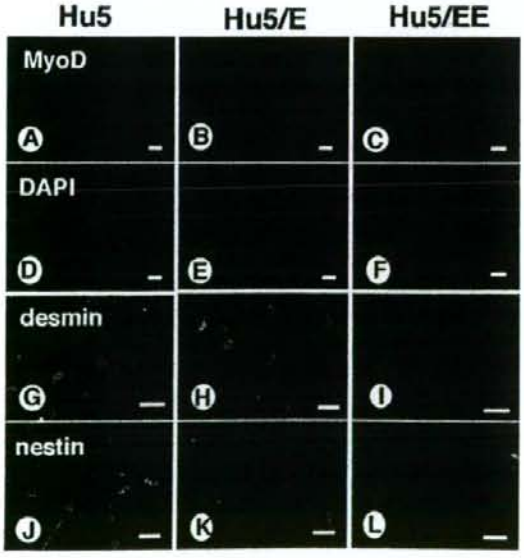


Fig. 2. Expression of myogenic proteins in primary and immortalized human myogenic cells. Undifferentiated Hu5 (A, D, G, and J), Hu5/E (B, E, H, and K), and Hu5/EE (C, F, I, and L) cells were subjected to immunofluorescence analysis with antibodies to MyoD (A through C), desmin (G through I), or nestin (J through L). Nuclei in the same fields as those in (A) through (C) were visualized by DAPI staining in (D) through (F), respectively. Nuclei in (G) through (L) were also revealed by DAPI staining (blue). Scale bars, 20 μm.

*Establishment of immortalized human myogenic cell clones*

The immortalized Hu5 cells, Hu5/E, and Hu5/EE cells contained multiple clones in which expression levels of the transduced genes varied. Furthermore, it is conceivable that differentiation-defective variant cells are included in the immortalized Hu5 cell population. To remove putative differentiation-defective cells from the culture, we isolated clones derived from individual Hu5/E cells, but not Hu5/EE cells, because a major fraction of Hu5/EE cells represented a nondifferentiating phenotype. First, the myogenic differentiation potential of each clone was determined by its ability to differentiate into myotubes in vitro on achieving confluence.

One of the Hu5/E-derived clones retaining myogenic differentiation potential, E18, was subjected to further analyses, although similar results were obtained with the eight other independent clones derived from individual Hu5/E cells. The doubling time of E18 cells was estimated at $34.7 \pm 4.1$ h based on their growth rate when cultured in pmGM. Karyotypic analyses of E18 cells were performed at 26 passages. All of the twenty E18 cells tested contained 46 chromosomes (Fig. 3). Therefore, E18 cells exhibited normal diploidy throughout in vitro culture and the immortalization process. The muscle lineage markers desmin and nestin were expressed in similar amounts in both parental Hu5 and immortalized E18 cells (data not shown).

Next, the expression of *MyoD* was examined in the immortalized Hu5 cell clone E18. MyoD was detected at a high level in less than 30–40% of E18 cells under the low cell density culture conditions (Figs. 4C and D) but at a lower level compared with that apparent in parental primary Hu5 cells (Figs. 4A and B). However, under the high cell density culture conditions, MyoD expression was up-regulated and detected at a high level in more than 80% of E18 cells (Figs. 4E and F). The immortalization process might result in the reduction of MyoD expression in E18 in a cell density-dependent manner. Taken together,

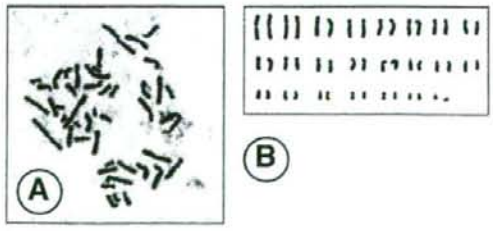


Fig. 3. Karyotypic analysis of immortalized human myogenic cell clone E18. (A and B) E18 cells were treated with colcemid (2 μM) for 10 h. Metaphase chromosomes were visualized by Giemsa staining (A). All of the twenty E18 cells tested exhibited 46 chromosomes (B).

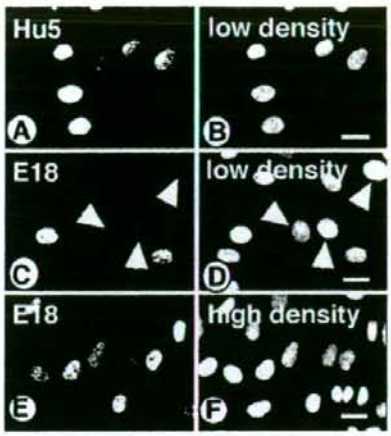


Fig. 4. Expression of MyoD in primary and immortalized human myogenic progenitor cell clones. Undifferentiated Hu5 cultured under a low-density condition (A and B) and E 18 cells cultured under low—(C and D) or high—(E and F) density conditions were subjected to immunofluorescence analysis with antibodies to MyoD (A, C, and E). Nuclei in the same fields as those in (A, C, and E) were visualized by DAPI staining in (B, D, and F), respectively. MyoD levels were at a reduced level in 60–90% of E18 cells under low-density conditions (arrowheads in C and D). Scale bars, 20 μm.

the results described here indicate that the immortalized line E18 largely retains the myogenic phenotype represented by parental Hu5 cells.

Mouse myogenic cells preserve the ability to undergo myogenic, osteogenic, and adipogenic differentiation [14]. E18 also underwent myogenic, osteogenic, and adipogenic terminal differentiation under the appropriate culture conditions that are somewhat different from those for mouse myogenic cells (Figs. 5A, B and C): induction of differentiation on day 2 of growing culture, medium containing 10% FBS, and higher concentration of γ-linolenic acid for adipogenesis. Hu5 and Hu5/E cells also underwent osteogenic

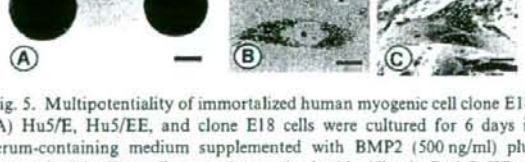


Fig. 5. Multipotentiality of immortalized human myogenic cell clone E18. (A) Hu5/E, Hu5/EE, and clone E18 cells were cultured for 6 days in serum-containing medium supplemented with BMP2 (500 ng/ml) plus βGP (10 mM). The cells were then stained with Alizarin Red S. Whole 35 mm dishes are shown. Scale bar, 10 mm. (B) E18 cells were cultured for 5 days in serum-containing medium supplemented with γ-linolenic acid (200 μM). Numerous lipid droplets were stained with oil red O. Asterisks represent nuclei. Scale bar, 20 μm. (C) E18 cells were cultured for 6 days under myogenic differentiation-inducing conditions. MHC was detected by immunostaining with a horseradish peroxidase reaction product (brown). Nuclei were detected by staining with hematoxylin (blue). Scale bar, 50 μm.

terminal differentiation, although Hu5/EE cells lost the ability to undergo any terminal differentiation (Fig. 5A).

In conclusion, we succeeded in establishing an immortalized human myogenic cell line that preserves the multipotentiality that the parental primary human myogenic cell retains. E18 would provide a useful culture system for analysis of the characteristics, differentiation mechanisms, and species-specific characteristics of human myogenic cells giving information for both basic biological research and therapeutic approaches.

## Acknowledgments

We thank S.-Y. Song and Y. Arimatsu for critical readings of the manuscript and Y. Nagai for his continuous encouragement throughout this study. This study was supported by a grant from the Ministry of Health, Labor, and Welfare of Japan and was performed in part as a contribution to the Ground Research for Space Utilization Program promoted by the Japan Space Forum.

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Original Article

# Immortalization of Epstein–Barr virus-negative human B lymphocytes with minimal chromosomal instability

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**The genes required for immortalization of human B cells infected by Epstein–Barr virus are multiple, and the precise mechanism of this process remains to be elucidated. In the present study HPV16 E6 and E7 were retrovirally transduced into human primary B cells stimulated by CD40–CD40L interaction, thereby establishing an Epstein–Barr virus negative immortalized human B cell line, which continued to proliferate for more than 2 years (100 population doublings). The established cell line had a high telomerase activity from the beginning of the culture period, and no shortening of the telomere length was observed. A chromosomal analysis revealed that a large portion of the HPV16E6E7 transduced cells had retained a normal karyotype. Similar to human epithelial cells, human B lymphocytes seem to require two steps for immortalization, namely, the inactivation of the p16/Rb pathway and the activation of telomerase, the latter that can be induced by the CD40–CD40L interaction. Furthermore, using this system, it is possible to analyze the role of individual genes in human B lymphocyte immortalization without the influence of a pre-existing Epstein–Barr virus genome.**

**Key words:** CD40–CD40L interaction, Epstein–Barr virus, HPV16 E6E7, human B cells, immortalization, telomerase

Normal cells cease to grow after a limited number of cell divisions when cultured *in vitro*.[1] This phenomenon is called replicative or cellular senescence, and senescence is now widely accepted as one of the important mechanisms in tumor suppression.[2] It has been suggested that senescence can be classified into two types. One is induced by telomere shortening. The telomere length is maintained by telomerase. Generally, somatic cells are devoid of any expression of telomerase reverse transcriptase (TERT), a key component of telomerase, and thus their telomere length becomes shortened after cell division. The other type is associated with the activation of the p16/Rb pathway or the p53 pathway by various types of extrinsic stress such as *in vitro* culture, although the exact activation mechanism remains to be elucidated.[3] The p16/Rb pathway or the p53 pathway is often abolished in tumor cells, and the activation of these pathways leads to the inhibition of cyclin-dependent kinase activity, thus resulting in cell cycle arrest or apoptosis.[4]

Overcoming senescence is called immortalization.[5,6] Because mouse cells have extremely long telomeres, murine cells tend to be spontaneously immortalized. In contrast, human primary cells are hardly ever spontaneously immortalized,[3,7] and some genetic manipulations are thus necessary to establish immortalized human cells. Human foreskin fibroblasts, retinal pigment cells[8] and, according to a most recent report, human fetal mesenchymal stem cells can be immortalized by the introduction of human TERT (hTERT) alone.[9] In contrast, both hTERT expression and the inactivation of the p16/Rb pathway are required for the immortalization of other fibroblasts and epithelial cells such as human mammary gland epithelial cells (HMEC) and human foreskin keratinocytes. E6 and E7 oncoproteins of human papillomavirus (HPV) type 16 or 18 can cooperatively immortalize HMEC, in that E7 directly binds and inactivates Rb, and E6 induces expression of hTERT.[10] Recently, several molecular mechanisms have been proposed for the induction of hTERT expression by E6.[11,12] In some other cells, a direct introduction of hTERT itself, instead of E6, is required for immortalization, probably due to the inefficient activation of endogenous hTERT expression by E6 in those cells.[13,14] SV40 T antigen, which inactivates both p53 and Rb, has also been used to expand the lifespan of human cells.[15]

Human B lymphocytes infected by Epstein–Barr virus (EBV) are called B-lymphoblastoid cell lines (LCL), which have long believed to be immortalized.[5] However, recent studies have suggested that LCL derived from normal

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individuals eventually reach senescence due to a shortening of their telomeres. Whether EBV immortalizes or transforms human B lymphocytes remains an unresolved and confusing problem.[16] Furthermore, at least five proteins of EBV are required for immortalization or transformation, while even more are involved in this process.[17–21] As a result, the exact molecular mechanisms involved in the immortalization of B cells remain to be clarified. Recently, normal somatic cells have been shown to have only a slight degree of telomerase activity.[22] In contrast, lymphocytes contain a high degree of telomerase activity, while also showing an elongation of telomere length during their proliferation, for example in the normal germinal center.[23–25] We therefore speculated that the steps required for human B lymphocyte immortalization may be different from those of other human cells such as HMEC.

In the present study we retrovirally transduced HPV16 E6 and E7 simultaneously into CD40L-stimulated human primary B cells, and attempted to gain some insight into the molecular mechanism of immortalization of human B lymphocytes.

## MATERIALS AND METHODS

### Long-term culture of human B lymphocytes

Peripheral mononucleocytes were obtained from the peripheral blood of a healthy donor by Ficoll gradation. Informed consent was obtained from the donor following the ethics standards of Aichi Cancer Center Research Institute. The cells were then cultured on 96 Gy-irradiated CD40 ligand (CD40L)-expressing NIH 3T3 cells (a kind gift from Dr Gordon Freeman, Dana-Farber Cancer Institute, Boston, MA, USA) as previously described.[26] The cells were analyzed by flow cytometry using Cy 5-labeled anti-CD19 antibody (Immunotech, Marseille, France), 1 week after the initiation of culture to confirm that >98% of the cells were CD19-expressing B lymphocytes, and thereafter were cultured further, with slight modification as described previously.[27]

Vector construction and retroviral transduction of HPV16 E6E7, enhanced green fluorescent protein (EGFP) and short hairpin RNA (shRNA) pCLMSCV-neo and pCLMSCV-puro comprise the CMV/LTR fusion promotor, packaging signal Psi+ and the multicloning site from pCLXSN (Imgenex, San Diego, CA, USA), followed by the PGK-neo cassette and 3′ long-terminal repeat of murine stem cell virus from pMSCVneo or pMSCVpuro (Clontech, Palo Alto, CA, USA). The Gateway system (Invitrogen, Carlsbad, CA, USA) was used for subcloning the genes into retroviral vectors. PCLMSCVneo-16E6E7 was constructed by recombining the segment of pCLXSN-16E6E7 containing full-length HPV16 E6 and E7 into the destination vectors as described previously.[13] For EGFP, a segment encoding a fusion protein of hygromycin-resistant gene and EGFP was polymerase chain reaction (PCR) amplified from pHygEGFP (Clontech), and then was inserted into the destination vector pDEST-CLMSCVpuro- as described here. shRNA E6Ri3 (GTATGGAACAACATTAGAA) and E7Ri2 (GAGATACACCTACATTGCA) were designed by selecting a 19-mer nucleotide sequence open reading frame (ORF) of HPV 16 E6 and E7, according to the criteria of Dharmacon siRNA Design Center (http://design.dharmacon.com/rnadesign/). E6Ri3 was chosen from the sequence inside the E6 ORF (122–306 nt), which is excised in two of the three splicing variants of the HPV 16 E6-E7 polycistronic transcript.[28] Sense and antisense oligonucleotides with the shRNA fragments, cohesive restriction sites, the stem loop sequence and the polyT signal were designed as follows: sense, GATCCCC (sense shRNA 19mer) TTCAAGAGA (antisense shRNA 19mer) TTTTTGGAAA; antisense, AGCTTTTCCAAAAA (sense shRNA 19mer) TCTCTTGAA (antisense shRNA 19mer) GGG. A total of 4.5 μmol/L each of sense and antisense oligonucleotides were mixed in 100 mmol/L NaCl and annealed by lowering the temperature from 95°C to 4°C in 2 h, and then were ligated into an entry vector with an H1 promotor region, and then were finally recombined into pCMSCV-puro by the Gateway system. The production of retroviruses has been described.[29] Briefly, retroviral vector and packaging vector encoding viral *gag-pol* gene, and pCL-GALV, which encodes envelope protein from gibbon ape leukemia virus[30] were cotransfected into 293T cells using Trans IT-293 (Mirus, Madison, WI, USA) according to the manufacturer's instructions, and the culture fluid was directly harvested at 48–72 h after transfection or sometimes harvested after centrifuging overnight for concentration. The titers of the recombinant viruses were $>1 \times 10^5$ drug-resistant colony-forming units/mL on HeLa cells. For retroviral infection, $1 \times 10^6$ B cells were placed in a 12-well plate, and centrifuged at 32°C, 1000 *g* for 1 h with 1 mL retroviral supernatant in the presence of 10 μg/mL polybrene. After infection, the cells were drug selected by either 0.8 mg/mL G418 or 0.5 μg/mL puromycin.

### Reverse transcription–polymerase chain reaction and western blot analysis

RNA was extracted from E6 and E7/or EGFP-transduced B cells using RNeasy kit (Qiagen, Hilden, Germany) combined with RNAse-Free DNase Set (Qiagen) according to the manufacturer's instructions. Next, cDNA was synthesized using the random hexamers provided in the SuperScript III First-Strand Synthesis System for reverse transcription–polymerase chain reaction (RT-PCR; Invitrogen). RT-PCR was performed as described previously[31] with the following primers: HPV16 E6-FW, GCAACAGTTACTGCGACGTG; HPV16 E6-RV, GGACACAGTGGCTTTTGACA; HPV16

E7-FW, GATGGTCCAGCTGGACAAGC; HPV16 E7-RV, GTGCCCATTAACAGGTCTTC; actinF, TCAGAAGGATTCCTATGTGG; actinR, TCTCCTTAATGTCACGCACG.

Western blot analysis was performed with 20 μg protein from whole-cell extracts separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis, and blotted on Immobilon P filters (Millipore, Bedford, MA, USA). The following antibodies were used: anti p16 (clone G175-405, BD Biosciences, San Jose, CA, USA), anti p53 (clone DO-1, Oncogene Science, Cambridge, MA, USA) and antimouse IgG, HRP-linked antibody (Cell Signaling, Danvers, MA, USA). Lumi-Light Plus Western Blotting Substrate (Roche, Manheim, Germany) was used for detection.

### Telomeric repeat amplification protocol assay and telomere length analysis by Southern blotting

Telomeric repeat amplification protocol (TRAP) assays were performed using the Trapeze telomerase detection kit (Intergen, Purchase, NY, USA) according to the manufacturer's protocol. DNA was extracted from B cells using the Qiamp DNA Blood Mini kit (Qiagen) according to the manufacturer's instructions, and the telomere length was analyzed using Southern blot analysis with a TeloTAGGG Telomere Length Assay (Roche), according to the manufacturer's protocol.

### Cell cycle analysis by flow cytometry

A total of $5 \times 10^5$ cells were washed with PBS, fixed on ice with 70% ethanol for 30 min, washed again with PBS, and incubated with 100 μg/mL RNAse A solution (Qiagen). The cells were centrifuged, washed again with PBS, and then incubated with propidium iodide (5 μg/mL: Pharmingen) on ice for 20 min. Finally, the DNA contents were analyzed by flow cytometry as previously described.

### Karyotype analysis

A karyotype analysis was performed at late passage (19 months) for each cell line. Routine karyotypic analysis was performed using preparations stained with 5% Giemsa solution. In order to identify possible rearrangements, chromosomes of metaphases were G-banded. For each cell line, more than 50 cells were scored for their chromosome number.

### Immunophenotyping and *in situ* hybridization for EBV

Immunophenotyping and *in situ* hybridization were also performed at late passage (19 months) for each cell line. Cells were fixed in 10% formaldehyde and embedded in paraffin. The avidin–biotin–peroxidase complex method was used for all immunohistochemical studies. Primary antibodies and the probe used were as follows: polyclonal CD3, L26 (CD20), mb-1 (CD79a), IgM, IgG, IgA, κ, λ, Bcl2 and Bcl6 (Dako, Copenhagen, Denmark), CD5, CD10, cyclin D1 and EBV probe for EBV-encoded RNA (Novocastra, Newcastle, UK). *In situ* hybridization was performed according to the manufacturer's protocol with slight modifications.

## RESULTS

### Culture, growth curve and expression of HPV16 E6 and E7

Co-cultured with CD40L-expressing and irradiated NIH3T3, primary human B lymphocytes entered into the cell cycle and then began to grow, forming cell clusters (Fig. 1a). Next, these cells were infected with retrovirus vectors expressing HPV16 E6E7 or control EGFP, and the culture was continued with CD40L-NIH3T3 cells. The efficient infection of retrovirus vectors into activated human B lymphocytes was demonstrated by EGFP signals (Fig. 1b). Without transduced genes, human B lymphocytes ceased to grow around 10 population doublings (PDL; around day 25–30; Fig. 1c) with the accumulation of p16 protein, an inhibitor of Cdk4/6 (Fig. 1d). In clear contrast, E6E7-transduced B cells were able to proliferate for more than 2 years (>100 PDL; Fig. 2). By RT-PCR, the expression of E6E7 mRNA was detected in the E6E7 vector-transduced cells in both early and late passages (Fig. 3). Surprisingly, EGFP-transduced cells were also able to proliferate for more than 2 years although at a slower proliferation rate (Fig. 2). We first considered that EBV infection might be attributable to the continuous proliferation of the EGFP-transduced B lymphocytes. However, *in situ* hybridization for EBV-encoded RNA was negative, and we could not detect any EBV-DNA even by real-time quantitative PCR (data not shown).

### Telomerase activity and telomere length

We investigated telomerase activity in these cells using a TRAP assay. Both E6E7-transduced cells and EGFP-transduced cells had a high telomerase activity both in the early and late passage (Fig. 4a). We then measured the telomere length by Southern blotting. The average telomere lengths of E6E7-transduced cells or EGFP-transduced cells were 8–10 kb, irrespectively of passage periods (Fig. 4b). Together, both E6E7-transduced cells and EGFP-transduced cells were considered to be immortalized.

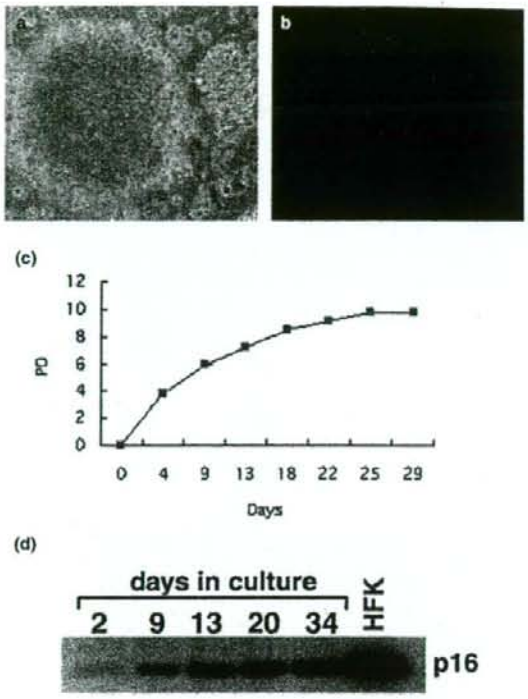


**Figure 1** p16-related growth arrest in long-term culture of human primary B lymphocytes. The morphological features of human primary B lymphocytes cultured on CD40L expressing NIH 3T3 cells. Cluster formation is seen. (**a**) Morphological features of enhanced green fluorescent protein (EGFP)-transduced B lymphocytes. (**b**) Growth curve of human primary B lymphocytes. The cells stopped proliferating at 9–10 population doublings (PD). (**c**) Western blot analysis of p16 protein. p16 accumulation is observed from day 9, and continues to increase until growth arrest at day 25–29. Cell lysate of human foreskin keratinocyte (HFK) was included for positive control.

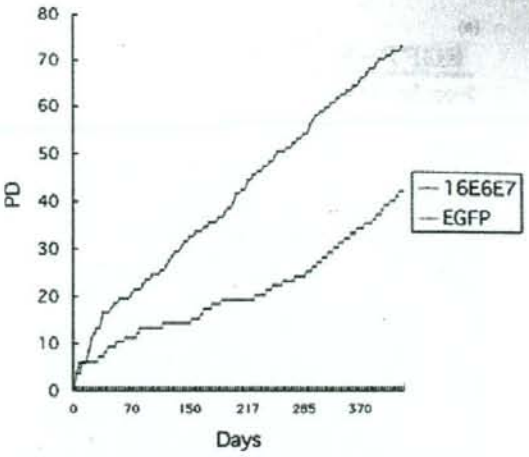


**Figure 2** Growth curve of 16E6E7- or enhanced green fluorescent protein (EGFP)-transduced B cells. PD, population doubling.


**Figure 3** Reverse transcription–polymerase chain reaction (RT-PCR) analysis of 16E6E7- or enhanced green fluorescent protein (EGFP)-transduced human B cells. PCR product of 243 bp and 143 bp for E6 and E7, respectively, are detected in 16E6E7-transduced cells.

### Expression of p16 protein

As described in the previous section, it is conceivable that the accumulation of p16 protein may cause cell cycle arrest in cultured primary B-cells (Fig. 1c,d). Therefore, we examined the p16 protein levels in E6E7- or EGFP-transduced immortalized B cells (Fig. 5). A comparable accumulation of p16 to that in presenescent B cells was observed in the E6E7-transduced immortalized B cells. This finding suggests that E7 directly binds and inactivates Rb and thereby cancels the inhibitory effect of p16 in B cells, and this finding is consistent with previous findings observed with other cell types. Interestingly, the p16 level decreased in the EGFP-transduced immortalized cells. We considered that cells in which p16 expression has been spontaneously downregulated are selectively immortalized. Such a phenomenon is observed also in HMEC, where the p16 mRNA expression is suppressed by the methylation of the promoter region.[32] We therefore performed a PCR-based analysis to investigate the methylation status of the p16 promotor region in the EGFP-transduced immortalized cells, but no methylation was found (data not shown). We also examined the p53 protein levels in these cells (Fig. 5). In the E6E7-transduced cells, the p53 protein levels decreased, which was an indication that p53 is also degraded by E6 in B lymphocytes. In EGFP-transduced B cells, p53 protein levels were unchanged, thus suggesting that the status of p53 protein was not associated with the spontaneous immortalization of EGFP-transduced cells.

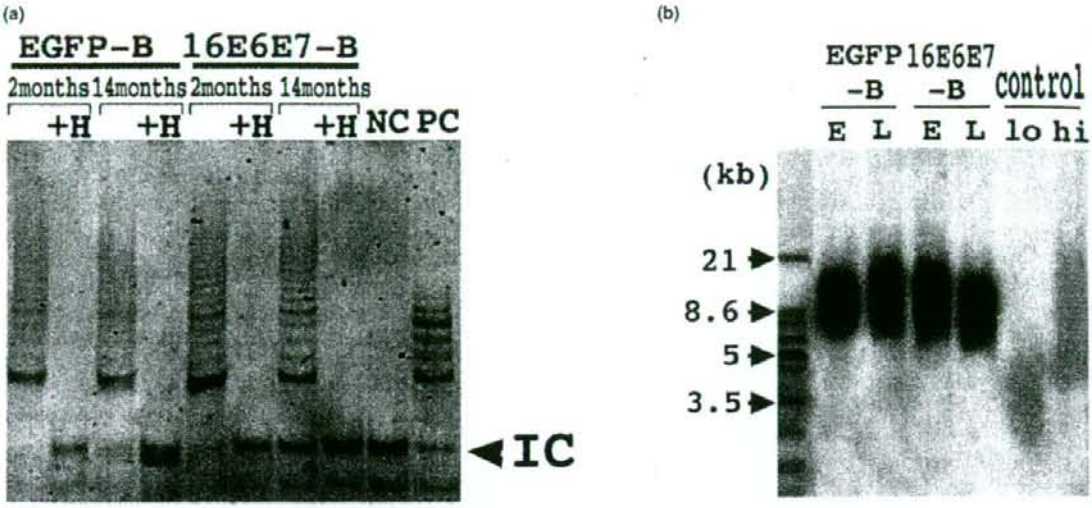


**Figure 4** Analysis of the telomerase activity and telomere length of 16E6E7- or enhanced green fluorescent protein (EGFP)-transduced B cells. (**a**) Telomeric repeat amplification protocol (TRAP) assay of early (2 months) or late (14 months) passage of 16E6E7- or EGFP-transduced human B cells. A high telomerase activity is seen in early/late passage and in EGFP/16E6E7-transduced cells. +H, heat-inactivated negative control for each sample; IC, internal control (to normalize the efficiency of PCR amplification); NC, negative control; PC, positive control. (**b**) Southern blot analysis of early (2 months) or late (14 months)-passage 16E6E7- or EGFP-transduced B cells. Terminal restriction fragments are visualized using a probe against the telomeric repeat sequence. The size standard is indicated at the left. E, early passage; hi, control DNA with high-molecular-weight telomeres; lo, control DNA with low-molecular-weight telomeres; L, late passage.

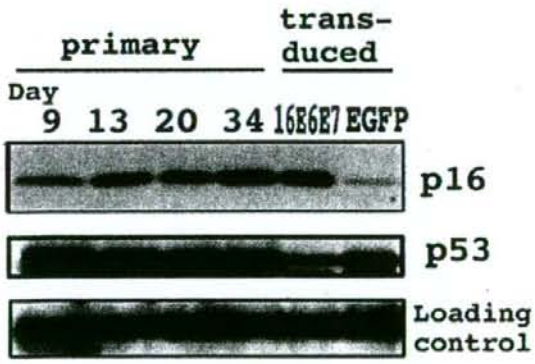


**Figure 5** Western bot analysis of p16 and p53 in primary or 16E6E7/enhanced green fluorescent protein (EGFP)-transduced B cells. A total of 20 μg protein from whole-cell extracts on day 9, 13, 20, 34 of the cultured primary B cells is loaded on the left side for control. And 20 μg protein from whole-cell extracts of late passage (14 months) 16E6E7- or EGFP-transduced B cells are loaded on the right side.

### RNA interference analysis

Three transcription variants are known to encode the E6 and E7 proteins in the HPV genome. E7 protein is generated from all three variants but, because two introns exist within the E6 coding sequence, full-length E6 protein is generated only from one variant that encodes both full-length E6 and E7.[28] We thus constructed shRNA expressing retroviral vectors that could silence the expression of either E6 alone or both E6 and E7 (Fig. 6a). CaSki cells expressing HPV16 E6 and E7 were first subjected to knockdown of E6 alone by expressing E6-specific shRNA (E6Ri3) or both E6 and E7 by expressing another shRNA (E7Ri2). Protein levels of E7 protein or p53 protein were analyzed by western blotting because the expression of p53 protein is normally reduced by E6 protein. Compared with Hela cells that do not contain the HPV16 genome, the upregulation of p53 was observed by the introduction of E6-specific shRNA or E7-shRNA but not by control vector, and reduction of E7 protein was observed by the introduction of E7-shRNA but not by E6-specific shRNA or control vector. We next infected E6E7-transduced B cells with the same set of retroviruses. After infection and drug selection by puromycin, control cells and E6 knocked-down cells were able to proliferate again, but the E6/E7 knocked-down cells all died within 1 week (Fig. 6). We infected EGFP-transduced B cells with the same set of viruses, but all cells remained healthy and proliferative after drug selection, thus indicating that the cell death in E6E7-transduced B cells was not caused by non-specific cell damage during retroviral infection and drug selection. To further investigate the mechanism of cell

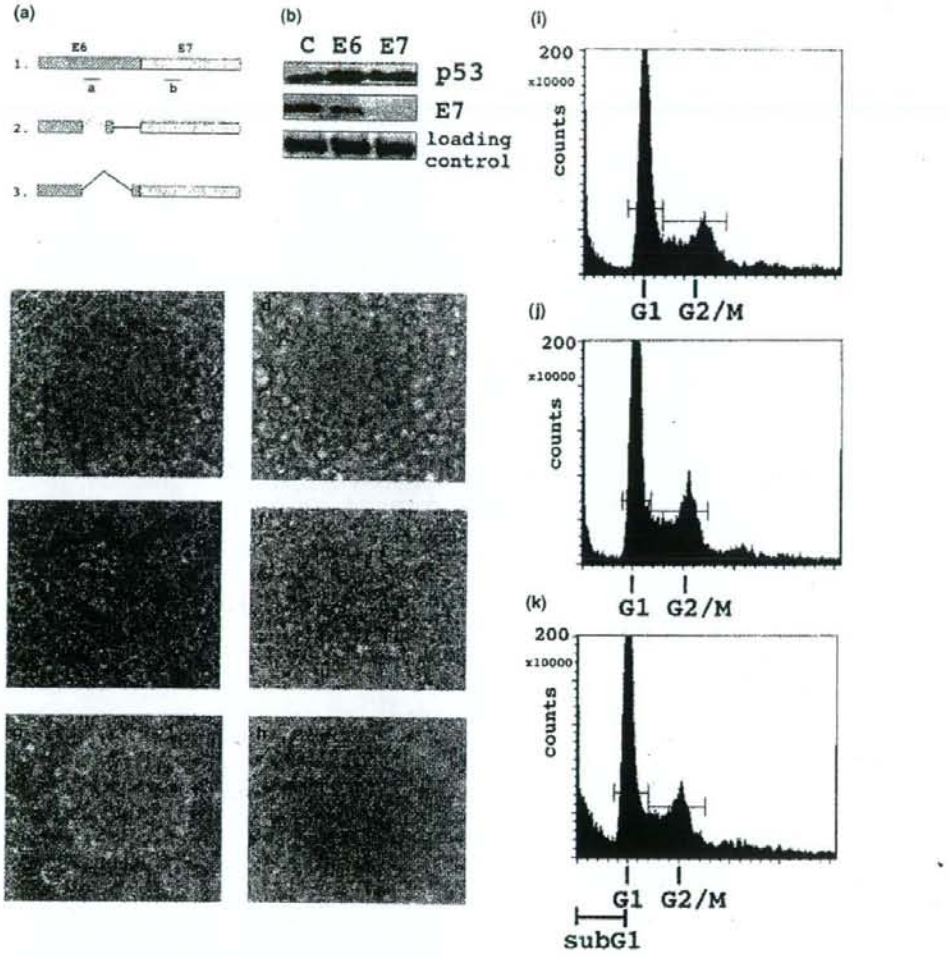


**Figure 6** RNA interference analysis of E6 alone or both E6 and E7 knockdown in 16E6E7- or enhanced green fluorescent protein (EGFP)-transduced B cells. (**a**) Three transcription variants (1–3) of HPV16E6E7 and the location of the target sequences for short hairpin RNA (shRNA; a: E6Ri3, b: E7Ri2). Knockdown of only variant 1 occurs by E6Ri3, and knockdown of all variants occur by E7Ri2. (**b**) Efficiency of shRNA constructs. ShRNA (E6Ri3 and E7Ri2) were transduced into SIHA cells by retroviral transduction. The upregulation of p53 by E6 silencing occurs in transduction of either E6Ri3 or E7Ri2, but knockdown of E7 occurs only in E7Ri2. C, control vector; E6, E6Ri3; E7, E7Ri2. (**c–h**) Retroviral transduction of shRNAs. Late-passage (12 months) 16E6E7- or EGFP-transduced B cells were further retrovirally transduced with shRNA constructs or retroviral vector. Drug selection using puromycin was done for 5 days after second infection. (**c**) 16E6E7-transduced B cells/negative control; (**d**) 16E6E7-transduced B cells/E6Ri3; (**e**) E7Ri2; (**f**) EGFP-transduced B cells/negative control; (**g**), EGFP-transduced B cells/E6Ri3; (**h**) EGFP-transduced B cells/E7Ri2. (**i–k**) Cell cycle analysis of shRNA transduced 16E6E7-transduced B cells. Late-passage (12 months) 16E6E7-transduced B cells were retrovirally transduced with shRNA constructs or retroviral vector: (**i**) control vector; (**j**) E6Ri3; (**k**) E7Ri2. No drug selection was performed, and flow cytometric analysis of DNA content stained by propidium iodide was performed 2 days after infection. A total of 30 000 cells were counted.

death by E6/E7 knockdown, a cell cycle analysis was performed. SubG1 fraction of E6/E7 knocked-down cells were more than twofold larger (17%) than the control cells (7.3%) and E6-only knocked-down cells (5.1%; Fig. 6), thus indicating that E6/E7 knockdown resulted in the apoptosis of E6E7-transduced B cells.

### Karyotype analysis

Although a karyotype analysis was performed in late passage cell lines, 50% of HPV16E6E7-transduced cells (25/50 cells) had the normal set of 46 chromosomes (Fig. 7). The chromosomal number of 20% of the cells (10/50 cells) was 47 and

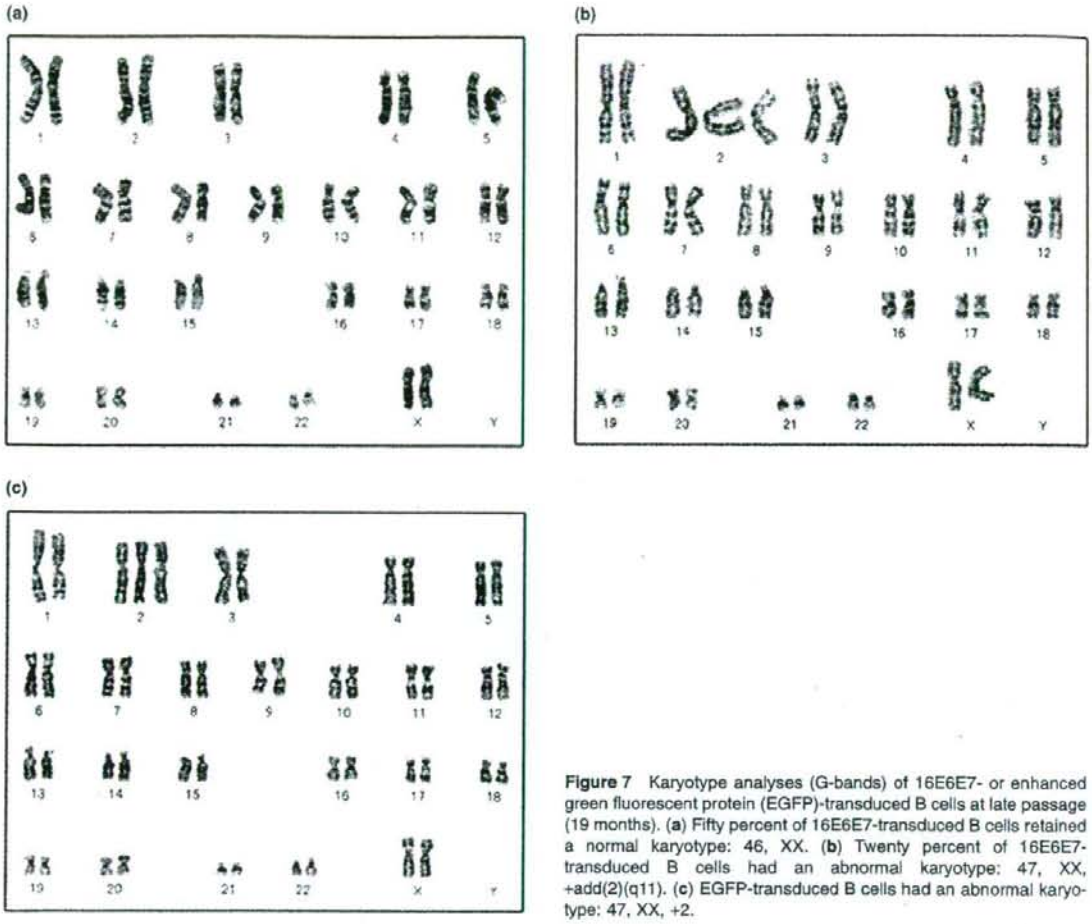


**Figure 7** Karyotype analyses (G-bands) of 16E6E7- or enhanced green fluorescent protein (EGFP)-transduced B cells at late passage (19 months). (**a**) Fifty percent of 16E6E7-transduced B cells retained a normal karyotype: 46, XX. (**b**) Twenty percent of 16E6E7-transduced B cells had an abnormal karyotype: 47, XX, +add(2)(q11). (**c**) EGFP-transduced B cells had an abnormal karyotype: 47, XX, +2.

that of residual cells varied from 90 to 95. G-banding revealed that cells with 46 chromosomes had a normal karyotype: 46 XX (10/10 cells); and cells with 47 chromosomes had a common abnormality: 47 XX add (2)(q11) (6/6 cells). All analyzed cells of EGFP-transduced cells (50/50 cells) had 47 chromosomes with a common karyotypic abnormality: 47 XX, +2 (10/10 cells).

### Immunophenotyping and *in situ* hybridization

Both E6E7-transduced cells and EGFP-transduced cells were positive for B-cell markers such as CD20 and CD79a, and negative for CD3, confirming that the immortalized cells retained the B-cell phenotype. Both cell lines were also positive for bcl-2, bcl-6, and CD10, and negative for either CD5 and cyclin D1, phenotypically similar to follicle center cells. Furthermore, E6E7-transduced cells were mostly positive for IgA and λ, and EGFP-transduced cells were mostly positive for IgG and λ. Both cells were negative for EBV-encoded RNA (data not shown).

## DISCUSSION

In the present study we successfully established EBV-negative cell lines derived from human primary B lymphocytes immortalized by HPV16 E6 and E7. This was unexpected, because human B cells were predicted to be difficult to immortalize, given the previous evidence that they require at least five EBV proteins for immortalization/transformation.[17–20] We also demonstrated for the first time that p16 protein is upregulated during the long-term culture of primary human B lymphocytes in a CD40-CD40L system.

This was understandable because the accumulation of p16 is a common feature observed in senescent fibroblasts, HMEC, keratinocytes, and T lymphocytes.[10,33,34] Although Herbert *et al.* proposed that p16 accumulation merely reflects an inappropriate culture condition (culture stress-induced growth arrest),[35] more recent experiments by Rheinwald *et al.* confirmed that p16-related arrest mechanism does exist in their system, with a p53-dependent component.[36] We therefore consider that whatever the cause of the p16-related arrest mechanism, the long-term culture of primary human B lymphocytes is also regulated by a telomere-independent, p16-related arrest mechanism that precedes the senescence induced by the shortening of telomere length.

Unexpectedly, EGFP transduced B cells also had a prolonged lifespan. We consider that this was because, similar to HMEC, p16 downregulated clones were selected and the activation of telomerase occurred by CD40–CD40L interaction. The telomerase activity is induced *in vitro* in B lymphocytes by stimulation via antigen receptor[37] or CD40–CD40L interaction, which mimics antigen–antigen receptor interaction.[38] In the present study we demonstrated that both cell lines showed a high telomerase activity in either the early or late passage, and we consider that an upregulation of the telomerase activity occurs in B lymphocytes cultured in this CD40–CD40L system, which is consistent with previous reports. It is therefore unlikely that clones were selected because of an upregulated telomerase activity. The reason for the downregulation of p16 protein is unclear. Because no methylation of the p16 promotor CpG island was detected, we speculate that an upregulation of the proteins that downregulate p16 may thus have occurred spontaneously. Further studies, however, are necessary to elucidate this point.

E7, and probably not E6, was therefore necessary for immortalization by HPV16 E6 and E7. This is explained by the aforementioned reasons. The upregulation of the telomerase activity occurs in B lymphocytes cultured in this CD40-CD40L system. E6 induces the telomerase activity in epithelial cells[39] and human T lymphocytes (Y Yamashita, T Kiyono, unpubl. obs. 2001), and degrades p53 in epithelial cells[40] and lymphocytes (Fig. 6). However, in the present system and in a previously reported system,[38] the telomerase activity was efficiently induced by CD40–CD40L stimulation. Interestingly, a recent report demonstrated that CD40–CD40L interaction is a critical effector in EBV-related cell survival and transformation.[41] We therefore consider telomerase induction by CD40–CD40L interaction to be a very important factor in B lymphocyte immortalization and, similar to epithelial cells, such as HMEC, it is an additional step that contributes to inactivation of the Rb pathway that is required for immortalization.

Although we started the culture in a bulk culture system with polyclonal B lymphocytes confirmed by a conventional nested PCR method using FrIII and LJH/VLJH primer,[42] clonal bands were observed by the same method, thus showing that established cell lines contained clonal populations (data not shown). This is explainable in the case of EGFP-transduced cells because in these cells, a clone with downregulated p16 was selected. The reason for the clonality in E6E7-transduced cells is unclear, but karyotypic analyses showed that HPV16 E6E7-transduced cells were polyclonal in contrast to clonal EGFP-transduced cells. Furthermore, according to a most recent report, human hematopoietic cells were immortalized by HPV16 E6E7 either alone or in concert with hTERT, and were either oligoclonal or clonal judged by a chromosomal analysis.[43] We speculate that in such long-term cultures, dominant clones are selected by subtle growth advantages.

Our findings should be a start for investigating precise steps required for B-cell immortalization, especially by EBV. *LMP1* perturbs the p16/Rb pathway in human fibroblasts and B cells by promoting the nuclear export of Ets2 and E2F4/5.[44] Recently, the expression of CD40L by EBV was found in LCL,[41] thus the CD40–CD40L interaction should occur stably in LCL. These findings together with our results suggest that LCL should easily be immortalized by EBV. However, this seems not to be true according to previous lines of evidence.[16] The reason for this discrepancy remains to be clarified. It will be interesting to determine whether LMP1 alone can immortalize B cells in the CD40–CD40L system.

In conclusion, we have showed that human primary B lymphocytes were successfully immortalized by HPV16 E6 and E7, with E7 playing the most important role by destroying the Rb pathway. CD40–CD40L interaction also participates in B-cell immortalization by activating telomerase. We thus conclude that the steps required for B-cell immortalization at least in culture are closely similar to HMEC in which two steps (the inactivation of the Rb pathway and the maintenance of the telomere length), are required for immortalization.

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# *Ex Vivo* Expansion of Human Cord Blood Hematopoietic Progenitor Cells Using Glutaraldehyde-Fixed Human Bone Marrow Stromal Cells

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**Human stromal cells were immortalized and fixed with glutaraldehyde to support an *ex vivo* expansion of human cord blood hematopoietic progenitor cells. In addition, this enabled glutaraldehyde-fixed stromal cells to be stored at 4°C. Although freeze-dried glutaraldehyde-fixed stromal cells did not increase the number of the progenitor cells, the percent decrease in the number of $CD34^+$ cells in the presence of freeze-dried glutaraldehyde-fixed stromal cells was less than that in the absence of the stromal cells. Thus, glutaraldehyde-fixed stromal cells can serve as a stabilizing device for hematopoietic cell expansion.**

[**Key words:** hematopoietic cells, *ex vivo* expansion, glutaraldehyde fixation, stromal cell, cord blood cells]

Human umbilical cord blood (CB) is an attractive source of cells for transplantation therapy and regenerative medicine because it contains hematopoietic progenitor and stem cells, is relatively immature in terms of immune responsiveness, and is easy to obtain from CB banks. However, it offers a limited number of cells for widespread medical applications. Many researchers have explored *ex vivo* expansion methods to increase the number of hematopoietic cells (1). A successful expansion of CB cells using hematopoietic growth factors or artificial substrata has been reported by several groups (2–6); however, the expansion efficiency is low in these systems. In contrast, since the establishment of Dexter-type long-term bone marrow (BM) culture (7), which attempts to mimic the BM environment, many coculture systems have been reported as promising systems for the maintenance and expansion of hematopoietic progenitor cells (8–10). However, because these systems mainly used murine cell lines and stromal media supplemented with calf and horse sera, they have been considered as sources of xenotransplantation for human hematopoietic cell expansion. Therefore, to reduce the risks of infectious disease, a culture system without the use of animal-derived stromal cells is desirable.

It has recently been reported that the number of human CB cells could be markedly increased in the presence of stem cell factor (SCF), thrombopoietin (TPO), and Flk-2/Flt-3 ligand (FL) by coculture with primary (11) or immortalized (12) human BM stromal cells. Although these techniques are useful, for the primary human CB cells, the use of the patient's own stromal cells is desirable so as not to induce undesirable immunoresponses. Furthermore, for the immortalized human BM stromal cells, contamination with undesirable genes or viral vectors must be considered. In addition, the preparation of stromal cells for each expansion is troublesome. Therefore, in this investigation, we prepared chemically fixed stromal cells, which can be cool-stored, for the expansion of human CB hematopoietic cells.

First, we constructed the retroviral plasmids pCMSCVpuro-16E6E7 and pCLXSN-hTERT as described previously (13) to prepare an immortalized stromal cell for a stable supply. The production of recombinant retroviruses has been described previously (13, 14). Briefly, the retroviral vector plasmids were cotransfected with pCL-10A1, which is a packaging construct encoding gag, pol, and env of the murine leukemia virus strain 10A1, into 293FT cells (Invitrogen, Carlsbad, CA, USA) using TransIT-293 (Mirus, Madison, WI, USA), in accordance with the manufacturer's instructions, and the culture fluid was harvested at 48 to 72 h post-transfection. The retrovirus, MSCVpuro-16E6E7 contains the human papillomavirus type 16 (HPV-16) genes E6 and E7, which inhibit p53 and Rb, respectively, and a puromycin resistance gene. The LXSN-hTERT retrovirus expresses the catalytic subunit of human telomerase reverse transcriptase, which inhibits the shortening of telomeres, and a neomycin (G418) resistance gene. The titer of recombinant retroviruses was greater than $3\times10^5$ drug-resistant colony forming units per milliliter in HeLa cells.

Second-passage primary human BM stromal cells (human mesenchymal stem cells, cat no. PT-2501, lot no. 3F0664)

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TABLE 1. Total number of human CB progenitor cells[a] after culture[b]

| Lot no. | Stroma free ($\times 10^4$ cells) | GA-fixed stroma ($\times 10^4$ cells) | GA-fixed stroma/Stroma-free | Culture time (d) | n |
|---|---|---|---|---|---|
| 1 | 28.0±1.8 | 58.0±13.5 | 2.1 | 14 | 6 |
| 2 | 17.7±1.5 | 24.2±3.3 | 1.4 | 14 | 6 |
| 3 | 74.0±9.8 | 123.3±22.5 | 1.7 | 14 | 6 |
| 4 | 275.8±30.4 | 395.8±30.1 | 1.4 | 14 | 6 |
| 5 | 35.7±4.4 | 62.5±10.0 | 1.8 | 14 | 6 |
| 6 | 114.0±18.8 | 188.7±25.8 | 1.7 | 14 | 6 |
| 7 | 31.0±5.7 | 76.5±6.0 | 2.5 | 14 | 6 |
| 8 | 57.2±3.5 | 85.5±2.9 | 1.5 | 10 | 6 |
| 9 | 115.3±10.5 | 189.4±24.3 | 1.6 | 10 | 6 |
| 10 | 27.3±2.6 | 46.2±5.8 | 1.7 | 10 | 6 |

[a] CB samples were obtained from the RIKEN BioResource Center (Tsukuba). CB mononuclear cells were washed with PBS containing 10% acid citrate dextrose-A, (ACD-A; Terumo, Tokyo) and 500 ml of 10% BSA (Sigma, St. Louis, MO, USA). $CD34^+$ cell-enriched populations were separated from mononuclear cells with a MACS Direct CD34 Progenitor Cell Isolation kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) following the manufacturer's instructions.

[b] Ten thousand $CD34^+$ cells were cultured on stromal cell layers in 1 ml of minimum essential medium α (α-MEM) supplemented with 20% fetal bovine serum (FBS), human SCF (10 ng/ml), human TPO (10 ng/ml), and human FL (10 ng/ml). Ideally, the addition of no serum was desired for the expansion; however, in this study, serum was added to the medium because it can be easily replaced with patient sera in the future. The culture was maintained at 37°C in a humidified atmosphere of 5% $CO_2$/95% air. After one week of culture, 1 ml of fresh medium was added and the coculture was continued for another week. At the end of the culture period, hematopoietic cells that did not adhere to stromal cells and those that adhered only weakly to stromal cells were collected by gentle pipetting.

TABLE 2. Total number and percentage of $CD34^+$ cells[a] of human CB progenitor cells cultured under same conditions as those described in Table 1 during 10 d

| | Stroma-free | GA-fixed stroma | GA-fixed/freeze-dried stroma | n |
|---|---|---|---|---|
| Total number of cells ($\times 10^4$) | 115.3±10.5 | 189.4±24.4 | 119.3±8.8 | 6 |
| Percentage of $CD34^+$ cells (%) | 4.6±0.1 | 7.0±0.1 | 8.0±0.4 | 3 |

[a] Cell suspensions were incubated with the fluorescein isothiocyanate-conjugated anti-CD34 antibody and the phycoerythrin-conjugated anti-CD45 antibody (Beckman Coulter, Tokyo) in PBS/5% FBS at room temperature for 10 min. The analysis was carried out using a Cytomics FC500 flow cytometer (Beckman Coulter). Dead cells were gated out with a forward versus side scatter window by 7-amino-actinomycin D staining.

were purchased from Cambrex (East Rutherford, NJ, USA) and the cells were maintained in mesenchymal stem cell growth medium (MSCGM, Cambrex, cat. no. PT-3001). Two days after $5 \times 10^4$ cells were plated on 35 mm-well of a six-well plate, 1 ml of the retrovirus culture fluid was added to each well in the presence of polybrene (4 μg/ml). Cells were first inoculated with LXSN-hTERT, and the infected cells were selected in the presence of 800 μg/ml G418. Then, the cells were inoculated with MSCVpuro-16E6E7, followed by selection in the presence of 0.4 μg/ml puromycin. Because the cells not transduced with E6 and E7 genes underwent premature senescence within a few passages, the cells with an extended lifespan were used as virtually immortal stromal cells. One hundred thousand stromal cells were plated in six-well plates and cultured for 6–7 d. The cultured stromal cells were fixed with 2.5% glutaraldehyde in phosphate buffered saline (PBS) for 30 min, following extensive washing with PBS. The prepared cells were stored for further experiments in PBS at 4°C. Freeze-drying was performed after washing with water, and the freeze-dried cells were stored for further experiments in air at room temperature.

The immortalized cells were prepared by the same method as that previously reported (15, 16) and considered to have similar properties. When compared with that of the cells established by Kawano *et al.* (12), the growth rate of the cells in our study was significantly high; it was difficult to decrease the growth rate using mitomycin C. Next, the cells were directly chemically fixed. Cells treated with glutaraldehyde had almost the same morphology as nontreated cells. Furthermore, glutaraldehyde-treated stromal cell layers maintained their morphology even after four weeks in a refrigerator, although they formed small interstitial spaces. Freeze-drying also did not affect the morphology.

We examined the ability of the fixed stromal cells to support hematopoiesis (Table 1). Ten thousand human CB $CD34^+$ cells were cultured in serum-containing medium supplemented with SCF, TPO, and FL. It was considered that cell viability differed among the CB preparations because the CB collected by the RIKEN Bioresource Center was obtained from various hospitals under different conditions. However, compared with stromal cell-free culture, glutaraldehyde-fixed stromal cell cuture increased the number of cells 1.7-fold over 10 d or two weeks, although the absolute number of cells significantly depended on the sample lot. We concluded that stromal cell layers clearly retained the ability to enhance CB cell expansion even after glutaraldehyde fixation.

Table 2 shows that the percentage of $CD34^+$ cells was greater in glutaraldehyde-fixed stromal cell culture than in stromal cell-free culture. In addition, in contrast with the glutaraldehyde-fixed stromal cells, the freeze-dried glutaraldehyde-fixed stromal cells did not support the expansion of human CB progenitor cells; however, they did not decrease

the percentage of CD34$^+$ cells, whereas the glutaraldehyde-fixed stromal cells did.

For clinical applications, the expansion system requires safety, reproducibility, and a stable source of cultured cells. To fulfill these requirements, it is desirable for the system not to use viable cells. Our results revealed that a substrate can be used as an expansion device instead of living cells. If bioactive molecules are extracted with their bioactivity preserved, a proper rearrangement on the matrix will provide a new useful expansion device. We previously attempted to immobilize bioactive molecules on a solid matrix by photoimmobilization and showed the effectiveness of this approach for cell expansion (17). The present method could also be useful for creating a cell expansion system.

In conclusion, glutaraldehyde-fixed stromal cells retain the ability to support hematopoiesis via the *ex vivo* expansion of human CB progenitor cells through a direct interaction between the hematopoietic cells and the fixed stromal cell surface, and this ability is maintained after simple refrigeration at 4°C.

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# Runx3 Negatively Regulates Osterix Expression in Dental Pulp Cells

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**Synopsis**

Osterix, a zinc finger-containing transcription factor, is required for osteoblast differentiation and bone formation. *Osterix* is also expressed in dental mesenchymal cells of the tooth germ. However, the transcritional regulation by osterix of tooth development is not clear. Genetic studies in osteogenesis place *Osterix* downstream to *Runx2*. *Osterix* was expressed in odontoblasts, overlapping with *Runx3* during their terminal differentiation in vivo. Runx3 down-regulated *Osterix* expression in the mouse dental pulp cells. Therefore, the regulatory role of Runx3 on *Osterix* expression in tooth development was investigated. Enforced expression of *Runx3* down-regulated the *Osterix* promoter activity in a cell line HEK293. When the Runx3 responsive element on the *Osterix* promoter, -713 bp to -707 bp (site 3, AGTGGTT) from the cap site, was mutated, this down-regulation was abrogated. Furthermore,