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 (Pittenger et al. 1999). 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 (Mori et al. 2005; Okamoto et al. 2002; Saito et al. 2005; Takeda et al. 2004; 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 (Mori et al. 2005; Takeda et al. 2004; 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 one year, 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, UCBTERT-21 (JCRB1107), UCB408E6E7TERT-33 (JCRB1110), UE6E7-3 (JCRB1136) and UBE6T-6 (JCRB1140) were obtained from the JCRB Cell Bank (Osaka, Japan). Two of them are cell lines which were 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 which were transformed with HPV16E6/E7 and hTERT genes (UE6E7T-3) (Mori et al. 2005) or with bmi-1, HPV16E6 and hTERT genes (UBE6T-6) (Mori et al. 2005; Takeda et al. 2004).

The UCBTERT-21 and UCB408E6E7TERT-33 were grown in PLUSOID-M medium (Med-Shirotori Co., Tokyo, Japan) or MSCGM BulletKit (Cambrex Co. USA). UE6E7T-3 and UBE6T-6 were cultured in POWEREDBY10 medium (Med-Shirotori Co., Tokyo, Japan) or MSCGM BulletKit (Cambrex Co. USA). $5x10^3$ cells / ml of each cell line were seeded and cultured for 7 – 10 days. When culture plate was subconfluent, cells were treated with 0.25% trypsin / 0.5mM EDTA solution (both from Invitrogen, Tokyo, Japan) and replated at a density of $5x10^3$ cells /ml.

All of the cells were maintained in a humidified incubator at 37 C and 5% CO_2. PDL were calculated using the formula: PDL= log (cell output/input)/log2. At the starting cultivation, PDLs of UCBTERT-21, UCB408E6E7TERT-33, UE6E7T-3 and UBE6T-6 were 42, 67, 60 and 56, respectively. The doubling time of the UCB408E6E7T-33 cell was 1.5 days, and that of UCBTERT-21, UE6E7T-3 or UBE6T-6 was 2.6 days, 2.0 days, 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 2h 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, USA) 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), and multi-color 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 0.07N NaOH or 70% formamide, hybridized at 37 C for 1-4 days, and counterstained with DAPI. FISH images were captured and analyzed on the Zeiss Axio Imaging microscope (Carl Zeiss Microimaging, GmbH) with Isis mBAND/mFISH imaging Software (MetaSystems, GmbH).

CGH Analysis.

Hybridization was carried out with the BAC Array (MAC ArrayTM Karyo 4000Component, Macrogen Co., USA) by the Hybstation (Genomic Solutions, USA). Briefly, test DNAs, which were isolated using an isolation kit (Amersham BioSciences, UK) and Spin Column (QIAGEN Co., Japan), and reference DNAs (Promega Co. USA) were labeled respectively, with Cy3 or Cy5 (BioPrimer DNA Labeling System, Invitrogen Co., Japan), 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), pH7), 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 48-72h. After hybridization, the slides were washed in a solution of 50% formamide - 2x SSC (pH 7.0) for 15 min at 50 C, in 2x SSC - 0.1% SDS for 15min 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, USA). Acquired images were analyzed with MacViewer (Macrogen Instruments, USA).

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, Camblex BioScience Waltervillo, Inc. USA) for adipocyte and NPMM Bullet kit (NPMM^{TM} BulletKit (B3209,

Camblex BioScience Waltervillo, Inc. USA) for neural progenitor cells. For osteoblast, cells were treated with 0.1 μM dexamethasone (Sigma Chemical Co., USA), 50 μg/ml L-ascorbic acid (Sigma Chemical Co., USA) and 10 mM β-glycerophosphate (Sigma Chemical Co., USA) in the PLUSOID-M medium (Med-Shirotori Co., Tokyo, Japan) or the POWEREDBY10 medium (Med-Shirotori Co., Tokyo, Japan) of culture medium.

After 2-4 weeks, the cells were washed in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde in PBS and stained with Oil Red-O (Sigma Chemical Co., USA) 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 Co. USA) or anti-neurofilament antibody NF-200 (Sigma Chemical Co., USA) and Texas Red-anti-mouse IgG (Southern Biotechnology Associates, Inc., USA) 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 non-transfecting original MSCs (Mori et al. 2005, Takeda et al. 2004, 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 constrast, 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 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* and *F*). A similar variation was also observed in UBE6T-6 cells (Fig. 1*C* and *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 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 2*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* and *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 non-random 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* and *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* and *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* and *F*).

Furthermore, a significant non-random 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* and *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 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 weeks. In adipocyte-specific culture medium, all cell lines accumulated lipid-rich vacuoles in their cytoplasm within 2 weeks, 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 weeks, UCB408E6E7TERT-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 days), the pseudo-neural shaped cells showed apparent increases in immunoreactivity to both antibodies (Fig. 4*Af* and *Ah*), whereas such changes were not evident with the flat-shaped cells before induction (Fig. 4*Ae* and *Ag*). Additionally, such cells did not undergo such differentiation in culture medium when cultured for as long as 30 days, 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 (Mori et al. 2005; Takeda et al. 2004; 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 extend 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 weakly 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, UE6E7-3 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, and therefore this gene transduction induces chromosomal abnormalities (Duensing et al. 2002; Patel et al. 2004; Schaeffer et al. 2004; Solinas-Toldo et al. 1997). 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 UCB408E6E7TERT-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 anueploid 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, though 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 Fig. 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 mechanism 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 (Pacary et al. 2006; Wislet-Gendebien et al. 2003; Wislet-Gendebien et al. 2005). 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 tumourigenicity 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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FIGURE LEGENDS

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 2n to near 4n in prolonged culture.

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. Multi color 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.

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 week positive response. +++ indicates a strong response shown by images of treated cells in Fig. 4*A*. (Bar indicates 20 μ meters.)

Fig 1

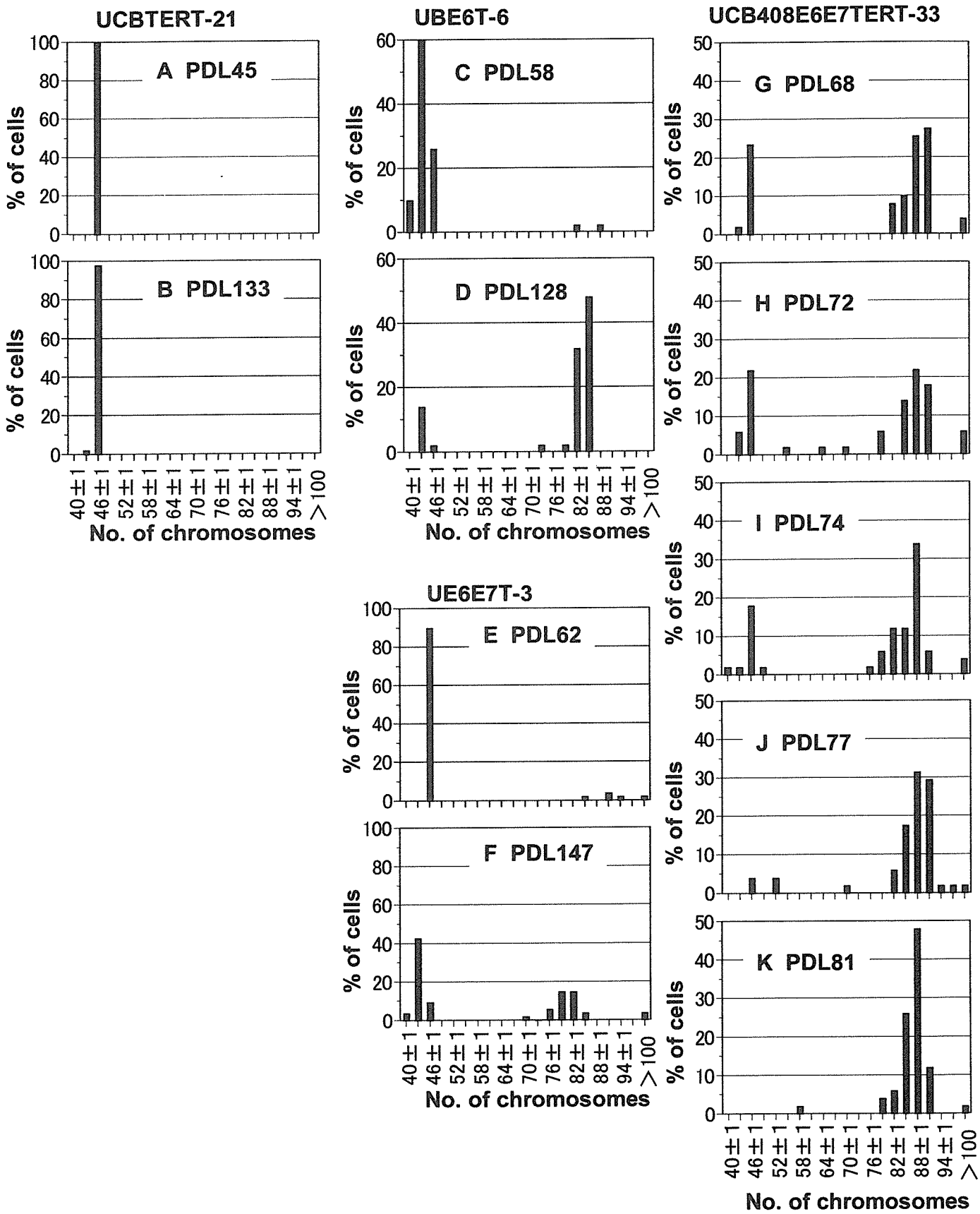
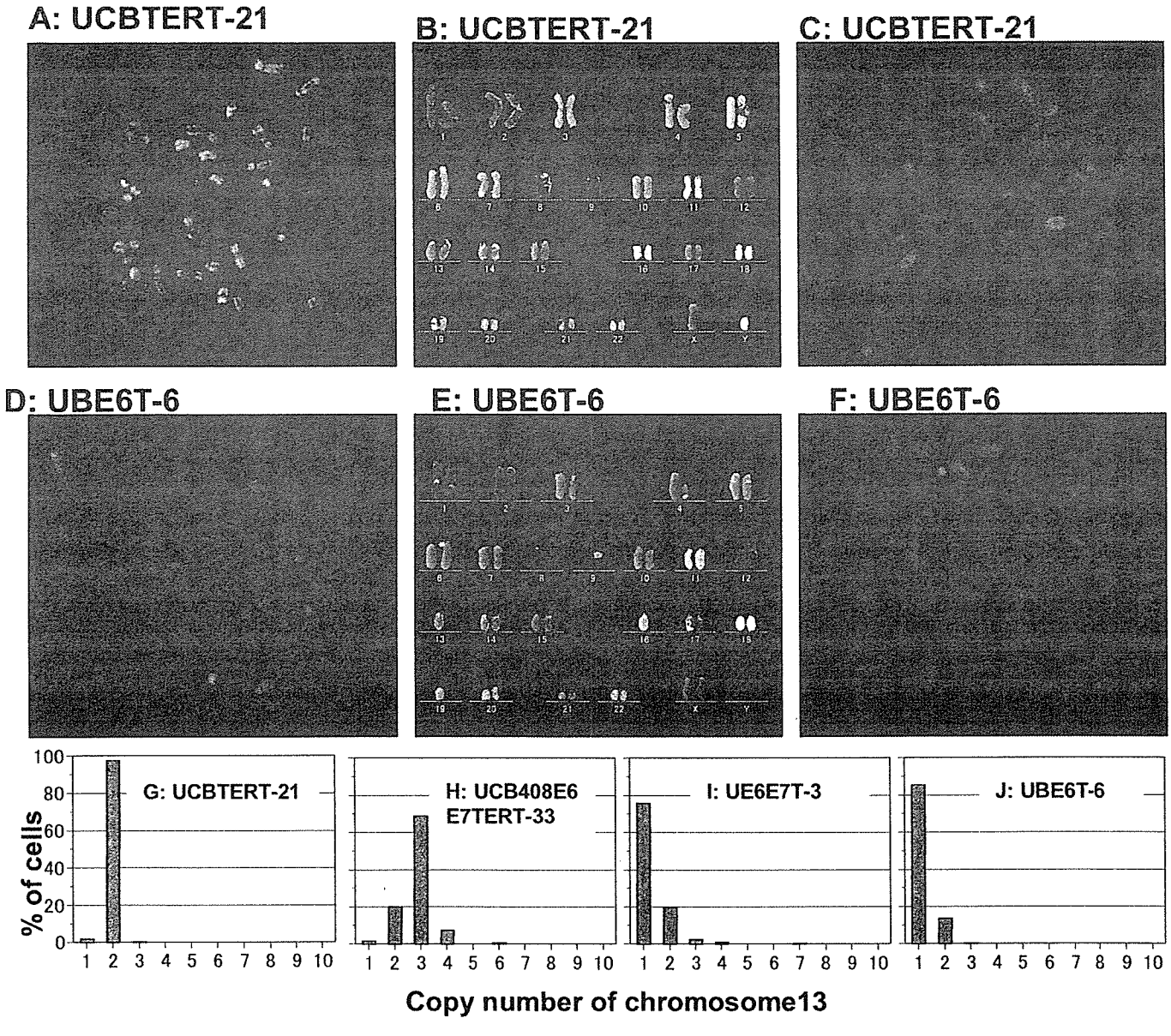
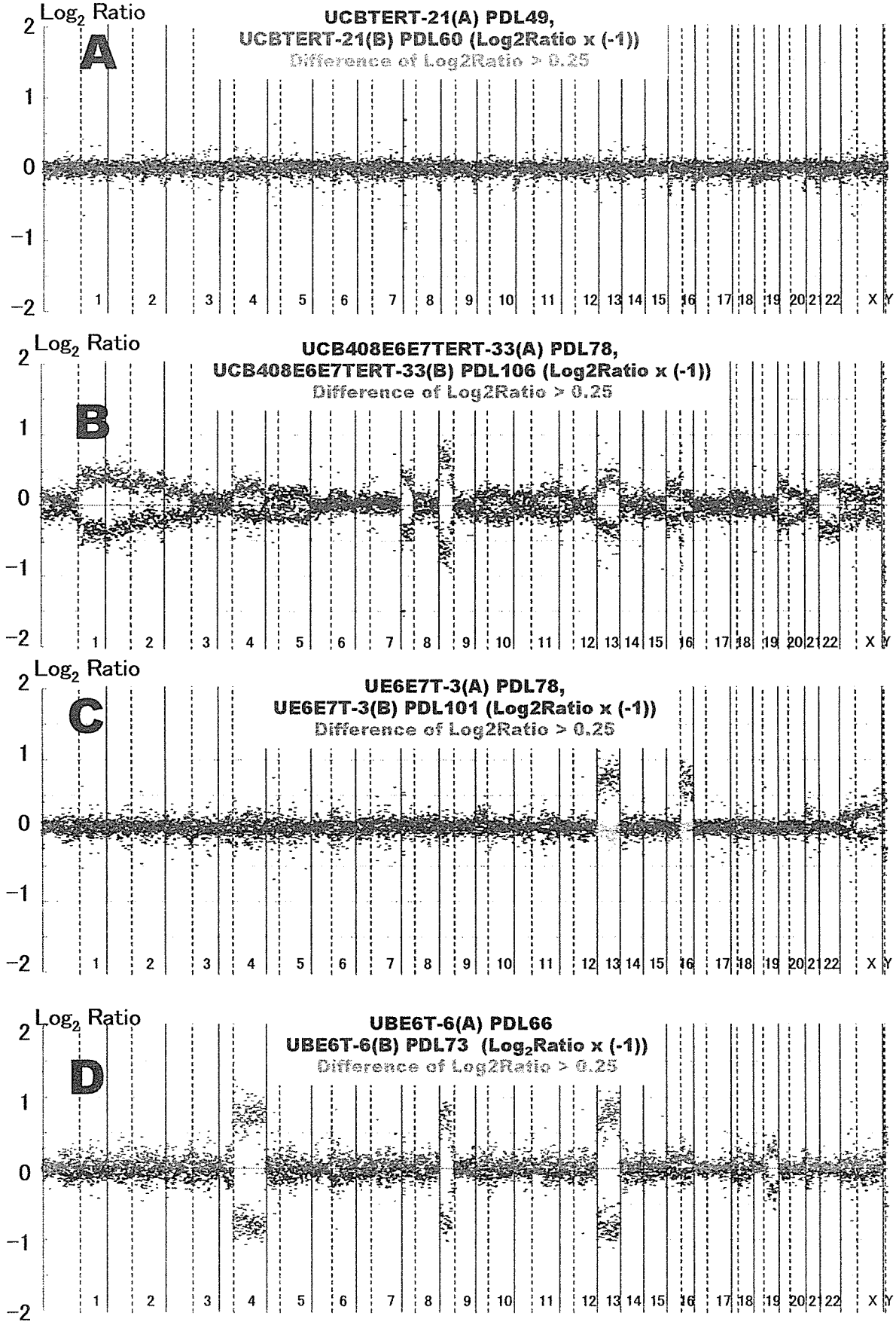
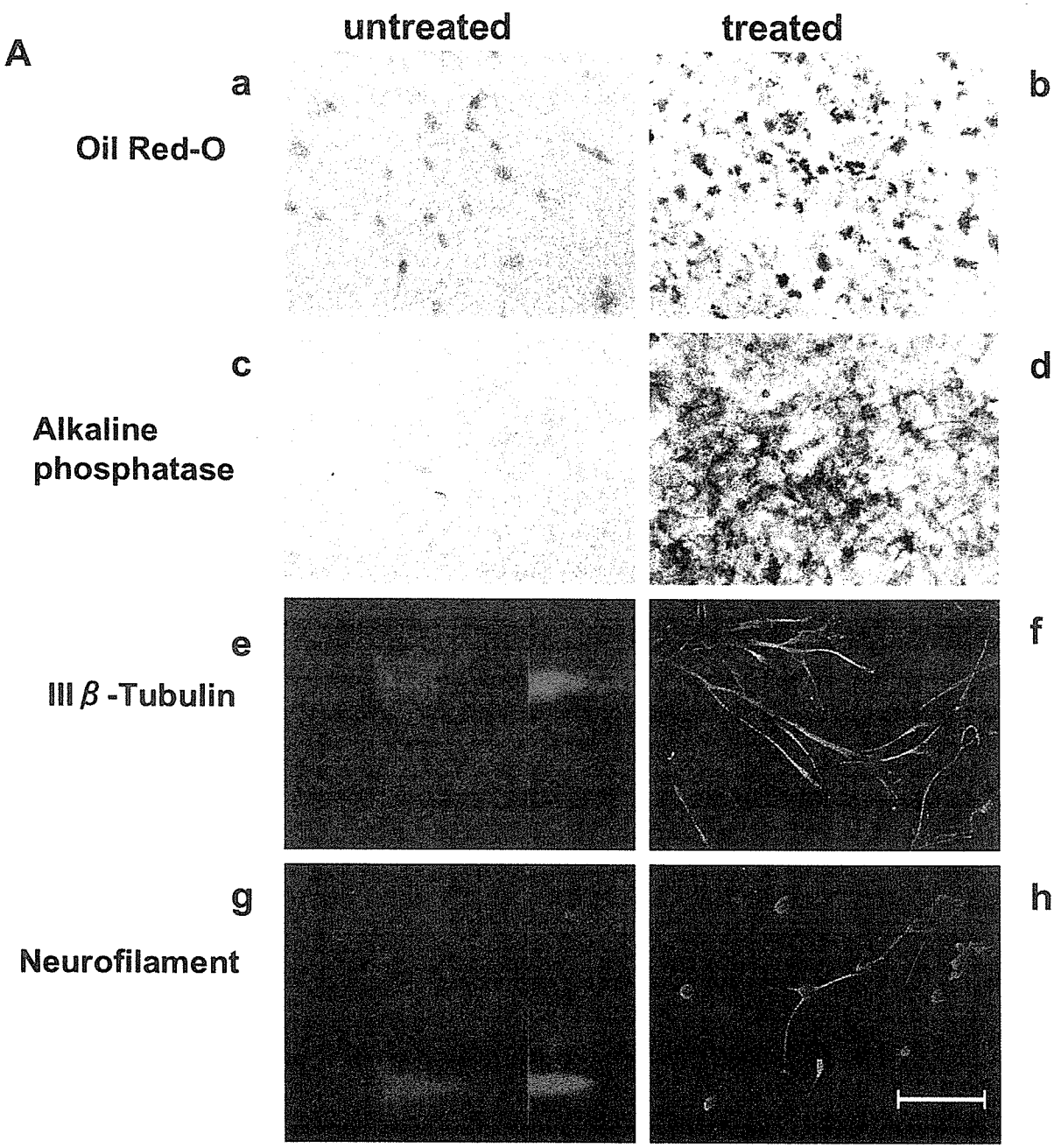

UCBTERT-21
A PDL45
B PDL133
UBE6T-6
C PDL58
D PDL128
UE6E7T-3
E PDL62
F PDL147
UCB408E6E7TERT-33
G PDL68
H PDL72
I PDL74
J PDL77
K PDL81
% of cells
No. of chromosomes
40±1
46±1
52±1
58±1
64±1
70±1
76±1
82±1
88±1
94±1
>100

Fig 2


A: UCBTERT-21
B: UCBTERT-21
C: UCBTERT-21
D: UBE6T-6
E: UBE6T-6
F: UBE6T-6
G: UCBTERT-21
H: UCB408E6
E7TERT-33
I: UE6E7T-3
J: UBE6T-6
% of cells
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10
Copy number of chromosome13


A
UCBTERT-21(A) PDL49,
UCBTERT-21(B) PDL60 (Log2Ratio x (-1))
Difference of Log2Ratio > 0.25
B
UCB408E6E7TERT-33(A) PDL78,
UCB408E6E7TERT-33(B) PDL106 (Log2Ratio x (-1))
Difference of Log2Ratio > 0.25
C
UE6E7T-3(A) PDL78,
UE6E7T-3(B) PDL101 (Log2Ratio x (-1))
Difference of Log2Ratio > 0.25
D
UBE6T-6(A) PDL66
UBE6T-6(B) PDL73 (Log2Ratio x (-1))
Difference of Log2Ratio > 0.25
Log2 Ratio
2
1
0
−1
−2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y

A



B

	UCBTERT-21	UCB408E6E7TERT-33	UE6E7T-3	UBE6T-6
Oil Red-O	+	++	+++	+
Alkarine phosphatase	+	+++	+	+
III β – Tubulin	—	+	+/-	+++
Neurofilament	—	++	—	++