Exploration of the genes responsible for unlimited proliferation of immortalized lung fibroblasts

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Running Title: Expression profiles of immortalized lung fibroblasts

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Abstract

Regulation mechanism of lung fibroblast proliferation remains unknown. To elucidate the key molecules in it, we here established mortal and immortal non-transformed lung fibroblast cell line/strains with elongated lifespan by telomerase reversetranscriptase gene transfection. Comparing the expression profiles of them, 51 genes were explored to be the candidates responsible for regulation of cellular proliferation of lung fibroblasts. This set of fibrobrast strains of same origin with different proliferative capacities may become useful model cells for research on lung fibroblast growth regulation and the candidate genes explored in this study may provide biomarkers or therapeutic targets of pulmonary fibrosis.

Key Words: lung fibroblast, microarray, growth regulation, proliferation, telomerase

INTRODUCTION

Interstitial lung diseases (ILDs) are heterogeneous pulmonary disorders including idiopathic interstitial pneumonias (IIPs), that further include idiopathic pulmonary fibrosis (IPF), nonspecific interstitial pneumonia (NSIP), and others, connective tissue disease–associated interstitial pneumonia (CVD-IP), hypersensitivity pneumonitis (HP), sarcoidosis, lymphangioleiomyomatosis (LAM), and others [1-3]. Although etiology, pathogenesis, disease activity, and patient prognosis vary among these diseases, there are common features: no radical treatment, few specific biomarkers, and unregulated proliferation of fibroblasts. Since fibrosis is much more intractable than inflammation in lung, understanding and control of fibroblast proliferation are the urgent needs to improve the patient prognosis.

What ever controls the proliferation of fibroblasts? Why ILDs principally occur in elderly rather than young people, and rarely in children? How we can obtain the answer? Recently, mutations in telomerase reverse transcriptase (*TERT*) or telomerase RNA component (*TERC*) gene encoding telomerase protein or RNA component, respectively, have been reported to be responsible for a part of familial interstitial

pneumonia [4, 5]. Telomerase is a key enzyme that can compensate for telomere degradation due to continuous cell division, providing elongation of cellular lifespan or immortalization in human somatic cells [6, 7], and these findings indicate that cellular lifespan in lung components plays an important role in the development of aging-related lung disorders. It has been reported that fetal normal lung fibroblasts are difficult to be immortalized even with overexpression of telomerase under hypoxic conditions, while adult lung fibroblasts can be easily immortalized with this condition [8]. From these facts, we hypothesized that there must be unknown regulators, in addition to telomere shortening, that inhibit lung fibroblasts from uncontrolled proliferation. Whereas several suggestive reports have demonstrated expression profiles of IPF, HP, familial IPF, et al. [9-11] comparing each other or with normal lung, their differences may be affected predominantly by difference of cell populations in the sample tissues rather than expression changes in each cell. Then, to clarify the putative additional factors regulating fibroblast proliferation, we established immortal and mortal, but with elongated lifespan, fetal lung fibroblast cell line/strains by *TERT* transfection. Since these fibroblasts with different lifespan and comparable *TERT* overexpression have

identical genetic background, the difference of their proliferative capacity could be derived from the putative additional factors. Under this assumption, we explored the genes differentially expressed among these nontransformed fibroblasts using Affymetrix GeneChip™ Human Genome U133 Plus 2.0 arrays. Representative data were confirmed in different platforms, together with further comparison with transformed fibroblasts developed from the same parent fibroblast TIG-1 by transfection of Simian virus 40 early antigens (SV40EA).

MATERIALS AND METHODS

Cell culture

Cell culture and gene transfection were carried out as previously described with slight modification [12]. Briefly, a normal human fetal lung fibroblast strain TIG-1 (Health Science Research Resources Bank, Tokyo, Japan) was cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM; Sigma-Aldrich Japan, Tokyo, Japan) containing 10% heat-inactivated fetal bovine serum (FBS; BioWhittaker, Verviers, Belgium) at 37°C in a humidified atmosphere of 5% CO_2. Population doubling

level (PDL) was calculated by the cell numbers counted each time of cell passage.

The *TERT* expression plasmid hTERTn2 [13] was kindly provided by Prof. F. Ishikawa (Kyoto University, Kyoto, Japan). SV40EA expression vector was kindly provided by Dr. N. Tsuyama (Hiroshima University, Hiroshima, Japan), by ligation of a 3.7 kb *Pvu*II-*Eco*RI fragment of Simian virus 40 genome (J02400) into a 5.3 kb *Eco*RI-*Bam*HI fragment of pSV2neo cloning vector (XXU02434) [14]. The 20 μg of plasmid hTERTn2 linealized by a single cut with a restriction enzyme *Nru*I and/or circular SV40EA was transfected into 10^7 TIG-1 cells at 44 PDL (population doubling level) by electroporation (Invitrogen, San Diego, CA, USA) at 330 V. After 24 hours, G418 (Wako, Osaka, Japan) was added to the medium with final concentration 400 μg/ml to select the clones harboring the transfected genes. G418 selection was continued at least one month, and exponentially growing cultured cells at various PDLs were collected using trypsin followed by rinse with PBS, and stored at -80°C until use.

For 5-azacytidine treatment, cultured cells were exposed to 2.5 μM 5-azacytidine and harvested on days 0, 3, 6, 9, 12, and 16. The 5-azacytidine was added to culture medium every 3 days.

Preparation of DNA and RNA

Genomic DNA and total RNA were extracted from the frozen cultured cell pellets using DNA Extractor WB^{TM} Kit (Wako) and QIAGEN RNeasy mini kit (QIAGEN, Inc., Valencia, CA, USA), respectively, according to the manufacturer's protocols. The quality of the high molecular genomic DNA was checked by 0.35-0.5% agarose gel electrophoresis without digestion, and that of RNA was checked using Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Southern blot analysis

Terminal restriction fragment (TRF) length, i.e. telomere length was determined by Southern blot analysis. Genomic DNA (5 μg) was digested to completion with *Hin*fI, subjected to electrophoresis on a 0.8% agarose gel, blotted onto a nitrocellulose filter, and then hybridized to a [γ-^{32}P]ATP labeled $(TTAGGG)_4$ probe at 50°C. The filter was washed four times in 4×SSC (1× = 0.15 M NaCl, 0.015 M sodium citrate) and 0.1% SDS at 55°C and exposed to a X-ray film as well as to an imaging plate. The mean length of TRFs was determined as the peak size of the smear signals by using BAS^{TM} 2000 (Fuji, Tokyo, Japan).

Flow cytometric analysis of the cellular DNA content

Cellular DNA content was analyzed as previously reported [15]. Briefly, cell pellets were resolved in trypsin to isolate bare nuclei and stained with propidium iodide using Cycletest^{TM} Plus DNA Reagent Kit (Becton Dickinson, Franklin Lakes, NJ, USA), and analyzed with a FACSCalibur^{TM} (Becton Dickinson) using peripheral mononuclear cells as control diploid cells.

Real-time RT-PCR for TERT expression

Total RNA (2 μg) extracted from each cell strain was reverse-transcribed using High-Capacity cDNA Archive^{TM} Kit (Applied Biosystems, Foster City, CA, USA). Two-hundredths aliquot of the cDNA (equivalent to 10 ng total RNA) was subjected to real-time RT-PCR using the designed *TERT* [12] and an internal control *GAPDH* TaqMan^{TM} probes (Applied Biosystems) . mRNA expression level was calculated by the ratio of *TERT* to *GAPDH*, and relative quantity was standardized using a pooled cDNA derived from 17 various cancer cell lines. Each reaction was carried out in duplicate using ABI PRISM^{TM} 7900 Sequence Detection System (Applied Biosystems).

Colony formation assay with soft agar

Anchorage dependency of the fibroblasts was evaluated by conventional colony formation assay with soft agar in triplicate, according to a previous report [16]. Briefly, 5×10^3 trypsinized cells were resuspended in DMEM or RPMI1640 containing 10% FBS and 0.4% SeaPlaque GTG agarose (Bioproducts), and poured onto bottom agar containing 10% FBS and 0.53% agarose in a 3-cm culture dish. After 21 days culture at 37°C with 5% CO_2, colony number was evaluated with crystal violet staining.

Tumorigenicity on NOD-scid mice

Immunodeficient NOD-*scid* mice were obtained from CLEA Japan (Tokyo, Japan). Two million cells of fibroblast clones, TERT6 at 326 PDL and ST11 at 189 and 211 PDL, or positive control cancer cell lines, LoVo and KYSE2270, in 50 μL of PBS were mixed with 50 μL of Matrigel Matrix (Becton Dickinson) and injected s.c. in the flank of animals. Development of tumor was evaluated every week until 12 weeks after injection. All work was done according to institutional animal care and use committee approval.

Microarray analysis

Three commercially available single-color oligonucleotide microarray platforms, i.e.

Affymetrix GeneChip™ Human Genome U133 Plus 2.0 array (~47,000 transcripts, Affymetrix, Santa Clara, CA, USA) for exploration of candidate genes, and Agilent™ Whole Human Genome Oligo Microarray (~41,000 transcripts, Agilent Technologies, Tokyo, Japan) and CodeLink™ Expression Bioarray System UniSet Human 20K I Bioarray (~20,000 transcripts, GE Healthcare, Tokyo, Japan) for confirmation, were used according to the manufacturers' protocols. Briefly, first-strand cDNA was generated from 0.5~1μg of total RNA of cell lines using reverse transcriptase and a T7 primer, and then second-strand cDNA was produced using DNA polymerase mix and RNase H supplied in each labeling kit, GeneGhip™ One-Cycle Target Labeling kit (Affymetrix), Agilent™ Low RNA Input Linear Amplification Kit PLUS, One-color (Agilent), and CodeLink™ Expression Assay Reagent Kit (GE Healthcare), respectively. cRNA was generated via an *in vitro* transcription reaction using T7 RNA polymerase, which was quantified by spectrometry and checked using Agilent™ Technologies 2100 Bioanalyzer. Twenty (Affymetrix array), 1.5 (Agilent array), or 10 (CodeLink array) μg of cRNA were then fragmented and hybridized to each array. After hybridization, Affymetrix arrays were rinsed and labeled automatically according to GeneGhip™

Expression Analysis Technical Manual (http://www.affymetrix.com/support/technical/manual/expression_manual.affx), and scanned using an Affymetrix GeneChip™ Scanner 3000 with Affymetrix GeneChip™ Operating System (GCOS) 1.2. The Agilent arrays were rinsed with Agilent Gene Expression Wash Buffer 1 at room temperature and with Buffer 2 at 37°C for 1 min according to the protocol. The CodeLink arrays were rinsed with 0.75 × TNT buffer at 46°C for 1 h, labeled with Streptavidin-Cy5 at room temperature for 30 min, rinsed again with TNT buffer and 0.05% Tween 20, and dried by centrifugation. Both arrays were scanned using Agilent DNA Microarray Scanner™ (Agilent Technologies), then analyzed with Agilent™ Feature Extraction software ver. 9.1. or CodeLink™ Expression Analysis Software (GE Healthcare), respectively. Expression levels were normalized to the median expression value of the whole array spots using GeneSpring™ GX (Agilent Technologies). The microarray data were registered to the Gene Expression Omnibus under GE accession no. GSE9077 (http://www.ncbi.nlm.nih.gov.geo/).

Statistical analysis

Statistical tests were performed using StatView™ version 5.0 software (SAS Institute Inc., Cary, NC, USA). Differences of TRF lengths and *TERT* mRNA expression levels between immortal and immortal fibroblasts were analyzed by student *t* test.

RESULTS

Establishment of immortal lung fibroblast cell lines

After the transfection of the *TERT*-expressing hTERTn2 plasmid and/or SV40EA into the fibroblasts TIG-1 at PDL 44, clones harboring the plasmids were obtained by 400 μg/ml of G418 selection and named as TERT2, 5, 6, 7, and 8 with *TERT* transfection, SV7, 9, and 12 with SV40EA transfection, and ST11 with *TERT* and SV40EA cotransfection. Out of these 8 clones, clones SV7, 9, and 12 without *TERT* transfection fell into apoptosis at 72 PDL or before, almost same PDL with that of the parent cell TIG-1 (Figure 1). Out of 5 *TERT* alone transfected clones, only TERT6 indefinitely proliferated over 300 PDL, while remaining 4 clones stop dividing around 80-90 PDL with morphologic changes of apoptotic cells, and never overcame 100 PDL.

Fig. 1
Table 1

These proliferative capacities were reproducibly confirmed (Figure 1, Table 1). Meanwhile, clone ST11, cotransfected with *TERT* and SV40EA, showed rapid growth from just after transfection, and continued proliferation indefinitely. While morphology of ST11 was small and angular, quite different from that of the parent fibroblasts, clone TERT6 kept fibroblastic morphology, fibrous form, even after 300 PDL.

Telomere length (TRF length)

The TRF length of each clone was measured by Southern blot analysis. Not only immortalized clones but also all *TERT* transfected mortal fibroblasts with elongated lifespan showed elongated TRF, while SV40EA alone transfected mortal clones without elongated lifespan showed shortened TRF (Figure 2, $P = 0.0057$). Thus, elongation of TRF was associated with *TERT* mediated elongation of lifespan, but to the extent that *TERT* is activated, the TRF length does not determine their proliferative capacities.

Fig. 2

DNA ploidy

The DNA ploidy of all *TERT* transfected clones examined revealed to be diploid (2n), using peripheral mononuclear cells as diploid controls (Figure 3).

Fig. 3

TERT expression

The *TERT* mRNA expression levels were evaluated by real-time RT-PCR in parent TIG-1, *TERT* alone transfected non-transformed TERT2, 5, 7, 8, and 6 clones at various PDLs (Figure 4, left-hand), a *TERT* and SV40 cotransfected immortal transformed clone ST11 and human cancer cell lines (middle), and the immortal TERT6 clone with 5-azacytidine treatment up to 16 days (right-hand). The *TERT* was not expressed in parent TIG-1 cells at all as expected, and the expression levels in *TERT* transfected fibroblasts before 100 PDL were dozens of times higher than those in human cancer cell lines, regardless of their immortal or mortal capacity ($P = 0.7604$, mortal vs. immortal competent). However, the *TERT* expression level in the immortal nontransformed clone TERT6, but not in the immortal transformed clone ST11, gradually reduced as they grow after 100 PDL ($P < 0.0001$), approaching to the level of human cancer cell lines. The reduced expression level was partially recovered after 5-azacytidine treatment ($P = 0.0193$, before vs. day 16).

Fig. 4

Maintenance of anchorage dependency

Anchorage-dependency was evaluated by a soft agar colony formation assay. The

Fig. 5

immortal fibroblast clone TERT6 transfected with *TERT* alone showed no colony at all, while another immortal fibroblast clone ST11 cotransfected with both *TERT* and SV40EA showed considerable number of colonies (Figure 5).

Lack of tumorigenicity

When injected on the flank of severe combined immunodeficient (NOD-*scid*) mice, both immortal fibroblast clones, TERT6 and ST11, were not tumorigenic as long as 12 weeks regardless of their colony formation capacity in soft agar, while human cancer cell lines, LoVo and KYSE2270, developed tumors within shorter period (data not shown).

Genes differentially expressed in immortalized fibroblasts

To explore the genes differentially expressed between immortal and mortal *TERT* transfected nontransformed fibroblasts, expression profiles in 6 samples, i.e. parent lung fibroblast TIG-1 at 45 PDL, mortal *TERT* overexpressing fibroblasts TERT5 at 71 PDL and TERT8 at 70 PDL, immortal-competent (before 100 PDL with capacity to proliferate over 100 PDL) *TERT* overexpressing fibroblasts TERT6 at 65 PDL, and immortal nontransformed TERT6 at 121 PDL and 217 PDL, were obtained using

Affymetrix GeneChip™ Human Genome U133 Plus 2.0 array (Figures 6 and 7, Affymetrix). After global normalization by using GeneSpring™ GX, 19 genes were selected as expressed 3 fold or higher in the immortal clone TERT6 both at 121 and 217 PDLs than any of the parent TIG-1 and mortal TERT5 and TERT8 clones, with positive calls for immortal TERT6-121 and -217 (Figure 6, Affymetrix; Table 2). During this selection, a few genes showing discrepancies among the plural probes were excluded, as artifacts of microarray analysis. To confirm the reproducibility with different platforms, same RNA samples derived from parent TIG-1, immortal-competent TERT6 at 65 PDL, and immortal TERT6 at 217 PDL were also subjected to Agilent™ Whole Human Genome Oligo Microarray with an additional sample, immortal transformed ST11 at 196 PDL (Figure 6, Agilent). Similarly, same RNA samples derived from parent TIG-1 and immortal-competent TERT6 at 65 PDL were also subjected to CodeLink™ Expression Bioarray System UniSet Human 20K I Bioarray with additional samples, the immortal-competent TERT6 at 85 PDL, mortal transformed SV12 at 68 PDL and 72 PDL, immortal-competent transformed ST11 at 65 PDL, and immortal transformed ST11 at 106 PDL (Figure 6, CodeLink). The probes that were issued by the company as

Fig.6
Table 2

representing multiple genes were not evaluated. As shown in Figure 6, expression patterns of all genes selected by Affymetrix array were consistent with other platforms among the commonly analyzed samples, TIG-1, TERT6-65, and TERT6-217, as long as the gene probes were evaluable on others. While upregulation of several genes among them, e.g. *IFI30, CTSS, LGR4, APOL1, EPSTI1,* and *IFI27*, was observed also in immortal transformed clone ST11, other genes showed no change or even downregulation in ST11 (Figure 6).

Similarly, 32 genes were selected as one third or lower expressed in the immortal clone TERT6 both at 121 PDL and 217 PDLs than any of parent TIG-1 and mortal TERT5 and TERT8 clones, with positive call for TIG-1 (Figure 7, Affymetrix; Table 3). As observed in upregulated genes, these downregulated genes showed no discrepancies among the different platforms. While downregulation of several genes among them, e.g. *EPPB41L3, CXCL12, NPTX1, HAS2, GABRB1, ZNF667, KCNK2, CUBBP2, MGC9913, INSR, MGMT, SOX11, FST, H2AFY2,* and *CORO2B,* was observed also in immortal transformed clone ST11, other genes showed no change or even upregulation in ST11 (Figure 7).

Fig. 7
Table 3

DISCUSSION

We established a set of model lung fibrobrast cell lines/strains of identical genetic background with different proliferative capacity: mortal and immortal nontransformed lung fibroblasts maintaining anchorage dependency without tumorigenicity, as well as mortal and immortal transformed fibroblasts with loss of anchorage dependency. With *TERT* overexpression, there was no significant difference in telomere length between the mortal and immortal lung fibroblasts ($P = 0.4237$). Moreover, *TERT* mRNA expression level was not associated with this immortality ($P = 0.7604$). *TERT* was even downregulated in clone TERT6 after immortalization, to the level of human cancer cell lines (in which average of *TERT/GAPDG* = 1). Since 5-azacytidine treatment somewhat recoverd this reduction ($P = 0.0193$), it is unlikely to consider that this reduction is due to a clonal selection of low *TERT* expressing clones. It may indicate that expression level of *TERT* in human cancer cell lines in general is enough to endow somatic cells with immortality to the extent that other required conditions are met, and higher expression level does not provide an advantage on proliferative capacity. Thus we

propose that the differntially expressed genes among these fibroblasts with different proliferative capacity are the candidates that critically regulate the proliferation of lung fibroblasts.

We found that 19 and 32 genes were characteristically up and downregulated, respectively, in immortal nontransformed lung fibroblasts compared with mortal lung fibroblasts of same origin. As widely accepted, maintenance of telomere length by telomerase is a key factor to elongate the cellular lifespan, and in fact we found that telomerase-negative fibroblasts with SV40EA transfection had shorter telomeres (P = 0.0057) and fell into apoptosis earlier than any of the *TERT* transfected clones, as the parent TIG-1 cells did. However, when the fibroblasts escaped from this proliferative limitation by ectopic expression of telomerase, they still confronted another critical limitation, and most of them could not overcome the 100 PDL border. From previous reports [8, 17], we speculated that this mechanism regulating the proliferation of lung fibroblasts is more strict in fetal lung than in adult, and possibly be related to the increase of lung fibrosis incidence with age.

Among these genes, placenta-specific 8 (*PLAC8*), also known as onzin, was dramatically and reproducibly upregulated in immortal non-transformed fibroblasts, but not in transformed fibroblasts (Figure 6). Mouse counterpart of this gene was reported to be downregulated in response to c-Myc overexpression, and its overexpression in fibroblasts was associated with an increased growth rate, resistance to apoptotic stimuli, and loss of the G2/M checkpoint, possibly through inability to induce p53 in response to apoptotic stimuli [18]. Since repression of *MYC* in immortal non-transformed lung fibroblast clone TERT6 was reproducibly observed in our present data (GSE9077 in GEO), this repression may have induced *PLAC8* overexpression promoting its continuous proliferation. Meanwhile, several genes upregulated in immortal nontransformed fibroblasts have been reported to be upregulated in malignant cells, e.g. *HSD17B6* in prostate cancer [19], *EPSTI1* in breast cancer [20], *IFI27* in epithelial cancers [21], *GPNMB* in glioblastoma with poor prognosis [22], and *MAGEA6* and *MAGEA12* in various cancer cell lines [23]. Among these reportedly cancer-associated genes, only *EPSTI1* and *IFI27* practically showed higher expression in transformed than nontransformed immortal fibroblasts, while the remainings were upregulated

specifically in nontransformed immortal fibroblasts. These genes may be involved in cell growth rather than transformation. Mouse counterpart of *SHOX2* was also reported to be associated with growth of chondrocytes [24].

On the contrary, among the genes downregulated in immortal nontransformed fibroblasts, *EPB41L3* has been reported to act as a tumor suppressor gene in several cancers [25, 26], and practically it was suppressed also in immortal transformed fibroblasts. *HES1* was reported to be repressed in mesenchymal stem cells with self-renewal capacity [27], follistatin encoded by *FST* was reported to attenuate bleomycin-induced fibrosis by blocking the actions of activin and TGF-β [28], and *H2AFY2* was reported to be upregulated in aged cells [29]. Downregulation of these genes in immortal fibroblasts is consistent with their reported functions.

Interestingly, *HSD17B6, IFI27, GATA3, and EPSTI1* among the 19 genes upregulated in immortal fibroblasts and *CYBA* and *SOX11* among the 32 downregulated genes had been reported to be highly expressed in HP tissues compared to IPF tissues [10]. On the contrary, *FST* among the 32 downregulated genes was reportedly higher in IPF. The chronic overproduction of TGF-β and TNF-α by activated macrophages as a

consequence of long-term antigen exposure is considered to be the key factors for the development of pulmonary fibrosis in chronic HP, while sequential alveolar epithelial cell microinjuries or apoptosis, possibly through oxidant stress, fas-signalling pathway, and/or short telomeres especially in familial IPF, followed by abnormal epithelial-mesenchymal interactions and an aberrant host response to wound healing are considered to be the crucial event in the pathogenesis of IPF [3-5, 30, 31]. It is natural that different factors may play a key role in unregulated proliferation of fibroblasts in different pathogenesis where different profibrotic mediators are activated. These findings may indicate that some of the genes we presently identified, e.g. overexpression of *HSD17B6, IFI27, GATA3, and EPSTI1* or suppression of *FST*, may be responsible for unregulated proliferation of lung fibroblasts in HP and some others, e.g. suppression of *CYBA* and *SOX11*, may be responsible in IPF.

In conclusion, we established a set of model lung fibrobrast cell lines/strains of identical genetic background with different proliferative capacity, mortal or immortal, and found 51 candidate genes possibly responsible for regulation of lung fibroblast proliferation. Elucidation of the key genes and their regulating mechanism for each

fibrotic disease may promote disease understanding and development of molecular targeting therapy for presently intractable fibrotic lung diseases.

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FIGURE LEGENDS

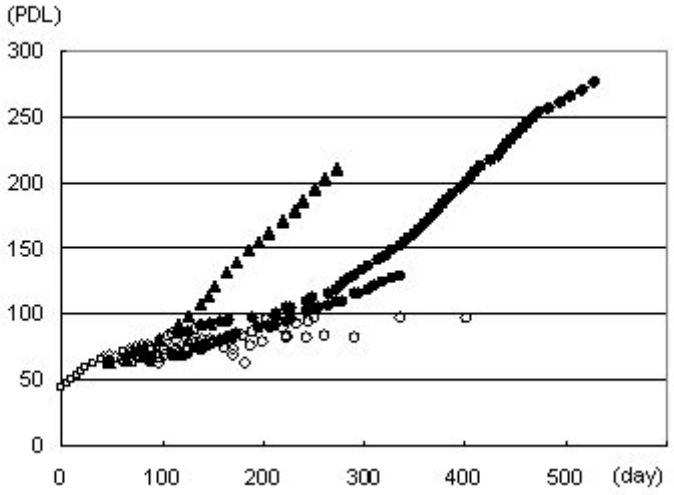

Figure 1. Growth curves of the fetal lung fibroblast TIG-1 (□) and its *TERT* (circle), SV40EA (diamond), or both (triangle) transfected mortal (open) or immortal (closed) subclones. The *TERT* alone transfected TERT6 (●) continued cell divisions unlimitedly, while other *TERT* alone transfected clones TERT2, 5, 7, and 8 (○) stopped dividing before 95 PDL, reproducibly. However, their lifespan was substantially elongated compared with those of parent TIG-1 or SV40EA alone transfected SV7, SV9, and SV12 (◇), which all fell into apoptosis before 75 PDL. The *TERT* and SV40EA cotransfected clone ST11 (▲) continued rapid proliferation indefinitely.

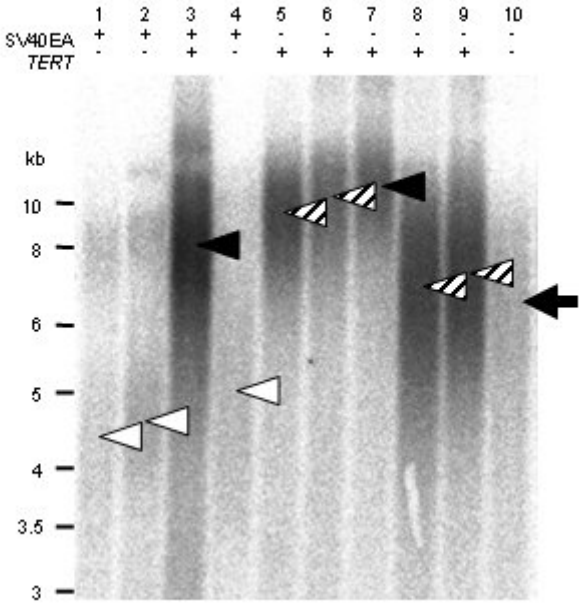

Figure 2. Southern blot analysis for TRF length. The peaks of the TRF smear signals are indicated by an arrow in parent TIG-1 cells at 47 PDL (lane 10), closed arrow heads in immortal-competent ST11 and TERT6 at 65 PDL (lanes 3 and 7), open arrowheads in SV40 alone transfected mortal SV7 at 63 PDL, SV9 at 70 PDL, and SV12 at 68 PDL (lanes 1, 2, and 4), and hatched arrowheads in *TERT* alone transfected mortal with elongated lifespan TERT2 at 71 PDL, TERT5 at 67 PDL, TERT7 at 71 PDL, and TERT8 at 72 PDL (lanes 5, 6, 8, and 9, respectively).

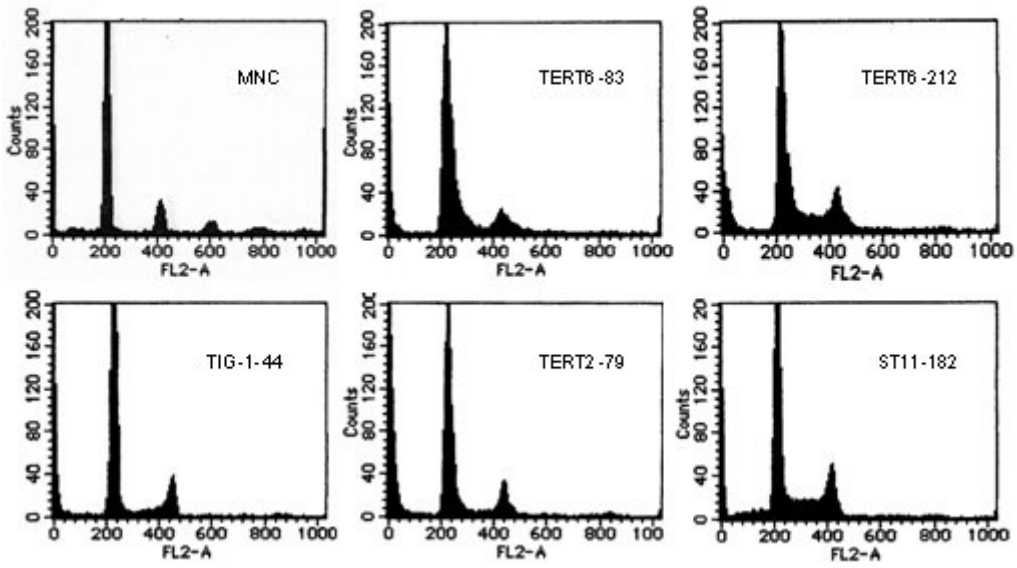

Figure 3. Flow cytometric analysis of DNA content of propidium iodide stained fibroblasts using peripheral mononuclear cells (MNC) as the diploid control. All samples examined including parent TIG-1 at 44 PDL, *TERT* alone transfected mortal TERT2 at 79 PDL and immortal TERT6 at 83 and 212 PDLs, and *TERT* and SV40EA cotransfected ST11 at 182 PDL revealed to be diploid.

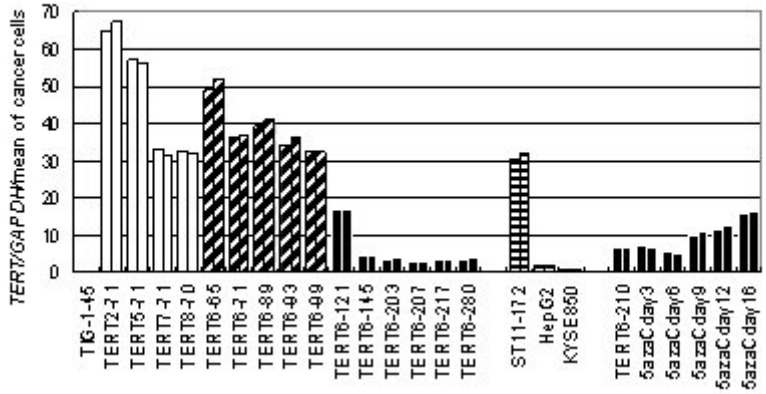

Figure 4. The values of the *TERT* mRNA expression levels in duplicate real-time RT-PCR experiments with *GAPDH* adjustment. Using a mixture of 17 kinds of various human cancer cell line cDNA as standard, 1 in Y axis represents the average of *TERT* mRNA expression levels in human cancer cell lines. Open bars are *TERT* alone transfected mortal clones, hatched bars are *TERT* alone transfected immortal-competent clone TERT6 before 100 PDL, closed bars are *TERT* alone transfected immortal clone TERT6 after 100 PDL, and horizontal-striped bars are transformed fibroblasts and human cancer cell lines. Fibroblast clone names with PDL numbers (left) and days of 5-azacytidine treatment on immortal TERT6 clone at 210 PDL (right) are listed.

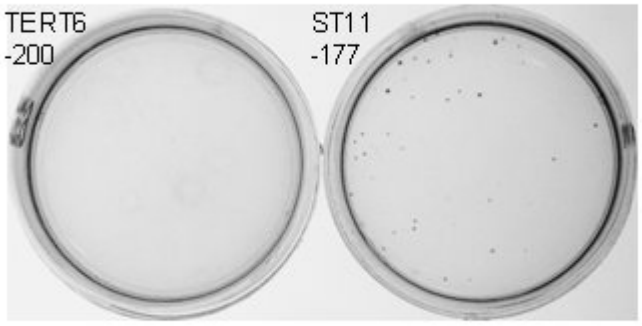

Figure 5. Colony formation assay with soft agar for *TERT* alone transfected clone TERT6 at 200 PDL and *TERT* and SV40EA cotransfected clone ST11 at 177 PDL. With crystal violet staining, the latter, but not the former, showed colonies.

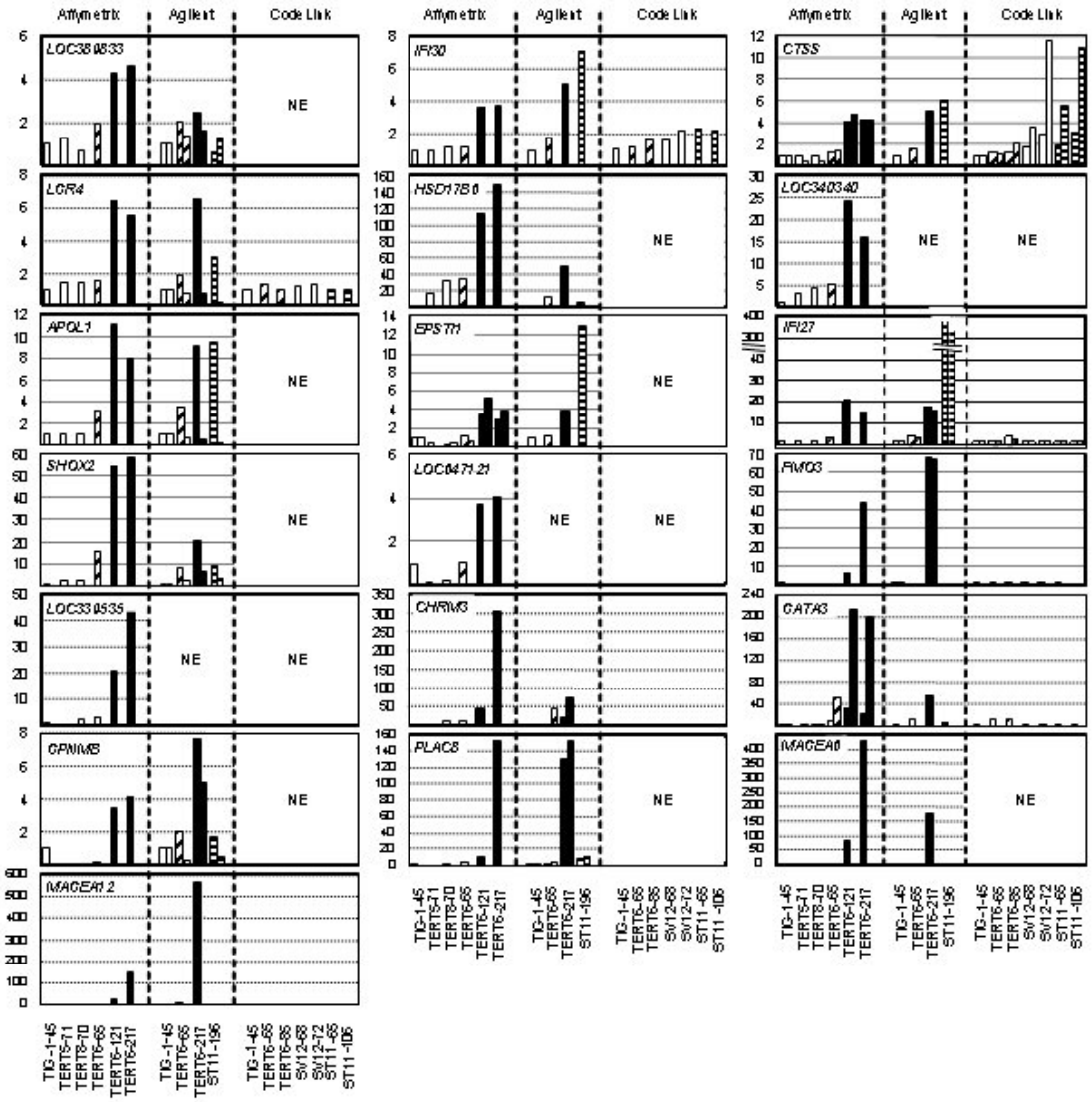

Figure 6. The ratio of mRNA expression levels in various fibroblast clones to that in parent TIG-1 fibroblasts at 45 PDL for the 19 genes selected as upregulated in immortal nontransformed TERT6, evaluated by Affymetrix GeneChip^{TM} Human Genome U133 Plus 2.0 array (Affymetrix), Agilent Whole Human Genome Oligo Microarray (Agilent), and CodeLink™ Expression Bioarray System UniSet Human 20K I Bioarray (CodeLink,

on the top) with global normalization. Open bars represent mortal fibroblasts, i.e. parent TIG-1 at 45 PDL, *TERT* alone transfected TERT5 and TERT8, and SV40EA alone transfected SV12, hatched bars represent *TERT* alone transfected immortal competent clone TERT6 before 100 PDL, closed bars represent immortal clone TERT6 after 100 PDL, and horizontal-striped bars represent immortal-competent or immortal transformed fibroblasts with *TERT* and SV40EA cotransfection. Presentation of two bars for one sample means that there are two probes for that gene in microarray platforms.

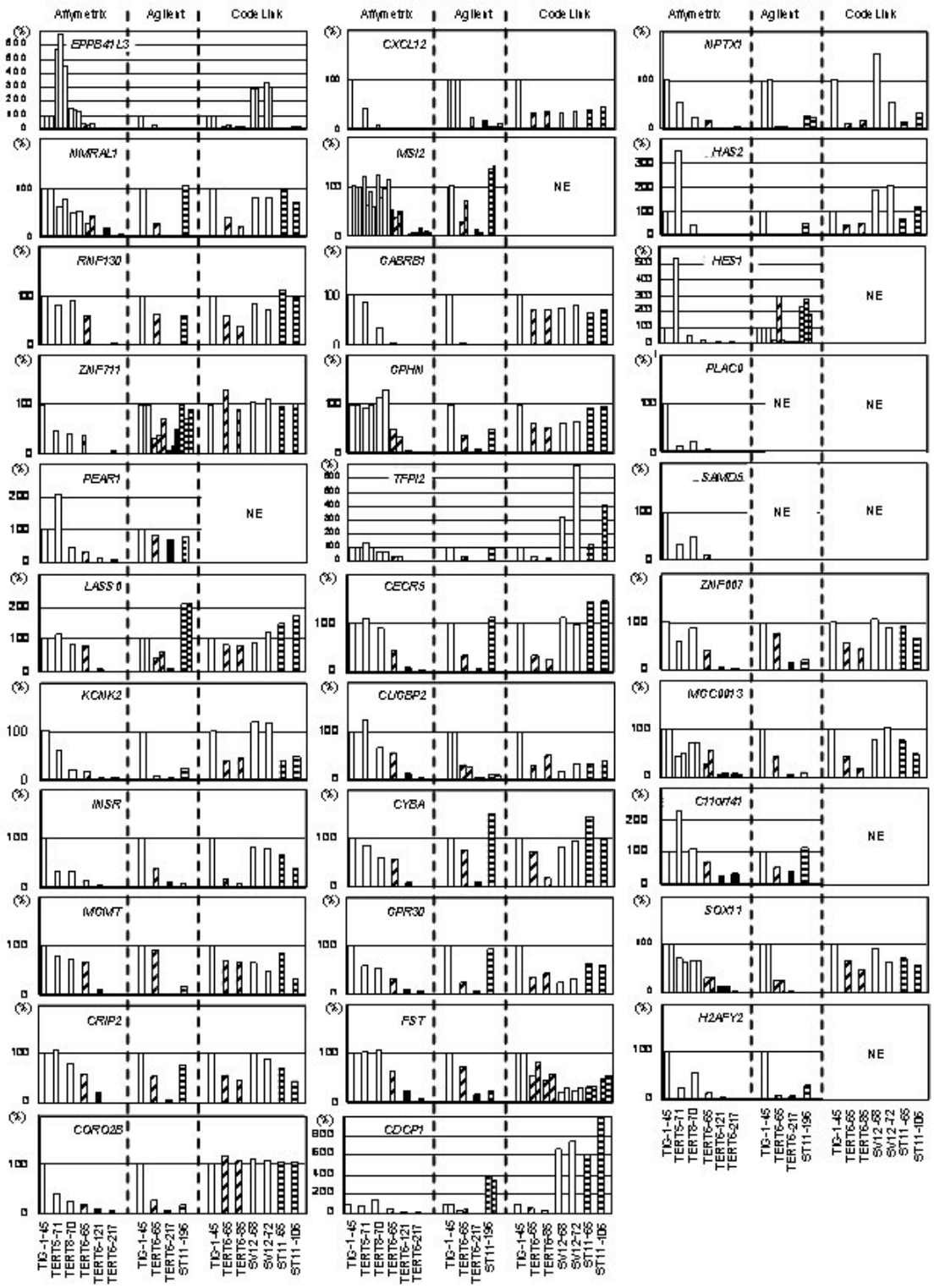

Figure 7. The percentage of mRNA expression levels in various fibroblast clones to that

in parent TIG-1 fibroblasts at 45 PDL for the 32 genes selected as downregulated in immortal nontransformed TERT6, evaluated by Affymetrix GeneChip™ Human Genome U133 Plus 2.0 array (Affymetrix), Agilent Whole Human Genome Oligo Microarray (Agilent), and CodeLink™ Expression Bioarray System UniSet Human 20K I Bioarray (CodeLink, on the top) with global normalization. Open bars represent mortal fibroblasts, i.e. parent TIG-1 at 45 PDL, *TERT* alone transfected TERT5 and TERT8, and SV40EA alone transfected SV12, hatched bars represent *TERT* alone transfected immortal competent clone TERT6 before 100 PDL, closed bars represent immortal clone TERT6 after 100 PDL, and horizontal-striped bars represent immortal-competent or immortal transformed fibroblasts with *TERT* and SV40EA cotransfection. Presentation of two or more bars for one sample means that there are two or more probes for that gene in microarray platforms.

Table 1. Lifespan of *TERT* and/or SV40EA transfected clones derived from lung fibroblast TIG-1

	Transfection			
Clone name	*TERT*	SV40EA	Anchorage dependency	Mortality
TIG-1	-	-	+	mortal
TERT2	+	-	+	mortal
TERT5	+	-	+	mortal
TERT6	+	-	+	immortal
TERT7	+	-	+	mortal
TERT8	+	-	+	mortal
SV7	-	+	ND	mortal
SV9	-	+	ND	mortal
SV12	-	+	ND	mortal
ST11	+	+	-	immortal

ND: not done (due to early apoptosis)

Table 2. Upregulated genes in immortal nontransformed lung fibroblast clone TERT6.

Gene Symbol	GenBank Acc#	Gene Name
LOC389833	NM_001033515	similar to hypothetical protein MGC27019
IFI30	NM_006332	interferon, gamma-inducible protein 30
CTSS	NM_004079	cathepsin S
LGR4	NM_018490	leucine-rich repeat-containing G protein-coupled receptor 4
HSD17B6	NM_003725	hydroxysteroid (17-beta) dehydrogenase 6 homolog (mouse)
LOC340340	BC040593	hypothetical protein LOC340340
APOL1	NM_003661	apolipoprotein L, 1
EPSTI1	NM_001002264	epithelial stromal interaction 1 (breast)
IFI27	NM_005532	interferon, alpha-inducible protein 27
SHOX2	NM_003030	short stature homeobox 2
LOC647121	XR_017692	similar to embigin homolog

FMO3	NM_001002294	flavin containing monooxygenase 3
LOC339535	XM_001132951	hypothetical protein LOC339535
CHRM3	NM_000740	cholinergic receptor, muscarinic 3
GATA3	NM_001002295	GATA binding protein 3
GPNMB	NM_001005340	glycoprotein (transmembrane) nmb
PLAC8	NM_016619	placenta-specific 8
MAGEA6	NM_005363	melanoma antigen family A, 6
MAGEA12	NM_005367	melanoma antigen family A, 12

Table 3. Downregulated genes in immortal nontransformed lung fibroblast clone TERT6.

Gene Symbol	GenBank Acc#	Gene Name
EPB41L3	NM_012307	erythrocyte membrane protein band 4.1-like 3
CXCL12	NM_000609	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
NPTX1	NM_002522	neuronal pentraxin I
NMRAL1	NM_020677	NmrA-like family domain containing 1
MSI2	NM_138962	musashi homolog 2 (Drosophila)
HAS2	NM_005328	hyaluronan synthase 2
RNF130	NM_018434	ring finger protein 130
GABRB1	NM_000812	gamma-aminobutyric acid (GABA) A receptor, beta 1
HES1	NM_005524	hairy and enhancer of split 1, (Drosophila)
ZNF711	NM_021998	zinc finger protein 711
GPHN	NM_020806	gephyrin

PLAC9	NM_001012973	placenta-specific 9
PEAR1	NM_001080471	platelet endothelial aggregation receptor 1
TFPI2	NM_006528	tissue factor pathway inhibitor 2
SAMD5	NM_001030060	sterile alpha motif domain containing 5
LASS6	NM_203463	LAG1 homolog, ceramide synthase 6 (S. cerevisiae)
CECR5	NM_017829	cat eye syndrome chromosome region, candidate 5
ZNF667	NM_022103	zinc finger protein 667
KCNK2	NM_014217	potassium channel, subfamily K, member 2
CUGBP2	NM_006561	CUG triplet repeat, RNA binding protein 2
MGC9913	NM_198879	Hypothetical protein MGC9913
INSR	NM_000208	insulin receptor
CYBA	NM_000101	cytochrome b-245, alpha polypeptide
C11orf41	XM_941447	chromosome 11 open reading frame 41
MGMT	NM_002412	O-6-methylguanine-DNA methyltransferase
GPR30	NM_001505	G protein-coupled receptor 30
SOX11	NM_003108	SRY (sex determining region Y)-box 11

CRIP2	NM_001312	cysteine-rich protein 2
FST	NM_006350	follistatin
H2AFY2	NM_018649	H2A histone family, member Y2
CORO2B	NM_006091	coronin, actin binding protein, 2B
CDCP1	NM_022842	CUB domain containing protein 1