might be essential for adipocyte differentiation from ES cells, and this might be why trypsinized EBs showed lower differentiation. Therefore, EBs in the sphere form were then treated with each Ad vector by triple transduction, and GPDH activity was measured after cultivation with adipogenic supplements. The levels of GPDH activity in the cells transduced with Ad-CA-LacZ or Ad-CA-C/EBPα were similar to those of untransduced EBs. On the other hand, it was significantly increased by Ad vector-mediated PPARγ (PPARγ1 and PPARγ2) expression at days 12 and 24 (Figure 4A). Moreover, Oil-red O staining revealed that 70–80% of the cells transduced with PPARγ were Oil-red O positive, whereas 50–60% were LacZ- or C/EBPα-transduced cells or untransduced cells (data not shown). In particular, many large lipid droplets accumulated in the cells transduced with Ad-CA-PPARγ1 or Ad-CA-PPARγ2 compared to untransduced EBs (Figure 4B, data not shown). We also confirmed the expression of marker genes of adipocyte differentiation by semi-quantitative RT-PCR analysis. As shown in Figure 4C, PPARγ, C/EBPα, adipocyte-specific fatty acid binding protein (aP2), and adiponectin expression were up-regulated in PPARγ1- and PPARγ2-transduced EBs (Figure 4C). Furthermore, when we measured the expression levels of LacZ mRNA to examine whether Ad vector-mediated transduction still continued, its expression was undetectable in Ad-CA-LacZ-transduced EBs (Figure 4C), suggesting that expression of PAPRγ or C/EBPα, which was observed in PPARγ- or C/EBPα-transduced EBs, would not be derived from the Ad vector but from endogenous genes.

Next, we examined whether Ad vector-mediated transduction into EBs could increase the differentiation efficiency even in the absence of adipogenic supplements. Adipogenesis from EBs was promoted by transduction of PPARγ, although the levels of GPDH activity and lipid droplet accumulation could not achieve the adipogenic supplements-treated levels (Figure 5 and data not shown). These results indicate that Ad vector-mediated transduction of the PPARγ gene into EBs could improve the efficiency of adipocyte differentiation from ES cells with or without adipogenic supplements.

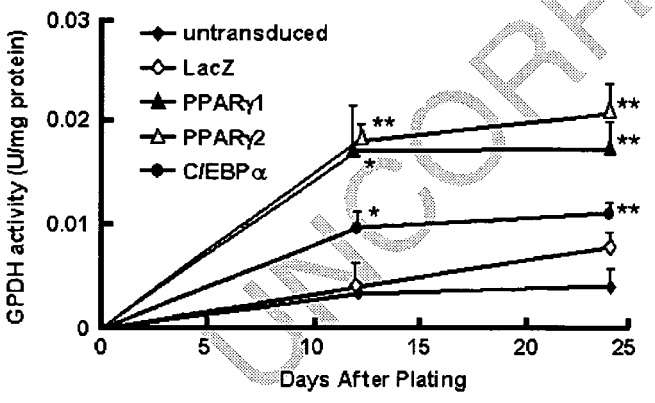

Figure 5. Ad vector-mediated transduction into EBs promotes adipogenesis in the absence of adipogenic supplements. EBs were transduced in triplicate with 10 000 VP/cell of each Ad vector, and then GPDH activity was measured after cultivation in differentiation medium without adipogenic supplements. The data are expressed as mean ± S.D. (n = 3). *$p < 0.05$ and **$p < 0.01$, respectively, as compared with untransduced EBs

Discussion

In the present study, we compared the transduction efficiency of four types of promoters (RSV, CMV, CA, and EF-1α), which are widely used in transduction experiments, in EBs by using Ad vector, and demonstrated that the CA promoter could robustly drive transgene expression in EBs (Figures 2A and 2B). We concluded that the CA promoter was the most appropriate promoter for transduction into EBs. We also showed that in trypsinized EBs, more than 90% of the cells were transduced with the Ad vector containing the CA promoter, and that a transgene could be successfully expressed in the interior of EBs by triple transduction (Figure 3). We and other groups have demonstrated that the CA promoter is potentially active in mES cells [11,32], human $CD34^+$ cells [33,34], and embryos of transgenic mice [35], suggesting that the CA promoter is active particularly in immature cells including stem cells. EBs are thought to be composed of immature cells because of the presence of Oct-3/4 and Nanog, although their expression levels are moderate (Figure 1). Thus, the CA promoter is useful in attaining high levels of transgene expression in EBs. Interestingly, the CMV promoter, which is one of the strongest promoters known so far, had little activity not only in mES cells [11,32], but also in EBs (Figures 2A and 2B). This might be due to the defense response against the transcription of foreign genes using a non-cellular promoter in immature cells. Rust *et al.* [36] reported that the CMV promoter was active in cardiac myocytes derived from mES cells in spite of being inactive in undifferentiated ES cells. These results suggest that the CMV promoter, in contrast to the CA promoter, does not work in both EBs and ES cells, and it is possible that transcriptional silencing might occur through some mechanism such as the DNA methylation of the CMV promoter [37]. However, Rufaihah *et al.* recently showed that about 90% of the human 7d-EB-derived single cells were transduced with an Ad vector containing the CMV promoter [38]. Although it is unknown why the CMV promoter has potent activity in human EBs, the transcriptional silencing using the CMV promoter might occur in murine but not human cells. Thus, the silencing mechanism in the CMV promoter in immature cells should be further investigated.

Differentiation procedures from ES cells by gene delivery have been performed using long-term constitutive expression systems such as those involving retrovirus vector [4,7]; however, these procedures might be not suitable for therapeutic use. Ad vectors could be useful because of their transient expression. However, few studies have been performed to differentiate ES cells into functional cells using transient expression systems. In the

present study, we showed that transient PPARγ transduction into EBs by using an Ad vector could enhance adipocyte differentiation in the presence or absence of adipogenic supplements (Figures 4 and 5). Adipocyte differentiation, however, was not enough in the absence of adipogenic supplements compared with that in the presence of it (Figure 5), suggesting that it is important to combine PPARγ transduction and treatment with adipogenic supplements to attain efficient adipcyte differentiation. Analysis of semi-quantitative RT-PCR revealed that the LacZ mRNA expression level by the Ad vector was quite low in EBs at day 24, indicating that Ad vector-mediated transduction is sufficient to trigger differentiation into functional cells even though its expression is transient and could not be introduced into all the cells composed of EBs. These observations lead to the expectation that an Ad vector-mediated transient gene expression system could be applied to differentiate ES cells into other cells such as osteoblasts, hematopoietic cells, and so on.

We also showed that both PPARγ1 and PPARγ2 significantly promoted the adipocyte differentiation from EBs (Figures 4 and 5). Our results indicate that overexpression of PPARγ1 and PPARγ2 was capable of accelerating adipogenesis in EBs, and that the N-terminal domain of PPARγ2 is not necessarily required for its adipogenic activity in EBs since both have similar activities. These results are consistent with the previous report [13]. In the absence of adipogenic supplements, C/EBPα-transduced EBs also exhibited a higher GPDH activity than LacZ-transduced EBs or untransduced EBs (Figure 5), indicating that C/EBPα as well as PPARγ has the potential to promote adipogenesis from EBs. However, in the presence of adipogenic supplements, C/EBPα could not increase the efficiency of adipocyte differentiation (GPDH activity) as much as could PPARγ, despite the increased expression of aP2 and adioponectin mRNA and a slight accumulation of lipid droplets in the EBs (Figure 4). C/EBPα has been shown to play important roles in adipogenesis, but its role is limited to the induction and retention of PPARγ levels [16]. It is possible that because C/EBPα could not robustly elicit endogenous PPARγ expression in the adipogenic supplements treated condition (Figure 4C) or adipogenic supplements might conceal the effect of C/EBPα, the efficiency of adipocyte differentiation in EBs transduced with C/EBPα might be similar to untransduced EBs. These results suggest that transient gene delivery into EBs using an optimized Ad vector could not only facilitate the efficiency of differentiation into functional cells, but could also be useful for the analysis of gene functions.

In summary, we have shown that an Ad vector containing the CA promoter has superior transduction efficiency for EBs and that the Ad vector system has potential use in basic research, particularly that regarding stem cell differentiation. Thus, this system might be a valuable tool for the molecular switching of cellular differentiation and could be applied to regenerative medicine based on ES cells.

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Chromosomal instability in human mesenchymal stem cells immortalized with human papilloma virus E6, E7, and hTERT genes

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Abstract Human mesenchymal stem cells (hMSCs) are expected to be an enormous potential source for future cell therapy, because of their self-renewing divisions and also because of their multiple-lineage differentiation. The finite lifespan of these cells, however, is a hurdle for clinical application. Recently, several hMSC lines have been established by immortalized human telomerase reverse transcriptase gene (hTERT) alone or with hTERT in combination with human papillomavirus type 16 E6/E7 genes (E6/E7) and human proto-oncogene, Bmi-1, but have not so much been characterized their karyotypic stability in detail during extended lifespan under in vitro conditions. In this report, the cells immortalized with the hTERT gene alone exhibited little change in karyotype, whereas the cells immortalized with E6/E7 plus hTERT genes or Bmi-1, E6 plus hTERT genes were unstable regarding chromosome numbers, which altered markedly during prolonged culture. Interestingly, one unique chromosomal alteration was the preferential loss of chromosome 13 in three cell lines, observed by fluorescence in situ hybridization (FISH) and comparative-genomic hybridization (CGH) analysis. The four cell lines all maintained the ability to differentiate into both osteogenic and adipogenic lineages, and two cell lines underwent neuroblastic differentiation. Thus, our results were able to provide a step forward toward fulfilling the need for a sufficient number of cells for new therapeutic

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applications, and substantiate that these cell lines are a useful model for understanding the mechanisms of chromosomal instability and differentiation of hMSCs.

Keywords Human cord blood mesenchymal stem cell · Long-term culture · Karyotype analysis · mFISH CGH · Differentiation

Introduction

Tissue-specific stem cells in various adult tissues are known to be an important source in the regeneration of damaged tissue and maintenance of homeostasis in the tissues in which they reside. Among these stem cells, human mesenchymal stem cell (hMSC) has recently become of great interest in regenerative medicine, not only to replenish their own tissues, but also to give rise to more committed progenitor cells, which can differentiate into other tissues. MSCs in bone marrow have been shown to differentiate into several types of cell such as osteoblasts, adipocytes, chondrocytes, myocytes, and probably also neuronal cells (Okamoto et al. 2002; Takeda et al. 2004; Mori et al. 2005; Saito et al. 2005; Terai et al. 2005). Because of these properties, it is expected that hMSCs are an enormous potential source for future cell therapy. The goal of our study is to establish cell lines with long lifespan and with parental properties for clinical application. However, clinical application using these cells has been met with enormous difficulty, e.g., isolation of a cell population with specific criteria, expansion in vitro system for obtaining a sufficient number of cells without affecting their genomic characteristics and differentiation properties, and their storage in higher viability.

At present, there is a little evidence suggesting whether changes in these properties occur during expansion. Human normal MSCs have a limited capacity to replicate in the 40- to 50-population doubling level (PDL) at the most. To extend their lifespan, we have previously established human mesenchymal cell lines from human umbilical cord blood or bone marrow by immortalization with human telomerase reverse transcriptase (hTERT), human papillomavirus high-risk type 16 E6/E7 genes (HPV16E6/E7) or polycomb gene, Bmi-1 (Takeda et al. 2004; Mori et al. 2005; Terai et al. 2005).

hTERT-immortalization without affecting biological characteristics, despite extensive proliferation, has been reported in bone-marrow-derived hMSCs (Burns et al. 2005), human fibroblast (Milyavsky et al. 2003), and human keratinocyte (Harada et al. 2003), although it has been indicated that there is the possibility that prolonged culture of hTERT-immortalized fibroblasts may favor the appearance of clones carrying potentially malignant alterations (Milyavsky et al. 2003). HPV16, which encodes oncogenes (E6 and E7), can also immortalize hMSCs in vitro. Both E6 and E7 proteins act through their association with tumor suppressor gene products, p53 and retinoblastoma family members (pRb), respectively. E6 accelerates the degradation of the p53 protein, which is essential for cell arrest at the checkpoint in G_1/S and at the mitotic checkpoint when tetraploidy occurs (Cross et al. 1995), as well as at the G_2 phase under damaging conditions. E7 protein binds to pRb and abrogates the repressive function of these cell cycle regulations (Zheng et al. 2001). Thus, both p53 and pRb play a multitude of important roles in cell-cycle-progression checkpoints as reported in human keratinocytes (Patel et al. 2004), and fibroblasts (Khan et al. 1998). As a consequence, the disruption of the checkpoints that govern accurate cell division leads to abnormal segregation of chromosome and genomic instability, as shown in the cells immortalized with HPV16E6/E7 genes (Duensing et al. 2002).

In this paper, we report on the chromosomal instability and the differentiation activity during prolonged culture (cell expansion) using four mesenchymal stem cell lines. These results indicate that an umbilical cord blood-derived clone immortalized with hTERT (UCBTERT-21) showed normal karyotype for a period of 1 yr, whereas three other cell lines immortalized with HPV16E6/E7 and hTERT or HPV16E6, Bmi-1 and hTERT showed chromosomal instability but maintained the ability to differentiate.

Materials and Methods

Cell culture. Human mesenchymal stem cell lines, UCB TERT-21 (JCRB1107), UCB408E6E7TERT-33 (JCRB1110), UE6E7T-3 (JCRB1136), and UBE6T-6 (JCRB1140) were obtained from the JCRB Cell Bank (Osaka, Japan). Two of them are cell lines obtained by immortalizing human umbilical cord blood mesenchymal stem cells (UCB) with hTERT alone (UCBTERT-21; Terai et al. 2005) or with HPV16E6/E7 in combination with hTERT (UCB408E6E7TERT-33; Terai et al. 2005), and the two others are human bone-marrow-derived mesenchymal stem cell lines transformed with HPV16E6/E7 and hTERT genes (UE6E7T-3; Mori et al. 2005) or with bmi-1, HPV16E6 and hTERT genes (UBE6T-6; Takeda et al. 2004; Mori et al. 2005).

The UCBTERT-21 and UCB408E6E7TERT-33 were grown in PLUSOID-M medium (Med-Shirotori Co., Tokyo, Japan) or MSCGM BulletKit (Cambrex Co., East Rutherford, NJ). UE6E7T-3 and UBE6T-6 were cultured in POWEREDBY10 medium (Med-Shirotori Co.) or MSCGM BulletKit (Cambrex Co.); 5×10^3 cells/ml of each cell line were seeded and cultured for 7–10 d. When culture

plate was subconfluent, cells were treated with 0.25% trypsin/0.5 mM EDTA solution (both from Invitrogen, Tokyo, Japan) and replated at a density of 5×10^3 cells/ml.

All of the cells were maintained in a humidified incubator at 37° C and 5% CO_2. PDLs were calculated using the formula: PDL= log (cell output/input)/log2. At the starting cultivation, PDLs of UCBTERT-21, UCB408E6E7 TERT-33, UE6E7T-3, and UBE6T-6 were 42, 67, 60, and 56, respectively. The doubling time of the UCB408E6E7T-33 cell was 1.5 d, and that of UCBTERT-21, UE6E7T-3, or UBE6T-6 was 2.6, 2.0, or 4.0 days, respectively.

Measurement of chromosome number and fluorescence in situ hybridization. Metaphase chromosome spreads for measurement of chromosome number and fluorescence in situ hybridization (FISH) were prepared from exponential growing cells at various PDL. The cells were treated in a hypotonic solution after exposure to 0.06 μg/ml colcemid (Invitrogen, Carlsbad, CA) for 2 h and fixed in methanol/acetic acid (3:1). The cells were spread on a microscope slide.

To count the number of chromosomes, the cells were stained with DAPI (4′-6-diaminido-2-phenylindol; Vector Laboratories, Inc. Burlingame, CA) and examined under an Axioplan II imaging microscope (Carl Zeiss, GmbH) equipped with Leica QFISH software (Leica Microsystems Holding, UK). To examine statistically significant chromosome numbers, we have allowed ±1 deviation and 50–100 metaphase spreads were scored for each assay.

Painting probes specific for chromosome 13 (XCP13-kit, FITC; MetaSystems, GmbH) and chromosome 17 (XCP17-kit; Texas Red) (MetaSystems GmbH, Altlussheim, Germany), and multicolor probes (mFISH-24Xcyte-kit, DAPI, FITC, TexasRed, Cy3, Cy5, and DEAC; MetaSystems GmbH) were used for FISH analysis. FISH was performed according to the manufacture's protocol (MetaSystems GmbH). Briefly, both the metaphase chromosome spread and the probe were denatured with 0.07 N NaOH or 70% formamide, hybridized at 37° C for 1–4 d, and counterstained with DAPI. FISH images were captured and analyzed on the Zeiss Axio Imaging microscope (Carl Zeiss Microimaging GmbH, Jena, Germany) with Isis mBAND/mFISH imaging Software (MetaSystems GmbH).

CGH analysis. Hybridization was carried out with the BAC Array (MAC ArrayTM Karyo 4000 Component, Macrogen Co., Rockville, MD) by the Hybstation (Genomic Solutions, Ann Arbor, MI). Briefly, test DNAs, which were isolated using an isolation kit (Amersham BioSciences, Little Chalfont, UK) and Spin Column (QIAGEN Co., Tokyo, Japan), and reference DNAs (Promega Co., Madison, WI), were labeled, respectively, with Cy3 or Cy5 (BioPrimer DNA Labeling System, Invitrogen Co.), precipitated together with ethanol in the presence of Cot-1 DNA, redissolved in a hybridization mixture (50% formamide, 10% dextran sulfate, 2xSSC, 4% sodium dodecyl sulfate [SDS], pH 7), and denatured at 75° C for 10 min. After incubation at 37° C for 30 min, each mixture was applied to an array slide and incubated at 42° C for 48–72 h. After hybridization, the slides were washed in a solution of 50% formamide—2× SSC (pH 7.0) for 15 min at 50° C, in 2× SSC—0.1% SDS for 15 min at 50° C, and in a 100-mM sodium phosphate buffer containing 0.1% Nonidet P-40 (pH 8) for 15 min at room temperature, then scanned with GenePix4000A (Axon Instruments, Union City, CA). Acquired images were analyzed with MacViewer (Macrogen Instruments).

Differentiation ability. To evaluate the differentiation potential of each cell line, cells were cultured on a coverslip in each induction medium, that is, hMSC Differentiation BulletKit-Adipogenic (PT-3004, Cambrex BioScience, Inc., Walkersville, MD) for adipocyte and NPMM Bullet kit (NPMM™ BulletKit (B3209, Cambrex BioScience) for neural progenitor cells. For osteoblast, cells were treated with 0.1 μM dexamethasone (Sigma Chemical Co., St. Louis, MO), 50 μg/ml L-ascorbic acid (Sigma Chemical), and 10 mM β-glycerophosphate (Sigma Chemical) in the PLUSOID-M medium (Med-Shirotori Co.) or the POWER-EDBY10 medium (Med-Shirotori Co.) of culture medium.

After 2–4 wk, the cells were washed in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde in PBS and stained with Oil Red-O (Sigma Chemical) for detection of adipocyte, and with alkaline phosphatase staining solution containing 0.25 mg/ml naphthol AS-BI phosphate and 0.25 mg/ml Fast violet LB salt for detection of alkaline phosphatase-positive osteoblast. In immunostaining for neuron-like cells, the cells fixed with paraformaldehyde were permeabilized with methanol at −20° C for 10 min and stained with an anti-IIIβ tubulin antibody (Sigma Chemical) or anti-neurofilament antibody NF-200 (Sigma Chemical) and Texas Red-anti-mouse IgG (Southern Biotechnology Associates, Inc., Birmingham, AL) as previously described (Takeuchi et al. 1990).

Results

Changes in chromosomal number in human mesenchymal stem cell lines in prolonged culture. Immortalization of cultured cells frequently induces an abnormal chromosome number as shown in cancer cells (Duensing et al. 2000; Munger et al. 2004; Patel et al. 2004), especially at higher frequency in long-term culture. We therefore examined four cell lines, human mesenchymal stem cell (hMSC) lines immortalized with combinations of bmi-1, E6, E7, and/or hTERT genes, for chromosome instability by counting metaphase chromosomes.

All of the lines were diploid, each containing 46 up to 40 PDL including the PDL numbers of nontransfecting original MSCs (Takeda et al. 2004; Mori et al. 2005; Terai et al. 2005). For UCBTERT-21 cell, no further changes in chromosome number have been observed up to date (for PDL 133) as shown in Fig. 1*A* and *B*. In contrast, although the UBE6T-6 cell and the UE6E7T-3 cell were near diploid, both cells exhibited considerable variation in chromosome number from PDL 70 after the culture started. For example, when the assay of UE6E7T-3 cells start at PDL 62 in culture, 90% of cell population had 46 chromosomes, but the population decreased with prolonged culturing and a population containing 44 chromosomes became dominant (43% of cell populations) at PDL 147 (Fig. 1*E*, *F*). A similar variation was also observed in UBE6T-6 cells (Fig. 1*C*, *D*).

To ascertain whether or not the changes observed were induced by transfection with HPV16E6E7, we assayed the chromosome numbers of UCB408E6E7TERT-33 cell in prolonged culture. The cell line showed similar chromosomal changes to those of the UE6E7T-3 cell, the rate of which was more rapid. At day 2 after culture by us changes became evident (PDL 68), the UCB408E6E7TERT-33 cells consisted of two distinct populations concerning chromosome number (near diploid [24%] and near tetraploid [53%]), shown in Fig. 1*G*. However, the near diploid population was unstable and decreased gradually. At PDL 81, the population became only near tetraploid, 80% of the

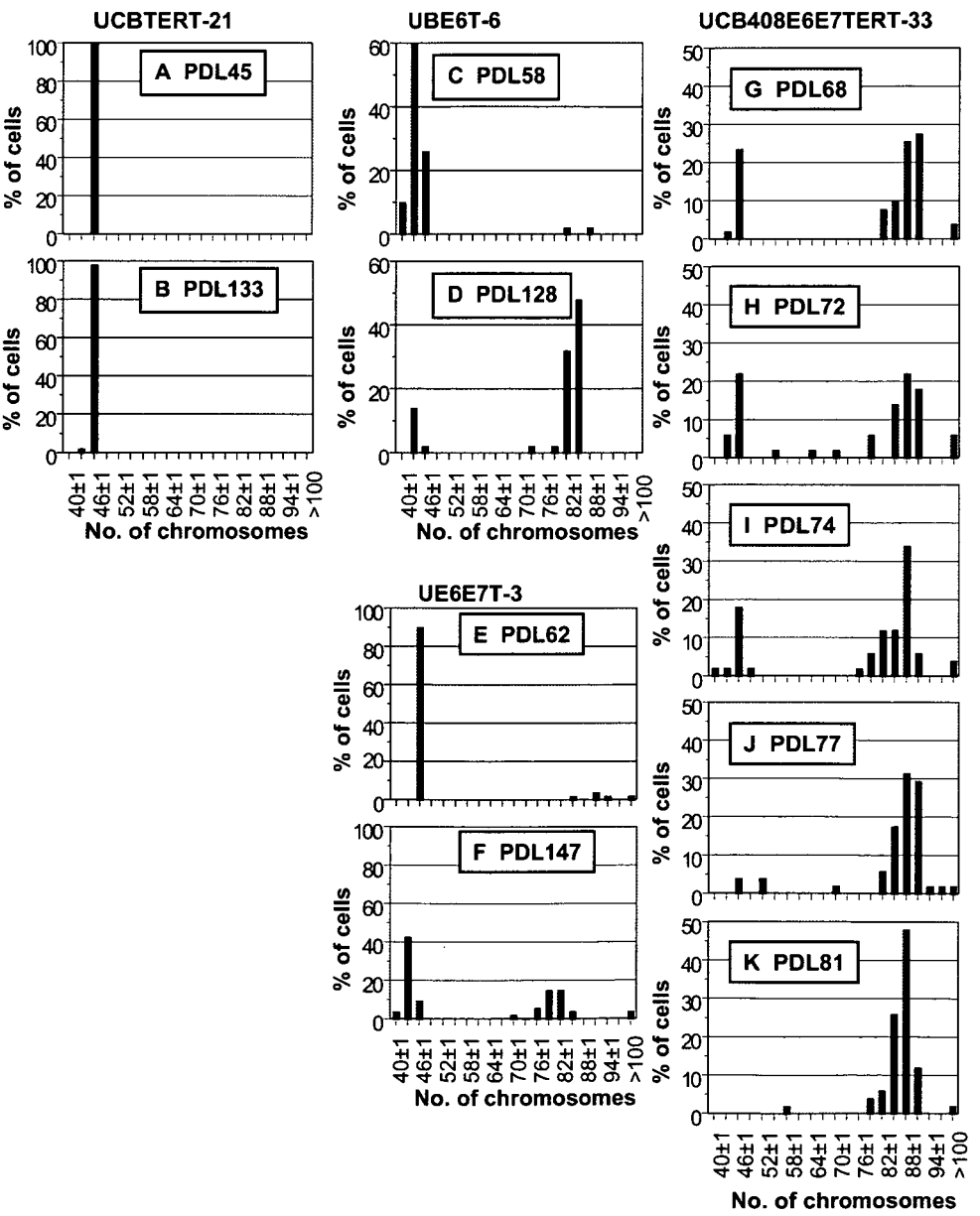

Figure 1. Changes in chromosomal numbers in prolonged cultures of four hMSC cell lines. (*A–K*) The chromosomal numbers at various culture stages were counted by DAPI staining. (*A*, *B*), (*C*, *D*), (*E*, *F*), and (*G–K*) represent the chromosomal numbers from UCBTERT-21, UBE6T-6, UE6E7T-3, and UCB408E6E7TERT-33, respectively. To examine statistically significant chromosomal numbers, we have allowed ±1 deviation, and 50–100 metaphase spreads were examined for each assay. *Note* the changes in chromosomal number from near 2*n* to near 4*n* in prolonged culture.

cells contain 85–92 chromosomes (Fig. 1*K*). The results indicate that UCBTERT-21 is relatively stable in chromosome number, whereas each of the oncogene-immortalized cells (UE6E7T-3, UBE6T-6, and UCB408E6E7TERT-33 cell) were unstable in chromosome numbers, which altered substantially during prolonged culture.

We next applied FISH and CGH analysis to characterize the chromosomal aberrations of the cell lines. All of the four cell lines passed for PDL 50 before examination by FISH. mFISH analysis of the UCBTERT-21 cell at PDL 52 showed normal chromosome composition (Fig. 2*A* and *B*) as observed in non-immortalized cells. The UBE6T-6 cell containing 43–45 chromosomes demonstrates losses of chromosome 13, 16, and 19 (marginal variation in chromosome 4 was observed among cells), but keeps on proliferating in chromosome number of 43–45 (Fig. 2*D*, *E*). In contrast, the UCB408E6E7TERT-33 cell showed more heterogeneity in chromosome composition with intrachromosomal and interchromosomal aberrations (data not shown). However, by mFISH analysis we were able to detect nonrandom losses of chromosome 13 in three cell lines except the UCBTERT-21 cell line. This was also confirmed by pFISH analysis using the probes specific for chromosome 13 and chromosome 17 (Fig. 2*C*, *F*). More than 97% of UCBTERT-21 cells showed two copies for chromosome 13, indicating the stability of the chromosomes in the cell line (Fig. 2*G*). The UE6E7T-3 and the UBE6T-6 cell lines with chromosome numbers of 43–45 showed only one copy of chromosome 13 in 76% of UE6E7T-3 cells and 86% of UBE6T-6 cells, respectively (Fig. 2*I*, *J*). A similar loss of chromosome 13 was also observed in 70% of UCB408E6E7TERT-33 cells, which showed three copies of chromosome 13 in near tetraploid (Fig. 2*H*). Other chromosomes, for example chromosome 17, were contained in the UCBTERT-21 and UBE6T-6 cell lines (Fig. 2*C*, *F*).

Furthermore, a significant nonrandom loss of chromosome 13 at the single cell-level observed by FISH was examined by array CGH, which samples the entire cell population. Figure 3 shows the array CGH profiles from early (*blue spots*) and late (*red spots*) stages of proliferating of each cell line. The UCBTERT-21 cell did not show any detectable differences in array CGH profiles between early and late stages (Fig. 3*A*). Although the loss of chromosome 13 had already occurred at early stages in the UBE6T-6 and the UCB408E6E7TERT-33 cell lines, in addition to the losses of chromosomes 4, 9, and 16 (Fig. 3*B*, *D*), in UE6E7T-3 the loss appeared between PDL 78 to 101 with loss of chromosome 16. The most compelling observation was that all three cell lines revealed a consistent whole loss of chromosome 13. These data are consistent with the results observed by FISH analysis. From these results, we

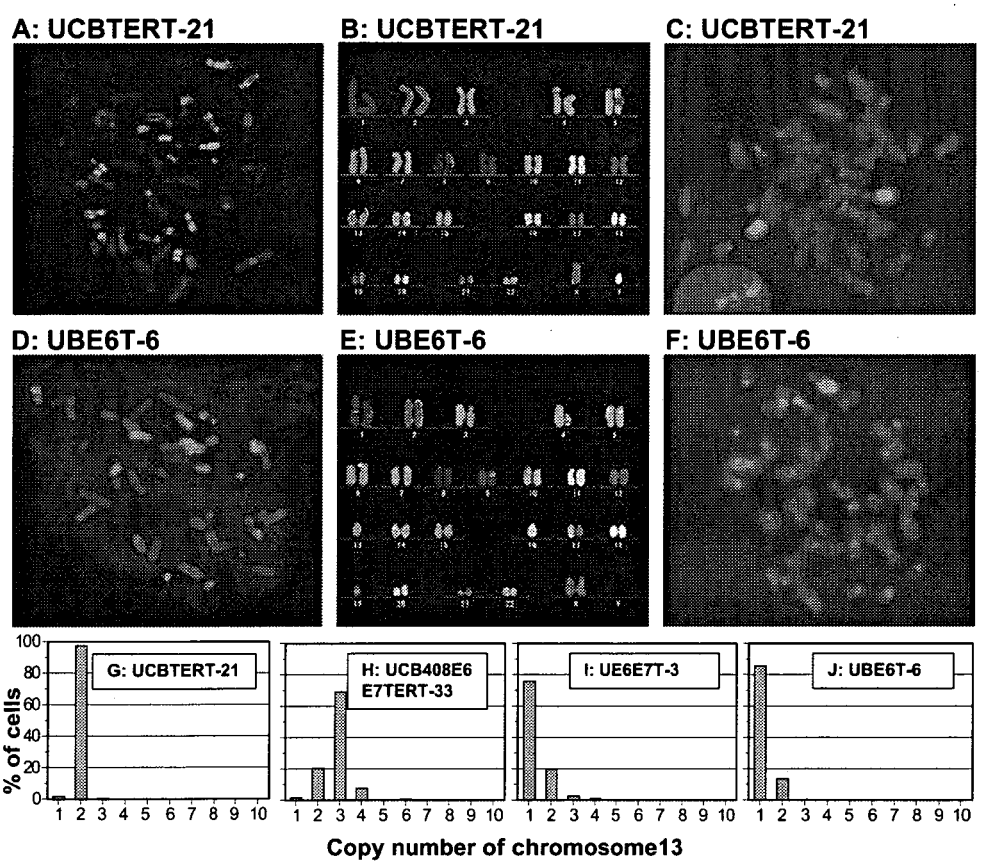

Figure 2. FISH analysis of human mesenchymal stem cell (hMSC) lines immortalized with hTERT alone, hTERT plus bm-1, HPVE6 or with hTERT plus HPVE6/E7. Multicolor FISH images of metaphase spreads (*A*, *D*), their karyotypes (*B*, *E*), and painting FISH images using DNA probes specific for chromosome 13 (*green*) and 17 (*red*) (*C*, *F*) of UCBTERT-21 (*A*, *B*, *C*) and UBE6T-6 (*D*, *E*, *F*). Quantity of chromosome 13 copy numbers in four cell lines (*G–J*). FISH signals were counted in 120~200 metaphase spreads plus interphase nuclei. UCBTERT-21 cells contained two copies of chromosome 13 and 17, and showed normal human karyotype, whereas other cells lost one copy of chromosome 13.

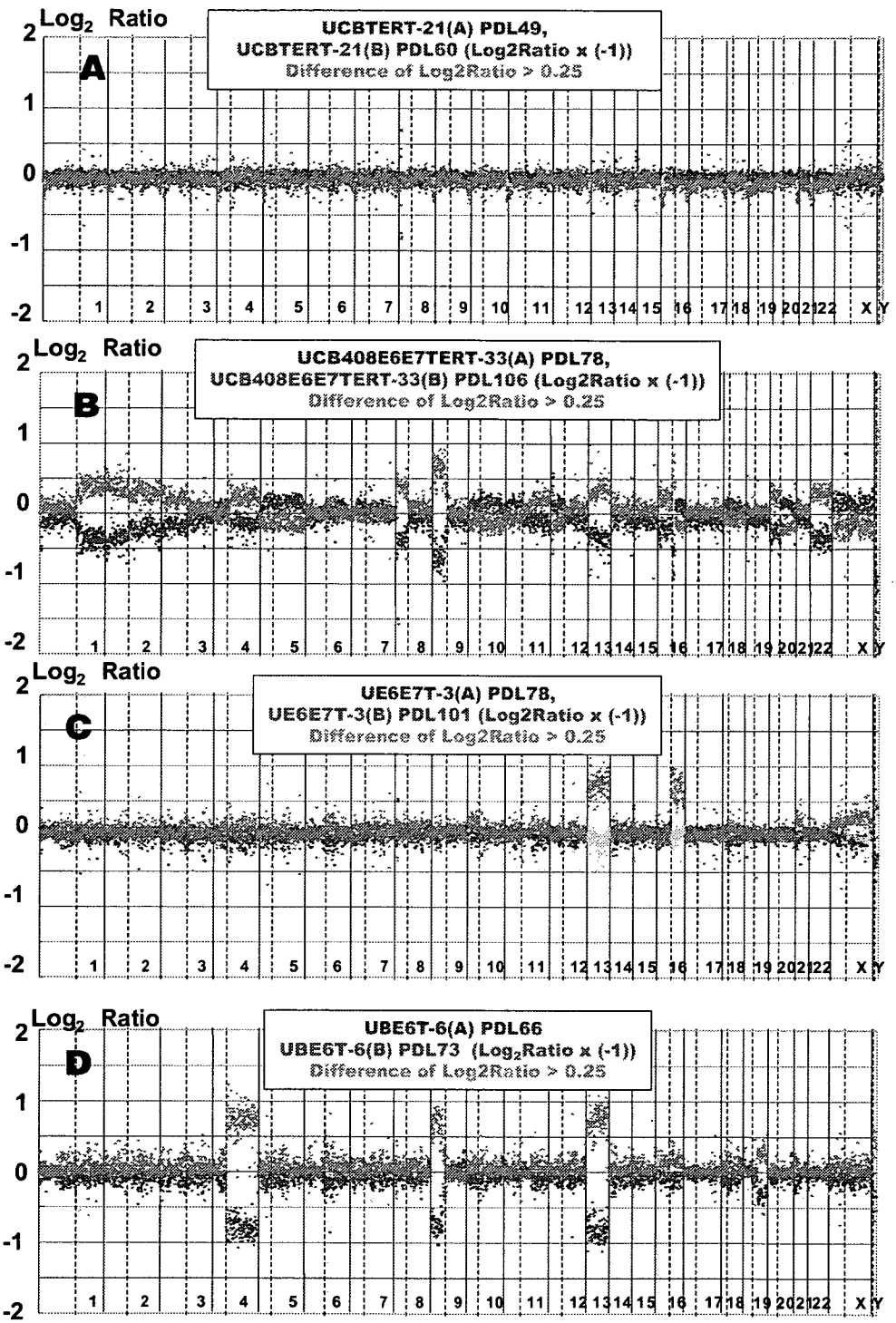

Figure 3. Array CGH profiles performed on four immortalized human mesenchymal stem cell lines at selected PDL. For each *panel*, the *X*-axis represents the 22 autosomes, the *X* and *Y* chromosomes, and the *Y*-axis shows the $\log_2$ of the fluorescence intensity ratio (cy3 [hMSCs]/cy5 [normal cell]) of all spots of the chromosome. Values above 0 (*red spots*) or values below 0 (*blue spots*) signify a loss of chromosome (chromosome regions). *Blue spots* in each *panel* indicate the $\log_2$ ratios observed at early stage in the culture of each cell line, which are overlaid with *red spots* indicated at the late stage. *Green spots* indicate the difference in value between *blue spots* and *red spot*. *Note* that in the UE6E7T-3 cell line, one copy of chromosome 13 and 16 were lost between PDL 78 and 101.

concluded that only hTERT-mediated immortalization induced little change in the chromosome numbers and chromosome structures of mesenchymal stem cells, but immortalization with Bmi-1, E6, and E7 in addition to hTERT results in chromosome instability.

Differentiation potential into lineages of immortalized mesenchymal stem cell lines. It has been reported that mesenchymal stem cells have the extensive potential to differentiate into multiple cell lineages including osteoblast, chondrocytes, adipocytes (Pittenger et al. 1999), cardiac myocytes (Makino et al. 1999), and neural cells (Pacary et al. 2006; Wislet-Gendebien et al. 2005). To evaluate whether chromosome instability of these cell lines in prolonged culture affects differentiation, cells of each cell line were stimulated in each induction medium for 2 to 4 wk. In

adipocyte-specific culture medium, all cell lines accumulated lipid-rich vacuoles in their cytoplasm within 2 wk, which were made evident by Oil Red-O staining. In particular, the UE6E7T-3 cell line showed a greater adipogenetic ability among the four cell lines (Fig. 4*Ab*). In osteoblast induction medium for 2 wk, UCB408E6E7 TERT-33 cells showed a marked increase in alkaline phosphatase expression, a marker of osteoblast, compared with those in the three other cell lines (Fig. 4*Ad*). In addition, UBE6T-6 cells in neuron induction medium reduced proliferation and displayed marked changes in morphology from being a flat-polygonal shape to taking on the characteristic neuron-like shape in which the cells develop long branching processes. Moreover, in comparing the expression patterns of characteristic neural antigens, i.e., neurofilament, III-β-tubulin, before and after induction (28 d), the pseudo-neural shaped cells showed apparent increases in immunoreactivity to both antibodies (Fig. 4*Af, Ah*),

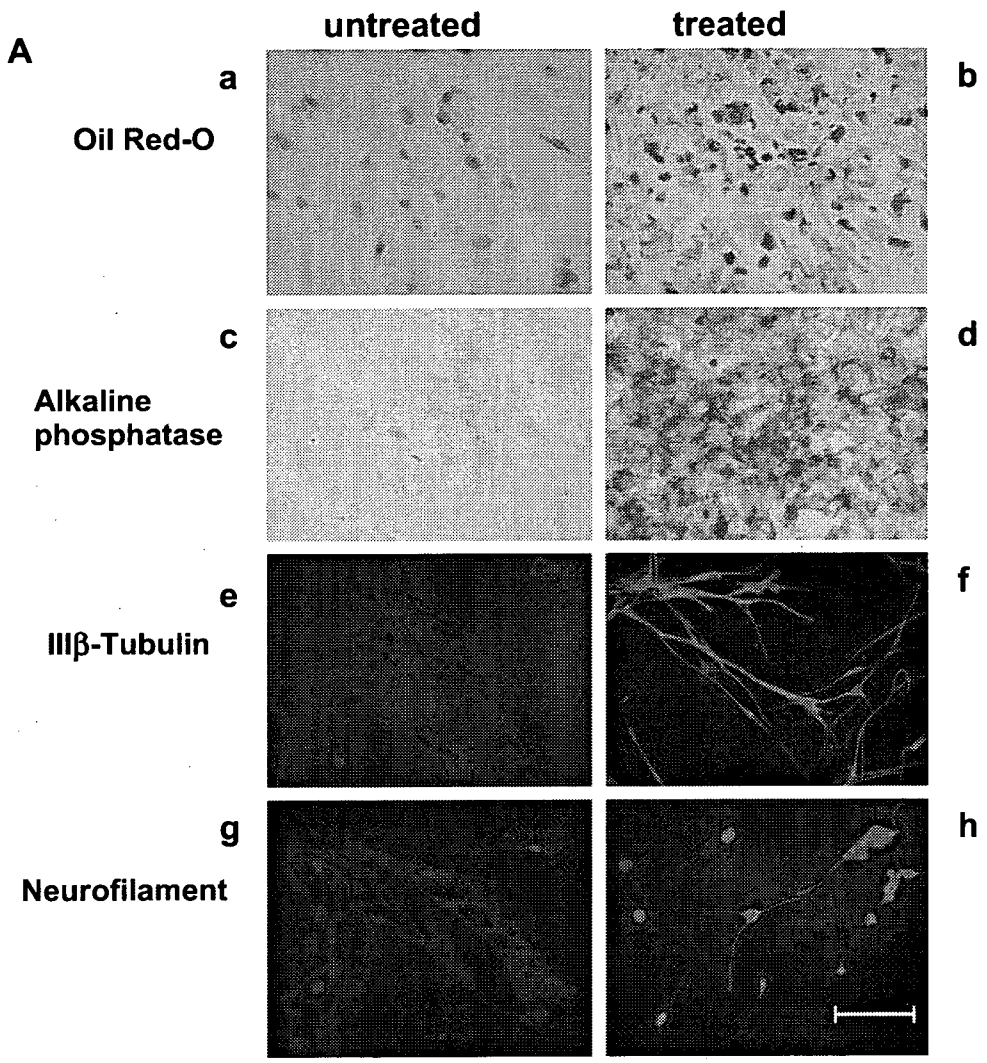

Figure 4. Differentiation potential of immortalized human mesenchymal stem cell lines into adipogenic, osteogenic, and neurogenic lineages. Adipogenesis was indicated by the accumulation of lipid stained with Oil Red-O (*Aa* and *Ab*, UE6E7T-3 cell line). Osteogenesis is indicated by the increase in alkaline phosphatase (*Ac* and *Ad*, UCB408E6E7TERT-33 cell line). Neurogenesis was shown by staining with two kinds of monoclonal antibodies to IIIβ-tubulin and neurofilament, and by shape changes of cell (*Ae–Ah*, UBE6T-6 cell line). *B*, Comparison of the differentiation potential of four cell lines whose responses to stimuli into differentiation were diverse among the cell lines. − and + indicate a response similar to an untreated cell and a weak positive response. +++ indicates a strong response shown by images of treated cells in Fig. 4*A*. (*Bar* indicates 20 μm).

B

	UCBTERT-21	UCB408E6E7TERT-33	UE6E7T-3	UBE6T-6
Oil Red-O	+	++	+++	+
Alkarine phosphatase	+	+++	+	+
IIIβ- Tubulin	-	+	+/-	+++
Neurofilament	-	++	-	++

whereas such changes were not evident with the flat-shaped cells before induction (Fig. 4*Ae*, *Ag*). Additionally, such cells did not undergo such differentiation in culture medium when cultured for as long as 30 d, although faint staining was observed. Figure 4*B* shows the overall results of differentiation potential of the four cell lines into adipogenic, osteogenic, and neurogenic lineages. These immortalized mesenchymal stem cell lines retained the ability to differentiate into three lineages, although among cell lines there are significant variations in response to lineage-specific induction.

Discussion

Attempts to clarify the mechanisms for extending the lifespan of tumor cells have been made for many years, and several genes that have effects on cellular proliferation and survival have become clear (Munger et al. 2002) in addition to the elucidation that the majority of tumor cells express telomerase (hTERT; Armanios et al. 2005). The goal of one of the series of our studies has been to establish cell lines with long lifespan and with parental properties, on the basis of genotypic and phenotypic characterizations, for application to cell-based therapy. We previously established several cell lines (Takeda et al. 2004; Mori et al. 2005; Terai et al. 2005), and the present study demonstrated that UCBTERT-21, the immortalized cell line derived from human umbilical cord blood-derived MSCs with hTERT, has a normal karyotype and has an extended lifespan by at least 133 population doublings, and has the differentiation potential into the adipocyte or osteoblast similar to parental MSCs (Terai et al. 2005), although the potential was weak but clearly positive in this study. The specific environmental cues to initiate the differentiation of hMSCs are not yet clear.

UCBTERT-21 immortalized with hTERT alone can be prolonged without inhibition of the $p16^{INK4A}$/RB pathway (Terai et al. 2005), the result of which is in agreement with reports that hTERT alone significantly extends the lifespan of human fibroblasts, epithelial, and endothelial cells (Bodnar et al. 1998; Chang et al. 2005), without the requirement for molecular alterations in p53/p21 and pRB/$p16^{INK4A}$ pathways (Milyavsky et al. 2003). However, other researchers have indicated that inactivation of the RB/p16 pathway by E7, or downregulation of p16 expression, in addition to increasing telomerase activities, is necessary for expanding the lifespan of human keratinocytes (Dickson et al. 2000; Kiyono et al. 1998). Thus, the possibility that a telomere-independent barrier may operate to prevent immortalization according to cell types has been indicated.

UCB408E6E7TERT-33, UE6E7T-3, and UBE6T-6 are hMSC-clones immortalized with HPV16E6/E7 or polycomb group oncogene Bmi-1, in combination with hTERT. Immortalization of human keratinocyte in vitro using virus-derived oncogenes such as E6 and E7 is based on initial inactivation of the p53 and/or Rb pathways, which are essential for controlling cell cycle progression in response to DNA damage or after induction tetraploidy; therefore, this gene transduction induces chromosomal abnormalities (Solinas-Toldo et al. 1997; Duensing et al. 2002; Patel et al. 2004; Schaeffer et al. 2004). The cell lines used in this study became completely immortal, yet underwent dynamic changes in their chromosome numbers in prolonged culture. Near diploid population in early passage of UCB408E6E7 TERT-33 became near-tetraploid population with prolonged culture without the appearance of intermediate populations (60–70 chromosomes/cell), and thereafter gave rise to a population having smaller numbers of chromosomes than tetraploid. Similar patterns existed, although at a slower rate, in UBE6T-6 cells and UE6E7T-3. These results suggest that HPVE6 and E7 proteins cause tetraploidy that precedes the chromosomal aberration to aneuploid in E6/E7-immotalized hMSCs, as is currently shown in several lines of evidence. For example, in vitro experiments in human cell lines (N/TERT-1 keratinocytes and HeLa cells) demonstrate that chromosome nondisjunction yields tetraploid rather than aneuploid, and that aneuploid may develop through chromosomal loss from tetraploid, although the mechanistic basis for the tetraploid formation still remains to be elucidated (Shi et al. 2005). This is also suggested from evidence that high frequency of tetraproidy is present with aneuploidy in human tumors (Olaharski et al. 2006; Sen 2000). A distinct pattern of aneuploid became apparent using dual-probe FISH and CGH analyses, in which UCB408E6E7TERT-33 cells predominantly exhibited triploid 13 and tetraploidy 17 together with other chromosomal changes as shown in Figs. 2 and 3. However, surprisingly, the loss of one copy of chromosome 13 was also seen in 70–80% of diploid UE6E7T-3 and diploid UBE6T-6 cells retaining two copies of chromosome 17. The loss occurred in PDL 50 in both UE6E7T-6 and UCB408E6E7TERT-33, and between PDL 78 and 101 in UE6E7T-3. Structural and numerical aberrations targeting chromosome 17 are often reported in tumors from various tissues (Olaharski et al. 2006), whereas the pattern that chromosome 13 is lost and chromosome 17 is stable, was common for the three cell lines in this study, indicating the possibility that the loss of chromosome 13 may play an important role in the chromosomal aberration of hMSCs to acquire growth advantages under the given culturing condition. Similar karyotypic changes were evident in cultured human embryonic stem cells, involving the gain of chromosome 17 or chromosome 12 (Carlson et al. 2000; Draper et al. 2004). It is thus conjectured that the aneuploidy developed through chromosomal loss from

diploid cells arises through different mechanisms from tetraploid intermediate.

An alternative explanation for aneuploid formation mechanism independent of tetraploid intermediate is loss of regulation in centrosome duplication, leading to abnormal centrosome amplification and multipolar spindles, resulting in aneuploidy. In addition, centrosome amplification caused by loss of p53 has been shown in cultured mouse cells (Fukasawa et al. 1996), but not in cultured human cells (Kawamura et al. 2004). However, loss of p53 and centrosome amplification has been revealed in human cancer tissue. Our preliminary examination has indicated a weak correlation between centrosome amplification and chromosome number (data not shown). Only 2.4% of UCBTERT-21 cells contained >3 centrosomes per cell, whereas 11.9% of UCB408E6E7TERT-33, 19.1% of UE6E7T-3 and 14.3% of UBE6T-6 cells contained >3 centrosomes per cell. Thus, further study is still needed to clarify the mechanism inducing chromosomal instability in immortalized hMSCs cultured over a long period.

Human mesenchymal stem cells are thought to be multipotent cells that can replicate stem cells and that can differentiate to lineages of mesenchymal tissues including bone, fat, tendon, and muscle. Our results indicated that immortalized hMSCs, except UCBTERT-21, induced changes in chromosome number over prolonged culture, but these cells have still retained the ability to both proliferate and differentiate. Immortalized UBE6T-6 cells also displayed neuron-like morphology and strong expression of the neuron-specific markers of neurofilament and III-β-tubulin. We previously demonstrated that hTERT, E7-immortalized hMSCs differentiate into neural cells in vitro on the basis of morphological changes, expression of neural markers such as nestin, neurofilament, MAP-2, Nurr1, and III-β-tubulin. Furthermore, the physiological function showed reversible calcium uptake in response to extracellular potassium concentration (Mori et al. 2005). Similar observations have been reported using rat MSCs (Wislet-Gendebien et al. 2003; Wislet-Gendebien et al. 2005; Pacary et al. 2006). In preliminary experiment of cell transplantation that 10^6 cells of UCBTERT-21 cell (PDLs 120) or UCB408E6E7TERT-33 cell (PDLs 200) were injected into nude mice subcutaneously, no tumorigenicity was observed (data not shown).

In conclusion, our study showed that the hTERT-immortalized cell line displayed normal karyotype and differentiation ability in prolonged culture. These results provide a step forward toward supplying a sufficient number of cells for new therapeutic approaches. In addition, oncogene-immortalized cell lines exhibited abnormal karyotype accompanying the preferential loss of chromosome 13 but without differential alteration during prolonged culture. Thus, the results could provide a useful model for understanding the mechanisms of the chromosomal instability and the differentiation of hMSC.

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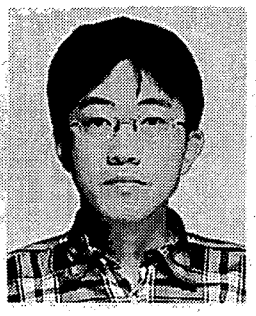
各種幹細胞への高効率遺伝子導入法の開発とその応用

川端健二　独立行政法人 医薬基盤研究所基盤的研究部遺伝子導入制御プロジェクト



1992年に京都大学薬学部を卒業後，同大学院薬学研究科薬剤学教室(橋田 充教授)に進学し，プラスミドDNAを用いた遺伝子治療に関する研究に従事．1997年に薬学博士の学位を取得．学位取得後，大阪府立母子保健総合医療センター研究所免疫部門(長澤丘司部長，現 京都大学教授)に流動研究員および日本学術振興会特別研究員して所属し，ケモカインSDF-1(CXCL12)およびその受容体CXCR4遺伝子欠損マウスにおける造血機能の解析研究に従事．

2002年，国立医薬品食品衛生研究所遺伝子細胞医薬部研究員(山口照英部長)として採用される．2004年より同 研究所大阪支所医薬基盤研究施設に異動，2005年4月より独立行政法人医薬基盤研究所基盤的研究部遺伝子導入制御プロジェクト主任研究員(水口裕之プロジェクトリーダー)，現在に至る．

現在の主な研究テーマは，改良型アデノウイルスベクターを用いた各種幹細胞の高効率分化法の開発，およびアデノウイルスベクターによる自然免疫応答の分子メカニズムの解明．

(かわばた けんじ)

幹細胞(stem cells)は，自己複製能と分化多能性という大きく二つの特長を有する細胞であり，過去には概念としてその存在が唱えられていたが，幹細胞マーカーの発見や機能アッセイ法の確立により，現在では実体として捉えられるようになった．幹細胞と一口に言っても，造血幹細胞，神経幹細胞，間葉系幹細胞，ES細胞など多種の幹細胞が発見されている．また最近では，腫瘍組織の一部の細胞が幹細胞様の性質を有しており，この細胞の存在が放射線治療や化学療法を困難にしているというがん幹細胞(cancer stem cells)仮説も提唱されている．

これらの幹細胞は，種々の細胞に分化可能であることから治療への応用が考えられ，そのなかでも造血幹細胞は実際に骨髄移植療法のなかで汎用されている．また，1998年にはヒトES細胞が胚盤胞内部細胞塊から樹立されたことにより，幹細胞を再生医療へ応用することが一層期待されている．しかしながら，幹細胞を直接生体に移植するには困難な場合も多く，たとえばES細胞はマウス生体に投与するとランダムに分化しテラトーマ(奇形腫)を形成する．したがって，治療目的には幹細胞を *in vitro* で目的の細胞に分化させたのち生体に移植することが望ましいと考えられる．幹細胞を骨，心筋，脂肪，血液などの目的の細胞に分化させるには，培養液に特定の液性因子を加える方法がとられているが分化効率は充分ではない．

そこで，筆者らは各種幹細胞に機能遺伝子を導入することにより効率よく分化させることが出来ないかと考え研究を進めている．一般に，幹細胞は遺伝子導入が困難であり，リポフェクション法やレトロウイルスベクター系など，通常用いられる方法では充分な導入効率が得られない．

アデノウイルスベクターは，CAR(coxsackievirus and adenovirus receptor)とよばれる膜蛋白質を受容体として感染し，高効率・一過性に外来遺伝子を発現させることが可能な系として遺伝子治療や基礎研究に汎用されているベクターであるが，多くの幹細胞のようにCARを発現していない細胞に対しては遺伝子導入することが出来ない．筆者の所属する研究室では，従来型(5型)アデノウイルスベクターだけでなく，CAR陰性の細胞に対しても高効率な遺伝子導入が可能となるように種々の改良型アデノウイルスベクターを開発してきた(図1)[1]．これらのベクターを用いることにより，ES細胞，間葉系幹細胞，造血幹細胞に対して，それぞ

若手研究者のひろば

Development of efficient gene delivery system into stem cells

Kenji Kawabata
Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation

本欄はDDS研究に携わる若手研究者の自己紹介を兼ねて日頃の研究内容，研究成果を広くアピールする欄です．人選は本誌編集委員，およびDDS学会役員の推薦によります．

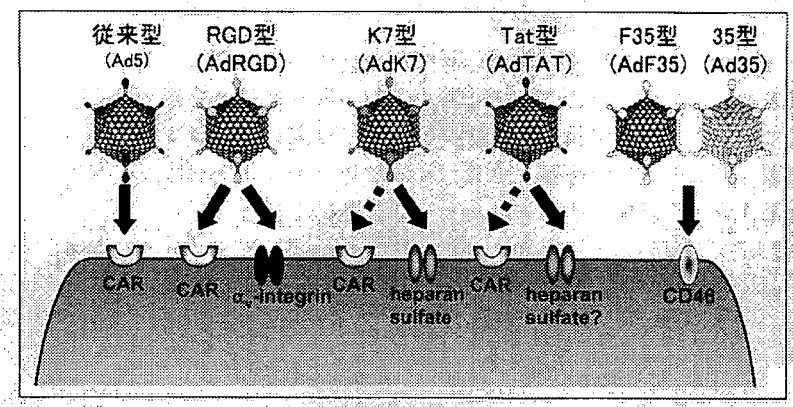


図1　筆者らが独自に開発した改良型アデノウイルスベクター

表1　改良型アデノウイルスベクターを用いた各種細胞への遺伝子導入効率

	従来型 Ad	改良型 Ad
ヒト造血幹細胞（CD34陽性細胞）	5%以下	50%以上（Ad35）
ヒト間葉系幹細胞	10%以下	100%（AdK7）
マウスES細胞	10%以下	90%以上（Ad5-EF1α プロモーター）

れ従来型アデノウイルスベクターの10倍以上の遺伝子導入効率が達成可能となった（表1）[2]．また，最適化されたアデノウイルスベクターを用いて幹細胞の分化を制御することも試みている．マウスES細胞は，LIF（leukemia inhibitory factor）の存在下で未分化を維持できることが知られている．LIFはその受容体に結合後，下流のアダプター分子であるSTAT3（signal transducer and activator of transcription 3）を介して未分化シグナルを伝達する．

そこで，アデノウイルスベクターを用いてSTAT3のdominant-negative変異体（STAT3F）cDNAを導入しSTAT3シグナルを遮断したところ，LIF存在下であるにもかかわらず，ES細胞は中胚葉，外胚葉，内胚葉いずれにも分化することが明らかとなった．さらに，STAT3F cDNAとNanog（ES細胞の未分化維持に必須の転写因子）cDNAを共導入したところ，ES細胞は未分化を維持しつづけた．したがって，アデノウイルスベクターを用いて機能遺伝子を導入することによりES細胞の分化・未分化を自由に制御できる可能性が示された[3]．現在までに，骨，筋肉，血液など，各分化系列への分化に必須のマスター遺伝子がつぎつぎと明らかにされており，アデノウイルスベクターを用いてこれらの遺伝子を各種幹細胞に導入することにより，目的の細胞に効率よく分化誘導することが可能になるものと期待される．

幹細胞を目的の細胞に分化させたあとは，副作用軽減の観点から遺伝子発現はそれ以上持続しないことが望ましい．したがって，遺伝子導入による幹細胞の分化誘導にアデノウイルスベクター用いることの長所は，その高い遺伝子導入効率だけでなく，発現が一過性であることもあげられる．

これまで述べてきたように，アデノウイルスベクターは幹細胞を用いた再生医療への応用に重要なツールとなる可能性を有している．現在，「つぎはどんな細胞に分化させたら面白いか」ということを考えながら研究を進めているところである．

文　献

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改良型アデノウイルスベクターを用いた各種幹細胞への遺伝子デリバリー

特集　遺伝子・核酸医薬品のデリバリー

川端健二・櫻井文教[*1)]，水口裕之[*1,2)]

Gene delivery into stem cells by modified adenovirus vectors

The application of adenovirus (Ad) vectors, which are widely used in gene therapy, depends on CAR (coxsackievirus and adenovirus receptor) expression on the cells. To overcome this problem, the capsid proteins of Ad vectors have been genetically modified. Here, we introduce several types of capsid-modified Ad vectors. Furthermore, we describe the application of capsid-modified Ad vectors into some kinds of stem cells for regenerative medicine.

アデノウイルスベクターは，遺伝子治療や基礎研究に幅広く用いられている．しかしながら，アデノウイルスベクターの受容体であるCARの発現が乏しい細胞では，アデノウイルスベクターによる遺伝子導入効率は低い．そこで筆者らは，CAR非依存的に遺伝子導入可能な種々のカプシド改変型アデノウイルスベクターを開発してきた．本稿では，これらのカプシド改変型アデノウイルスベクターの特徴と，その応用例として，近年，再生医療分野で注目を浴びている各種幹細胞への高効率遺伝子導入法について解説する．

Kenji Kawabata · Fuminori Sakurai[*1)]*, Hiroyuki Mizuguchi*[*1,2)]

key words : adenovirus vector, gene delivery, stem cells, regenerative medicine, gene therapy

ヒトアデノウイルスは，赤血球凝集活性の違いからAからFまでのサブグループにわけられ，計51種の血清型が存在する．遺伝子治療用ベクターとして繁用されているアデノウイルスベクターは，サブグループCに属するヒト5型アデノウイルスを基盤としている．5型アデノウイルスの感染は，カプシド蛋白質のファイバーが細胞表面に存在するCAR(coxsackievirus and adenovirus receptor)と結合することにより起こる．そのため，従来の5型アデノウイルスベクターは，CAR陽性細胞へは効率よく遺伝子導入可能であるが，CARの発現が乏しい細胞への遺伝子導入効率はきわめて低いことが課題であった．

CARの発現が乏しい細胞としては，造血幹細胞や間葉系幹細胞などの幹細胞，血液細胞，悪性度の高いがん細胞，血管内皮細胞などがあげられ，このような細胞へはアデノウイルスベクターの適用は不向きであった．

本稿では，CAR陰性細胞に対しても高効率遺伝子導入が可能な種々の改良型アデノウイルスベクターについて概説し，つぎにその応用例として，近年，再生医療への応用が期待されている幹細胞への高効率遺伝子導入法について紹介する．

改良型アデノウイルスベクター

1. ファイバー改変型アデノウイルスベクター

アデノウイルスのファイバーはノブ，シャフト，テール領域にわけられ，ノブ領域がCARと結合する(**図1a**)．アデノウイルスベクターの感染域を拡大するためのアプローチの一つとして，ファイバーノブのHIループやC末端領域に細胞接着活性などを有する外来ペプチドを遺伝子工学的に挿入することがあげられる．

筆者らは，これらの部位に外来ペプチドをコードした遺伝子をきわめて簡便に挿入できるファイバー

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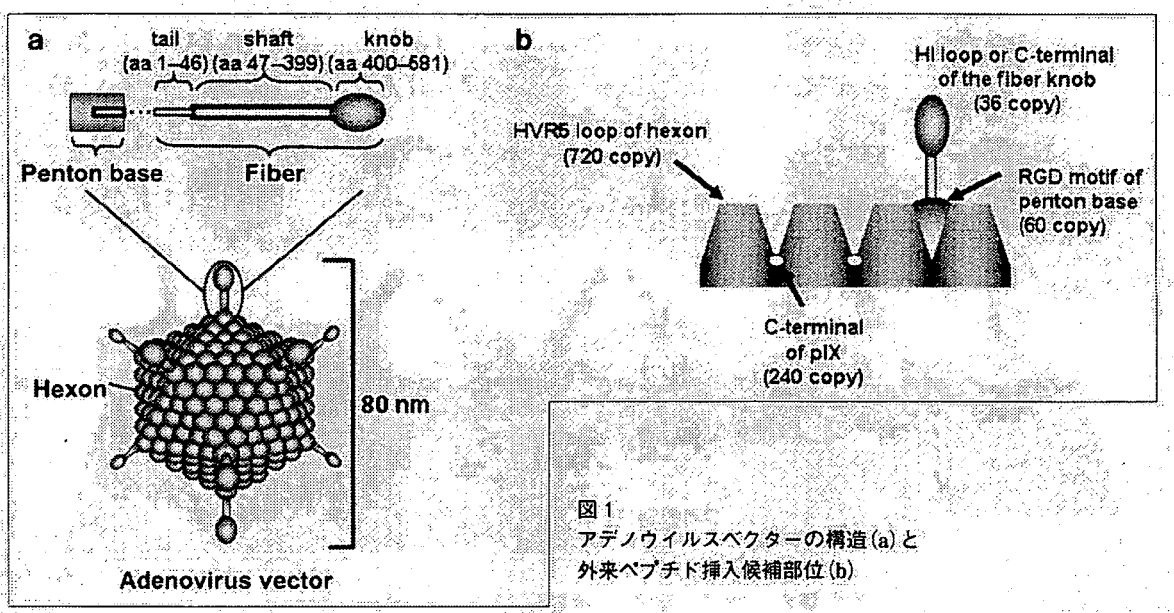

図1
アデノウイルスベクターの構造(a)と
外来ペプチド挿入候補部位(b)

改変型アデノウイルスベクター作製法を開発済みであり[1,2]，この技術と *in vitro* ライゲーションに基づいたE1欠損領域への外来遺伝子挿入法[3,4]を組み合わせることにより，CAR陰性細胞に対しても高効率に遺伝子導入が可能なアデノウイルスベクターを簡便に作製する方法を開発した(**図2**，**表1**)．ファイバーノブのHIループにRGD(リジン-グリシン-アスパラギン酸)からなるペプチドを挿入しインテグリンと親和性を保持させることにより(RGD型ベクター)，種々のがん細胞[5,6]や樹状細胞[7]，血管内皮細胞[8]に高効率な遺伝子導入が可能となった．また，ファイバーノブのC末端領域に七つのリジン残基からなるポリペプチドを挿入したファイバー改変型(K7型)アデノウイルスベクターでは，ヘパラン硫酸と親和性を有するようになり，種々のCAR陰性細胞に効率よく遺伝子導入が可能である[2,9]．

さらに筆者らは，最近，HIV(human immunodeficiency virus)由来のprotein transduction domain(PTD：蛋白質導入ドメイン)として知られているTatペプチド[10]をファイバーノブに付与することで，RGD配列やポリリジン配列を付与したベクターよりも，より広範に効率よく外来遺伝子を発現可能であることを見いだした．したがって，Tatペプチドを付与したアデノウイルスベクターは，遺伝子治療用ベクターや基礎研究におけるツールとしてきわめて有用であると考えられる．

2. ファイバー置換型アデノウイルスベクター

サブグループBに属する11型あるいは35型などのアデノウイルスはCARではなく，補体制御因子として知られているCD46を受容体として感染することが知られている[11,12]．

そこで筆者らは，5型アデノウイルスベクターのファイバー領域のみを35型アデノウイルスのものに置換したベクター(F35型ベクター)やすべての構造蛋白質を35型アデノウイルスからなるベクターを開発した(**図2**)[13~15]．ヒトにおいては，CD46は赤血球を除くほぼすべての細胞に発現していることが知られており，これらのベクターは，多くのヒト由来細胞だけではなく，たとえば5型アデノウイルスベクターでの遺伝子導入が困難で，遺伝子治療の重要な標的細胞であるCD34陽性ヒト造血幹細胞にも効率よく遺伝子導入可能であることが明らかとなった[14,16]．

3. ヘキソン，pIX改変型アデノウイルスベクター

ファイバーは，ウイルス1粒子当たり12分子存在するが(ファイバーは3量体を形成するため，ノブは36コピー存在する)，主要なカプシド蛋白質のヘキソンは240分子(同じく3量体をとっている

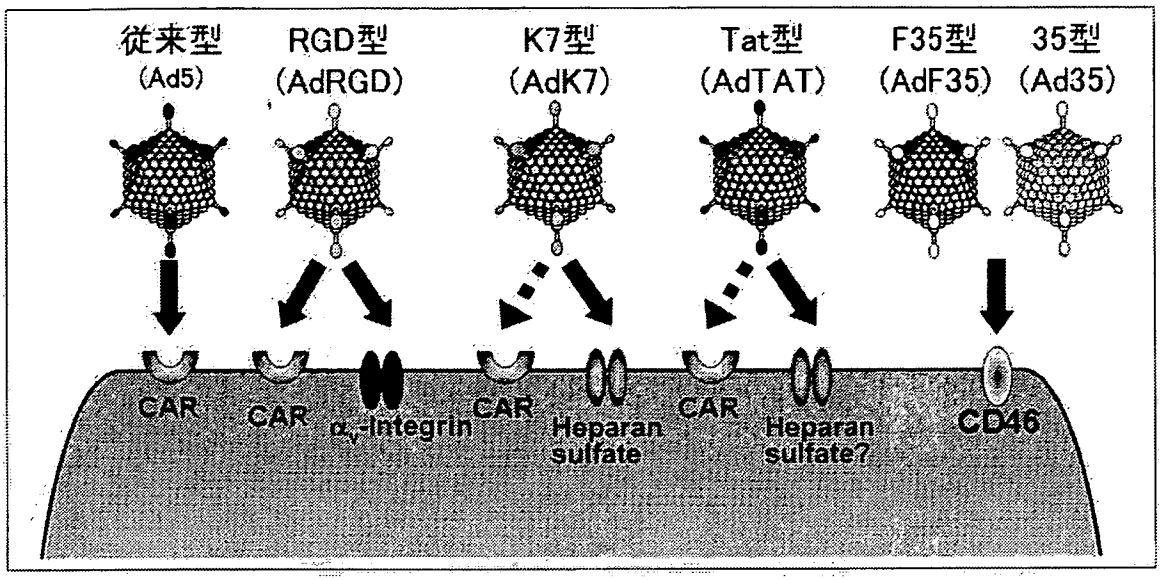

図2　筆者らが独自に開発した改良型アデノウイルスベクター

野生型のファイバーを持った従来の5型アデノウイルスベクターは細胞表面上の受容体であるCARを認識して感染するが，RGD配列やポリリジン配列をファイバーに有したファイバー改変型ベクターはCARだけでなく，αvインテグリンやヘパラン硫酸を認識しても感染できる．また，35型のアデノウイルスのファイバーを有したベクターや，すべての構造蛋白質が35型アデノウイルスからなるベクターは，CD46を認識して感染する．Tat型ベクターは未知の機構により(ヘパラン硫酸を介するという報告もある)細胞内に取り込まれる．

表1　改良型アデノウイルスベクターを用いた各種細胞への遺伝子導入効率

	従来型 Ad	改良型 Ad
ヒト造血幹細胞 (CD34 陽性細胞)	5%以下	50%以上(Ad35)
ヒト間葉系幹細胞	10%以下	100%(AdK7)
マウス ES 細胞	10%以下	90%以上 (Ad5-EF1α-プロモーター)
樹状細胞 (ヒト・マウス)	10%以下	90%以上(AdRGD)
CAR 陰性がん細胞 (ヒト・マウス)	10%以下	100%(AdRGD)
マウス脂肪細胞	10%以下	50%以上(AdK7)

ため，720コピー存在する)，pIX(プロテインIX)は240分子(240コピー)存在するため，これらの領域を改変できれば，より効率のよい遺伝子導入が可能になることが期待される(**図1b**)．ヘキソンは，ウイルス粒子の中で最も豊富に存在する蛋白質であり，カプシドの構造を維持する役割を有する．またpIXは，ヘキソンカプソマーの間に挟まれた形で存在し，ヘキソン同士の結合を補助する．

そこで，ヘキソンのhypervariable region 5 (HPV5)およびpIXのC末端に外来ペプチドを挿入できるベクター系を構築し，ファイバー改変型，ヘキソン改変型，pIX改変型各アデノウイルスベクターの遺伝子発現効率について比較検討した[17]．

各挿入部位にRGDペプチドを挿入した結果，ファイバーノブのHIループにRGDペプチドを挿入したファイバー改変型アデノウイルスベクターが最も高い遺伝子発現効率を示した．これは，ヘキソンやpIXと比較し，ファイバーノブは最も外側に位置するので，宿主細胞と結合しやすくなっていること，およびヘキソンやpIXに発現させたペプチドはファイバーによる立体障害のため，細胞表面に作用しにくくなっている可能性が原因として考えられる．

したがって，ヘキソンやpIXを改変する場合，ファイバーを遺伝子工学的に欠損させた(ファイバーレス)アデノウイルスベクターを基盤ベクターとすることにより遺伝子発現効率が改善する可能性が考えられ(この場合，同時にCAR経由による遺伝子導入も起こらないため，ターゲティングアデノウイルスベクターの開発にもつながる)，現在検討中である．

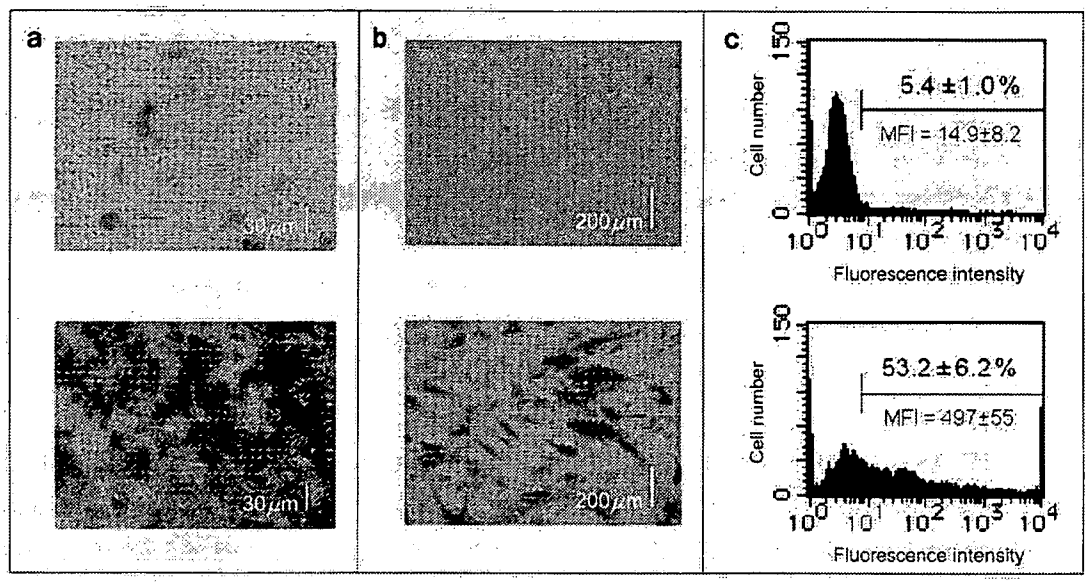

図3　改良型アデノウイルスベクターによる各種幹細胞への高効率遺伝子導入

a：マウスES細胞に対し，CMVプロモーター(上段)あるいはEF-1αプロモーター(下段)有するβ-ガラクトシダーゼ遺伝子を発現する従来型アデノウイルスベクターを感染させた．

b：ヒト間葉系幹細胞に対し，従来型(上段)あるいはK7型(下段)アデノウイルスベクターを用いてβ-ガラクトシダーゼ遺伝子を発現させた．

c：ヒト造血幹細胞に対し，従来型(上段)あるいは35型(下段)アデノウイルスベクターを用いてGFP遺伝子を発現させた．

ES細胞への高効率遺伝子導入法

ES細胞は胚盤胞内部細胞塊由来の細胞であり，無限に増殖するとともにすべての機能細胞に分化する性質を有する．近年，ヒトES細胞が樹立されたことにより，これを再生医療へ応用するための基礎研究が活発に行われている[18]．しかしながら，ES細胞の分化を自由に制御する技術はいまだ確立されておらず，その原因の一つとしてES細胞への効率よい遺伝子導入法が確立されていないことがあげられる．

これまで，ES細胞に対しては，プラスミドDNAを用いたエレクトロポレーション法(プラスミドDNAを電気的刺激により細胞内に導入し，染色体にわずかに目的遺伝子と薬剤耐性遺伝子が組み込まれた細胞を薬剤で選択する方法)[19]，レトロウイルスベクター[20]，レンチウイルスベクター[21]，ポリオーマウイルスの複製機構を利用したスーパートランスフェクション法(ポリオーマウイルスの複製起点を含んだプラスミドDNAがマウスES細胞ではエピゾーマルに増幅できる性質を利用した方法)[22]などが外来遺伝子導入法として用いられてきた．

しかしながら，これらは半永久的に導入遺伝子を発現しつづける方法であり，ES細胞の分化制御，特に医療目的などの細胞分化後には発現を停止させたい場合には好ましくない．アデノウイルスベクターは，導入遺伝子が宿主染色体へ組み込まれることなく，染色体外にエピゾームとして存在することから(増幅しない)，遺伝子発現が一過性であり，ES細胞を目的の機能細胞に分化させたあとは導入遺伝子の発現が消失するものと期待される．

そこで，筆者らは，マウスES細胞に最も適したアデノウイルスベクターによる遺伝子導入法の確立を試みた．その結果，マウスES細胞はアデノウイルス受容体CARを高発現しており，従来型アデノウイルスベクターが最適であることが明らかとなった[23]．また，RSV，CMV，CA(β-actin promoter/CMV enhancer)，EF-1αの4種のプロモーターを用いて検討した結果，ES細胞にはCAおよびEF-1αプロモーターを用いた場合にのみ遺

伝子発現がみられ，RSV や CMV プロモーターはほとんど機能しなかった(図 3a)．

これまでアデノウイルスベクターは，ES 細胞への遺伝子導入には不適と考えられてきたが，これは多くの場合，最も一般的に用いられている CMV プロモーターを用いて検討されてきたためであり，ウイルスの細胞へのエントリー自体には問題がないことが示された．ただし，CA プロモーターを用いた場合には，ES 細胞のみならずその支持細胞(フィーダー細胞)である胚線維芽細胞にも遺伝子発現がみられたのに対し，EF-1α プロモーターを用いた場合には，ほぼ ES 細胞特異的に遺伝子発現可能であった．これは，EF-1α プロモーターの活性が胚線維芽細胞にくらべ ES 細胞において相対的に高いことが原因と考えられる．したがって，目的により両プロモーターを使いわけることによって，再生医療への幅広い応用が期待できる．

つぎに，最適化されたアデノウイルスベクターを用いて ES 細胞に機能遺伝子を導入し，実際に ES 細胞の分化を制御できるかどうかについて検討した．マウス ES 細胞は，フィーダー細胞由来のサイトカイン LIF (leukemia inhibitory factor)がその未分化維持に必須であることが知られている．LIF は受容体に結合後，下流の STAT3 (signal transducer and activator of transcription 3)を介してシグナルを伝達する．

そこで，EF-1α プロモーターを有した従来型アデノウイルスベクターを用いて，STAT3 の dominant-negative 変異体(STAT3F) の cDNA をマウス ES 細胞に導入することにより，LIF の下流シグナルを阻害させたところ，LIF 存在下でも ES 細胞は三胚葉すべての細胞に分化することが明らかとなった．ES 細胞の未分化維持には，LIF 以外にも Nanog などの転写因子が必須であることが明らかとなっている．

そこで，先述のベクターを用いて STAT3F と Nanog を同時に発現させたところ，STAT3F による細胞分化シグナルが Nanog 発現により阻害され，ES 細胞は未分化状態を維持しつづけた．したがって，アデノウイルスベクターを用いることで ES 細胞の分化を自由に制御できる可能性が示された[23]．現在，筆者らは ES 細胞に対し分化に関与するマスター遺伝子などを導入することにより，特定の細胞への分化制御が可能かどうか検討中である．

間葉系幹細胞への高効率遺伝子導入法

間葉系幹細胞は骨髄由来のストローマ細胞であり，骨，軟骨，脂肪，心筋系列などの中胚葉系細胞に分化することができ，未分化状態で細胞を容易に増殖させることが出来る[24]．また，最近では，間葉系幹細胞は神経細胞，肝細胞，インスリン産生細胞などの外胚葉や内胚葉系の細胞へも分化するという報告もあり，再生医療や組織工学への応用が強く期待されている．

間葉系幹細胞の分化を制御する手段の一つとして，細胞分化に関与する遺伝子を導入することがあげられる．アデノウイルスベクターを用いた間葉系幹細胞への遺伝子導入も試みられてきたが，ヒト間葉系幹細胞は CAR を発現していないためにその導入効率はきわめて低く，遺伝子導入には高タイターのベクターを必要としていた[25,26]．

筆者らは，種々のファイバー改変型アデノウイルスベクターを用いて間葉系幹細胞にレポーター遺伝子を導入し，その発現効率を比較検討した．その結果，ヒト間葉系幹細胞には K7 型ベクターが最も適しており，従来型ベクターの 460 倍の遺伝子導入効率を示すことが明らかとなった(図 3b)[27]．RGD 型ベクターや F35 型ベクターは，従来型ベクターに比較しそれぞれ 16 倍，130 倍の導入効率を示した．また，種々のプロモーターを用いて比較検討したところ，CA プロモーターが最適であった．

したがって，間葉系幹細胞には CA プロモーターを有する K7 型アデノウイルスベクターを用いることにより，最も高効率に遺伝子導入できることが明らかとなった．間葉系幹細胞は，さまざまな系列の細胞に分化するというだけではなく，担がんマウスに投与された場合には腫瘍に集積する性質を有している[28]．したがって，間葉系幹細胞は分化させた細胞自身を治療に利用するだけでなく，抗腫瘍性サイトカインなどを発現する間葉系幹細胞を，がんに対する細胞治療薬として利用できる可能性があり，現