Materials and Methods

Cell line and culture conditions. A549 lung adenocarcinoma cells (JCRB0076) were obtained from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan), and cultured in ERDF medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS; Invitrogen) at 37°C in a 95% air/5% CO_2 atmosphere. A549 were subcultured every 3 days by 8-fold dilution. Recombinant transforming growth factor β1 (TGF-β; Austral Biologicals, San Ramon, CA) was added every other day to the culture at a final concentration of 10 ng·ml^{-1} where indicated.

RT-PCR. mRNAs coding for the human telomerase reverse transcriptase (hTERT) and telomerase-associated protein 1 (TEP1) were detected using the RT-PCR method.[13] Three μg of total RNA was used as the template for cDNA synthesis using Superscript II RNase H$^-$ reverse transcriptase (Invitrogen). hTERT cDNA was amplified using the primers LT5 (5′-CGGAAGAGTGTCTGGAGCAA-3′) and LT6 (5′-GGATGAAGCGGAGTCTGGA-3′) through 29 PCR cycles (94°C for 45 sec, 60°C for 45 sec, 72°C for 90 sec, 29 cycles), and TEP1 cDNA was amplified using the primers TEP1.1 (5′-TCAAG-CCAAACCTGAATCTGAG-3′) and TEP1.2 (5′-CCCGAGTGAATCTTTCTACGC-3′) through 27 cycles under these conditions. The reaction products were fractionated by polyacrylamide gel electrophoresis on an 8% gel and stained with SYBR Gold (Molecular Probes, Eugene, OR).

Measurement of telomere length. Genomic DNA was prepared using the DNA Extractor WB Kit (Wako, Osaka, Japan). The length of the TRF (terminal restriction fragment) for genomic DNA digested with *Eco*RI and *Hin*fI was measured by Southern blot analysis with a fluorescein-11-dUTP-labeled telomeric sequence probe $(TTAGGG)_4$. This method, used to estimate the length of the telomere, was described previously.[14] Signal detection was done with the Gene Images CDP-Star detection module (Amersham Biosciences Corp., Piscataway, NJ) and a LAS-1000 Luminoimage analyzer (Fujifilm, Tokyo, Japan). A fluorescein-labeled λ*Hin*dIII marker (Amersham Biosciences Corp.) was used for measuring the mean TRF length.

Senescence-associated β-galactosidase assay. Senescence-associated β-galactosidase (SA-β-Gal) staining was done by the method of Dimri *et al.* with some modifications.[15] Briefly, cells were fixed in 3% formaldehyde and then incubated with fresh SA-β-Gal staining solution (1 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) per ml of sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$). Staining was done at 37°C for 12 h.

Transfection. A549 cells were seeded at 1×10^5 cells·ml^{-1} and cultured over-night. A549 cells were transfected with an expression plasmid for Smad6 or Smad7 along with the pSV2bsr (Kaken Pharmaceutical, Tokyo, Japan) using the LipofectAMINE2000 reagent (Invitrogen) according to the manufacturer's instructions. Transfected cells were selected in the presence of 1 μg·ml^{-1} blasticidin S (Wako).

ELISA. The amount of IL-6 secreted by TGF-β-treated A549 cells was measured by the enzyme-linked immunosorbent assay (ELISA). Briefly, microtiter plates (Nunc, Roskilde, Denmark) were coated with anti-goat IgG antibody (Zymed, San Francisco, CA) and incubated at 37°C for 1 h. After the wells were washed with phosphate-buffered saline (PBS; 2.24×10^{-2} M phosphate, 1.37×10^{-1} M NaCl, pH 7.0) containing 0.05% Tween 20 (PBST), polyclonal goat anti-IL-6 antibody (R&D Systems, Minneapolis, MN) diluted with PBST was added and the plates were incubated at 37°C for 1 h. After the wells were washed with PBST, serially diluted supernatants and standard control samples (human IL-6; Genzyme, Cambridge, MA) were added and the plates were incubated at 37°C for 1 h. After the well were washed with PBST, biotinylated mouse anti-IL-6 monoclonal antibody (Genzyme) was added and the plates were incubated at 37°C for 1 h, then washed, then streptavidin-horseradish peroxidase conjugatge (Amersham Biosciences Corp.) was added and incubated at 37°C for 1 h. After the wells were washed, a solution of ABTS disodium salt (Wako) diluted in 0.2 M citrate buffer with 10^{-4} (w/v) H_2O_2 was added to the plate. Measurement at a wavelength of 405 nm with a reference at 492 nm was taken. Anti-hIL-6 monoclonal antibody was biotinylated by using *N*-hydroxysuccinimidobiotin (Sigma, St. Louis, MO).

Tumorigenicity assay. Four-week-old nude mice (BALB/c nu/nu, Charles River Labs., Wilmington, MA) were injected subcutaneously with 1×10^7 A549 cells cultured in the absence or presence of TGF-β for 211 days. Cells were scored as tumorigenic if a visible nodule (>0.5-cm diameter) appeared at the site of injection by 6 weeks after the injection. This experiment was done under the guidance for animal experiments in the Faculty of Agriculture and in the Graduate Course of Kyushu University and the Law (No. 105) and Notification (No. 6) of the Japanese Government.

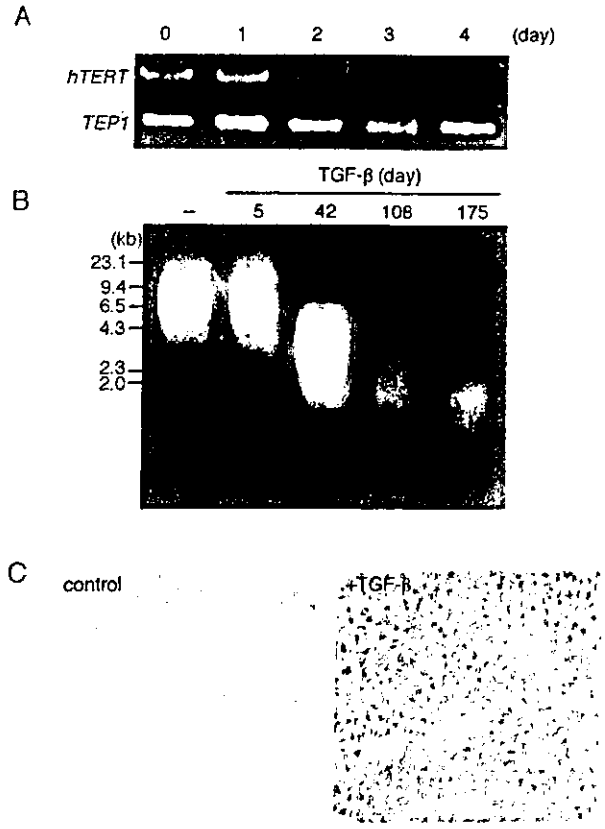

Fig. 1. TGF-β Triggers Two Independent Senescence Programs in Cancer Cells.

A, TGF-β represses the transcription of hTERT. A549 cells were treated with 10 ng·ml^{-1} of TGF-β for the indicated periods. Three μg of total RNA was used as the template for a cDNA synthesis reaction in a total volume of 20 μl. Subsequent PCR was done using 1 μl of the cDNA synthesis reaction mixture. hTERT cDNA was amplified using primers LT5 and LT6 through 29 cycles (94°C for 45 sec, 60°C for 45 sec, 72°C for 90 sec), and TEP1 cDNA was amplified using primers TEP1.1 and TEP1.2 through 27 cycles under the above-mentioned conditions. Reaction products were resolved on an 8% polyacrylamide gel and stained with SYBR Gold. B, Telomere length is shown to be shortened in A549 cells treated with TGF-β. Telomere shortening-dependent replicative senescence was induced in A549 cells treated with TGF-β. Prepared DNA was digested with *Eco*RI and *Hin*fI, and analyzed by Southern blot analysis using a fluorescein-dUTP-labeled probe $(TTAGGG)_4$. Signal detection was done using a Gene Images CDP-Star detection module. The fluorescein-labeled λ*Hin*dIII marker was used for estimating the length of TRF. C, Telomere shortening-independent premature senescence was induced in A549 cells upon treatment with TGF-β. SA-β-Gal activity was increased in A549 cells by treatment with TGF-β for 5 days, where the TRF length was almost the same as non-treated A549 cells (B).

Results and Discussion

TGF-β triggers cellular senescence programs in cancer cells

We have previously reported that TGF-β triggers two independent senescence programs in the A549 human lung adenocarcinoma cell line.[12] TGF-β represses transcription of hTERT in A549 cells, and has no effect on transcription of the ubiquitously expressed TEP1 (Fig. 1A). The regulations of hTERT transcription and telomerase activity are known to be coordinated in cancer cells. In A549 cells, it has been shown that the repression of hTERT leads to attenuated telomerase activity, which results in incomplete telomeric end replication and passage-number-dependent telomere shortening. As shown in Fig. 1B, TGF-β-treated A549 cells underwent telomere shortening in a passage-number-dependent manner. The telomere length for A549 cells treated with TGF-β for 175 days shortened to below 2 kb, whereas untreated A549 cells maintained a constant telomeric length (about 8 kb). These results suggest that TGF-β induces the replicative senescence program dependent upon telomere shortening in A549 cells. Furthermore, TGF-β triggers another cellular senescence program. TGF-β augmented senescence-associated β-galactosidase (SA-β-Gal) activity in A549 cells. This SA-β-Gal activity augmentation was observed from 5 days after addition of TGF-β (Fig. 1C), at which time A549 cells maintained almost the same telomeric length as non-treated A549 cells. This demonstrates that TGF-β also triggers the premature senescence program independent of telomere shortening in A549 cells. Taken together, these results indicate that TGF-β triggers two independent senescence programs in A549 cells; a replicative senescence program and a premature senescence program.

Senescence induction by TGF-β is dependent upon the Smad signaling pathway

The Smad pathway is well documented as a signal transduction pathway downstream from the TGF-β receptor. Smad2 and Smad3 are known to be phosphorylated by an activated TGF-β type I receptor, to form a complex with Smad4, and to regulate transcription of the TGF-β-responsive genes after translocation into nucleus. The Smad pathway has been reported to be inhibited by two inhibitory Smads, Smad6 and Smad7.[16,17] Here we tested for whether or not the Smad pathway is involved in TGF-β-induced cellular senescence.

First we established Smad6 or Smad7-expressing A549 cells by transfecting pcDNA3-Smad6 or pcDNA3-Smad7 into A549 cells and selected for recombinant transfectants. Then we treated these transfectants with TGF-β for 7 days, and the ability of TGF-β to induce cellular senescence was evaluated by detecting the cell's ability to repress hTERT and to increase SA-β-Gal activity. As shown in Fig. 2, the repression of hTERT expression in response to TGF-β was greatly attenuated in the Smad6 or Smad7-expressing transfectants. Furthermore, SA-β-Gal activity after a 7-day TGF-β treatment was greatly reduced in Smad6 or Smad7-expressing transfectants (Fig. 2). These results demonstrate that replicative and premature senescence induction by TGF-β are

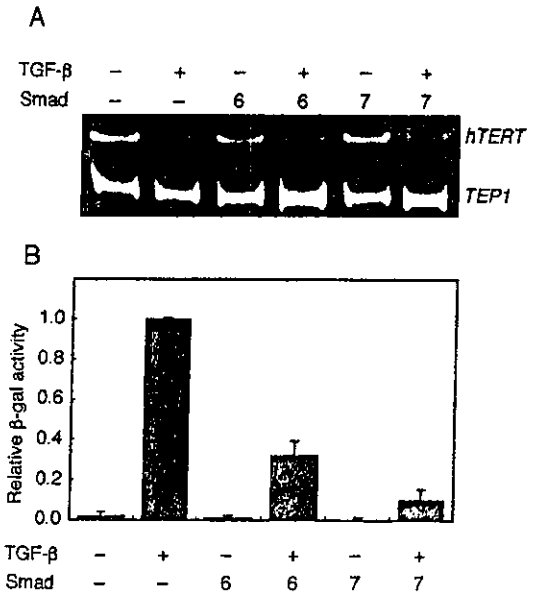

Fig. 2. Senescence Induction by TGF-β Is Dependent upon the Smad Pathway.

A, Repression of hTERT was canceled in Smad6- or Smad7-expressing transfectants. Smad6- or Smad7-expressing transfectants were treated with TGF-β for 7 days and assayed for the expression of hTERT. TEP1 was used as control. B, Augmentation of SA-β-Gal activity by treatment with TGF-β was greatly reduced in Smad6- or Smad7-expressing transfectants. Smad6- or Smad7-expressing transfectants were treated with TGF-β for 7days and assayed for SA-β-Gal activity. The number of SA-β-Gal positive cells was counted under a microscope.

both dependent upon the Smad pathway in A549 cells, and further suggest that activation of the Smad pathway is a prerequisite for the TGF-β induced cellular senescence. We can assume that genes under the control of the Smad pathway participate in the phenotypic changes leading to the senescent state, and further believe that identification of target genes in the Smad pathway may lead to the elucidation of the whole mechanism that controls senescence.

Phenotypic analysis of the TGF-β-treated A549 cells

As described above, we have demonstrated that TGF-β induces two independent senescence programs in A549 cells in a Smad-pathway-dependent manner. Next we investigated the phenotypic changes induced in long term TGF-β-treated A549 cells. Elongated A549 cells similar to senescent cells (Fig. 3B) were found to appear concurrently with the telomere shortening. Untreated A549 cells show a spherical morphology and continue to grow and divide, piling up upon each other even after reaching confluence (Fig. 3A). However, A549 cells treated with TGF-β for 182 days demonstrated arrested growth at confluence (Fig. 3B), and contact inhibition continued during a further 6 days of culture (Fig. 3C). These results indicate that TGF-β-treated A549 cells acquired contact inhibition at confluence.

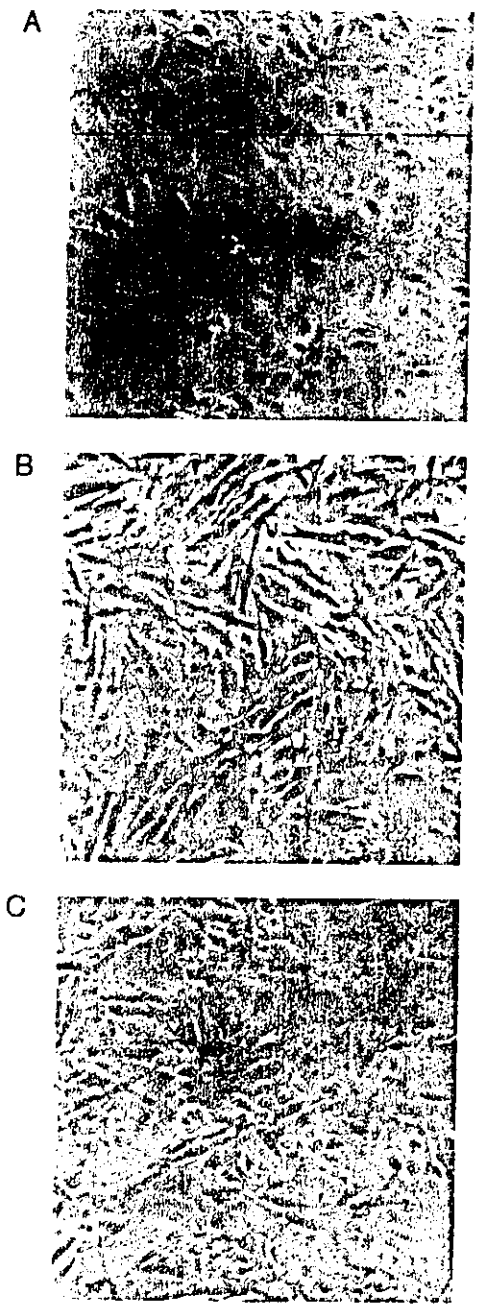

Fig. 3. TGF-β-Treated A549 Cells Show Contact Inhibition at Confluence.

A549 cells treated with TGF-β for 182 days were seeded at an initial density of 0.5×10^5 cells·ml^{-1} and cultured for 16 days. TGF-β-treated A549 cells arrested growth at confluence (B; day 10, 1.0×10^6 cells·ml^{-1}), whereas untreated A549 cells continued to grow by piling up upon each other (A). Contact inhibition at confluence was confirmed by culturing TGF-β-treated A549 cells for a further 6 days (C).

Considering together with the fact that TGF-β-treated A549 cells showed an elongated cell morphology resembling normal senescent cells (Fig. 3B), the TGF-β-treated A549 cells may have lost some tumor cell phenotypes and acquired some normal senescent cell phenotypes. To confirm that TGF-β is able to shift A549 cells into normal senescent cells, we measured the amount of IL-6 produced from TGF-β-

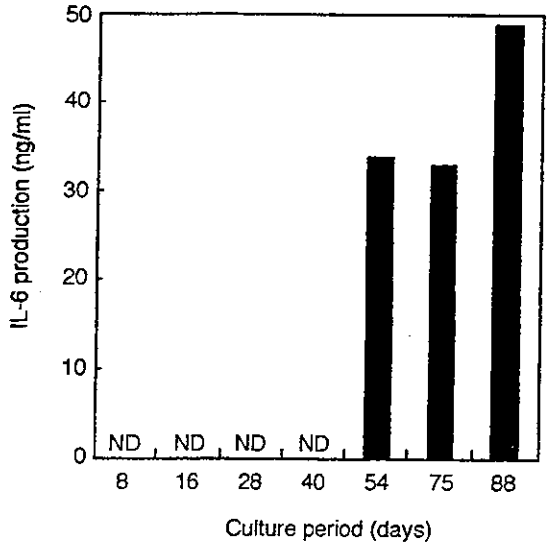

Fig. 4. TGF-β-Treated A549 Cells Secrete IL-6 in a Telomere-shortening Dependent Manner.

IL-6 secreted into the culture supernatant of A549 cells treated with TGF-β for a long time was measured by a sandwich ELISA. The amount of IL-6 secreted into the culture supernatant was expressed as that secreted from 1×10^6 cells per day. ND; not detected.

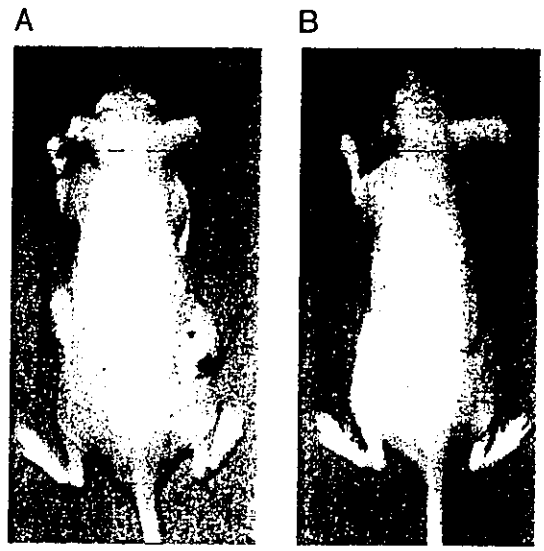

Fig. 5. Tumorigenicity of TGF-β-Treated A549 Cells.

Untreated A549 cells (A) and TGF-β-treated A549 cells (B) (1×10^7 cells) were injected subcutaneoously into the backs of 4 week-old nude mice ($n = 6$). We observed the progress of the tumor growth for 6 weeks and cells were scored as tumorigenic if a visible nodule (>0.5 cm diameter) appeared at the site of injection. Representative photographs are shown for each group, and the fraction in the photograph indicates the number of mice with a visible tumor at 6 weeks after the injection per the number of mice injected.

treated A549 cells. IL-6 is related to the onset of cellular senescence, and is suggested to be involved in age-related neural dysfunction, learning deficiency, and other pathological conditions.[18-21] Recently, the multifunctional cytokine IL-6 was reported to regulate growth and differentiation of many cell types *via* STAT3.[22,23] As shown in Fig. 4, A549 cells treated with TGF-β for a long time period (over 54 days) secreted IL-6 into the culture supernatant. These results suggest that TGF-β induces several phenotypic changes possibly related to cellular senescence in A549 cells, where IL-6 and a downstream signaling molecule might function in maintaining senescence. However, IL-6 alone did not induce cellular senescence in A549 cells (data not shown). Taken together, IL-6 might function in the maintenance, rather than induction, of these phenotypic changes. Actually, over 50 days of treatment with TGF-β is required for A549 cells to acquire the ability to inhibit the growth at confluence and secrete IL-6 into the culture supernatant. This is also when the telomere length in A549 cells shortened to about 4 kb upon the treatment of TGF-β and its concomitant repression of telomerase. These results indicate that these cell phenotypic changes are not directly induced by the TGF-β signal, but might be related to the telomere shortening, which in turn suggests that these phenotypic changes may be one of the consequences of telomeric positional effects. Taken together, we conclude here that TGF-β induces cellular senescence in A549 cells in two stages, one is the quick (within one week) repression of hTERT and augmentation of SA-β-Gal activity, while the other is dependent upon telomere shortening. Repression of hTERT leads to telomere shortening, thus the two stages of this induction process is thought to be inseparable and in fact sequential events. Next we investigated cancer cell phenotypes of senescent A549 cells with shortened telomeres upon a long term treatment with TGF-β *in vivo*.

Tumorigenicity of senesced A549 cells upon treatment with TGF-β

We tested for *in vivo* tumorigenicity of senesced A549 cells treated with TGF-β for 211 days by inoculation into athymic nude mice. A549 cells treated with TGF-β for 211 days were found to have a shortened telomere (about 2 kb) and demonstrated growth inhibition possibly due to the telomere shortening (data not shown),[12] thus we judged these cells to have fully undergone senescence. TGF-β-treated and untreated A549 cells (10^7 cells) were injected subcutaneously into the backs of 4 week-old nude mice. We observed the progress of tumor growth for 6 weeks and cells were scored as tumorigenic if a visible nodule (>0.5-cm diameter) appeared at the site of injection. One week after the injection, there was no detectable change in nude mice injected with untreated A549 cells, while scabs formed at the site of injection in nude mice injected with TGF-β-treated A549 cells. This suggests that senesced A549 cells may give rise to immune responses around the site of injection, the mechanisms of which are not yet clarified. Subsequently, small nodules gradually swelled to large

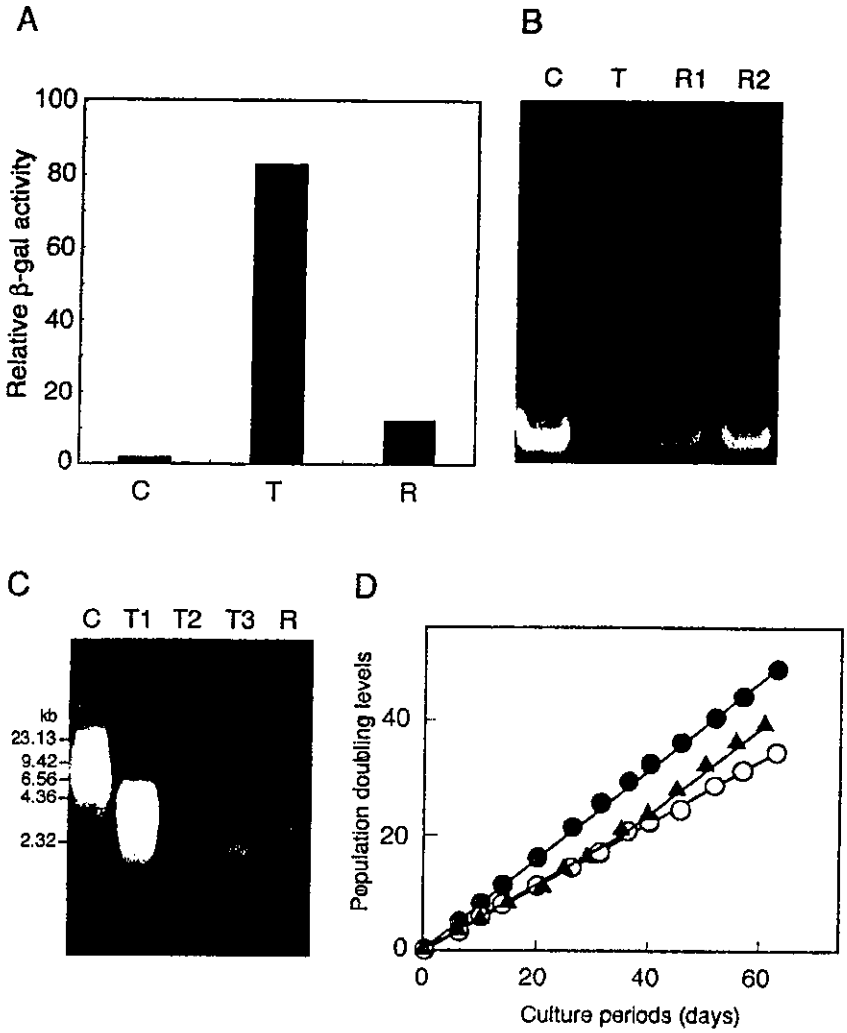

Fig. 6. Reversibility of TGF-β-Induced Senescence.

A, Reversibility of SA-β-Gal activity. Relative SA-β-Gal activities were assessed in A549 cells cultured in the absence (C) or presence (T) of TGF-β for 211 days, and in A549 cells treated with TGF-β for 211 days and subsequently cultured for 39 days after removal of TGF-β (R). B, Reversibility of telomerase activity. Reactivation of telomerase activity in A549 cells treated with TGF-β for 211 days and subsequently cultured for 20 days (R1) and 32 days (R2) after removal of TGF-β. C, non-treated A549 cells; T, A549 cells treated with TGF-β for 211 days. C, Reversibility of telomere length. Telomere lengths were measured in non-treaed A549 cells (C), A549 cells treated with TGF-β for 42 days (T1), for 108 days (T2), for 211 days (T3), and A549 cells treated with TGF-β for 211 days and subsequently cultured for 40 days after removal of TGF-β (R). D, Reversibility of growth rate. Growth rates were assessed in non-treated A549 cells (●), A549 cells cultured in the presence of TGF-β (○), and A549 cells cultured in the absence of TGF-β after the treatment with TGF-β for 211 days (▲).

tumor lumps (average diameter: 7.5 mm) in all nude mice injected with untreated A549 cells. In all the nude mice injected with TGF-β treated A549 cells, the scabs disappeared and no nodules could be detected (Fig. 5). At the end of the observation (6 weeks after the injection), tumor lumps formed in all nude mice injected with untreated A549 cells, but not in mice injected with TGF-β-treated A549 cells. These results demonstrate that senesced A549 cells by the treatment with TGF-β lost tumorigenicity. We could not clarify the mechanisms for how tumorigenicity is impaired in senescent A549 cells, however we can present several possibilities. One is that this tumorigenicity impairment is caused by forced mortalization of cancer cells with the repression of hTERT followed by the shortening of the telomere.

We then tested for the reversibility of TGF-β-induced senescence to investigate cell phenotypes of senescent A549 cells *in vivo* (Fig. 6). SA-β-Gal activity and telomerase activity were reversed by the removal of TGF-β (Fig. 6A and B), while 40 days, the time period spent in the tumorigenicity test, is not sufficient for senesced A549 cells to recover the telomere length and growth potential (Fig. 6C and D). Thus we assume that phenotypic changes dependent upon the telomere shortening and growth inhibition might be involved in the impaired tumorigenicity *in vivo*. Another possibility is that senesced A549 cells elicited immune responses possibly consisting of natural killer cells in nude mice *via* the acquisition of unanticipated antigenicity, or that these cells formed an anti-tumor environment by secreting senescence-

associated cytokines such as IL-6.

In conclusion, we showed that senescent A549 cells treated with TGF-β underwent several phenotypic changes, the major one of which is transformation into cells like normal cells both *in vivo* and *in vitro*. These results suggest that a forced induction into the senescence state in cancer cells is a novel and potentially effective method for anti-cancer therapy. Signal transduction pathways and signal mediators involved in cellular senescence may also be a novel target for anti-cancer therapy.

Acknowledgment

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Gene expression changes in BALB/3T3 transformants induced by poly(L-lactic acid) or polyurethane films

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Abstract: We performed DNA microarray analysis on two BALB/3T3 transformants (A5 and A6) induced by polyurethane (PU) film, two (L11 and L21) induced by biodegradable poly(L-lactic acid) (PLLA) film, and the parental cells. The transforming ability of the cells was in the order A5 < A6 < L21 < L11. In all, 1176 cancer-related genes were up- or down-regulated in at least one transformant. Those that were markedly up-regulated were c-*fos* protooncogene, FBJ osteosarcoma oncogene B, and Jun oncogene; those markedly down-regulated were pleiotrophin, histidine triad nucleotide-binding protein, protein kinase C iota, and large multifunctional protease 7. A common function of proteins encoded by genes that underwent marked expression changes was bone formation. The genes were c-*fos*, FBJ osteosarcoma, Jun, pleiotrophin, a disintegrin-like and metalloprotease with TS-1 motif protein 1. This finding was consistent with the tumor formation in the 2-year PLLA or PU subcutaneous implantation into rats. The number of genes that underwent marked expression change in each transformant was consistent with its malignancy. PLLA induced more malignant transformants than PU, especially in relation to osteosarcoma-like gene expression. © 2003 Wiley Periodicals, Inc. J Biomed Mater Res 68A: 376–382, 2004

Key words: BALB/3T3; transformation; PU; PLLA; DNA microarray analysis

INTRODUCTION

Polyurethanes (PUs) are widely used in medical devices because of their elasticity, high tensile strength, biocompatibility, and ease of handling. Poly(L-lactic acid) (PLLA) is used for bone screws and bone fixing plates because of its biodegradability. Some PUs, however, are unstable *in vivo* and induce tumors in rats.[1] Although there have been a number of *in vitro* studies on chemically induced transformation, few have analyzed the transformant DNA.

In the present study, we used DNA microarrays to analyze gene expression in transformants induced on high-molecular-weight polymer materials and related altered expression to the malignancy of the transformants, focusing on the consequences of transformation rather than on the process.

MATERIALS AND METHODS

Cells

Mouse Balb/3T3 clone A31-1-1 cells provided by Dr. T. Kuroki[2] (University of Tokyo) were maintained in minimum essential medium supplemented with 10% heat-inactivated fetal calf serum in 5% CO_2 in air at 37°C.

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Materials

PU [MDI/PTMO 1000/BD, weight-averaged molecular weight (M_w) 220,000] was obtained from Sanyo-kasei Co. Ltd. and PLLA (M_w 200,000) films (thickness 0.3 mm) were obtained from Shimadzu Corporation.

Coating of materials on the glass dishes

PU was dissolved in tetrahydrofuran. Half the surface area of glass dishes (diameter 6 cm) was coated with 320 mg of PU. After the dishes dried, they were sterilized by autoclaving at 121°C for 15 min. In the case of PLLA, the film was cut to fit the bottom of plastic dishes (diameter 6 cm) and attached with a small amount of acetone. The acetone was evaporated completely and the dishes were sterilized under UV-irradiation for 2 h.

The transformation assay

Cells were seeded at a density of 1×10^4 /plate (diameter 6 cm) on a coating and cultured in medium that was changed twice per week. After 6 weeks, transformants were isolated and stored at −80°C.

TABLE I
Transformation Assay of PU- and PLLA-Induced Transformants and Parental Controls

Cell Line	Cloning Efficiency (% of Control)	No. of Foci/Plate[a]
A31-1-1	100	0.3 ± 0.48
A5	106.3	2.3 ± 1.23
A6	110.8	47.0 ± 6.28
L11	144.8	114.1 ± 13.32
L21	126.2	84.5 ± 5.10
A31-1-1 + MC[b]	2.2	30.0 ± 5.15

[a]Average with standard deviation of 15 plates.
[b]0.5 μg/mL (positive control).

Two transformants induced by PU (A5 and A6) and two induced by PLLA (L11 and L21) were thawed and cultured for confirmation of transformation and for DNA microarray analysis, which were conducted in parallel. For the positive control assay, cells were treated with 3-methylcholanthrene (MC, 0.5 μg/mL) 24 h after seeding, washed with fresh medium 72 h later, and incubated in normal medium up to 6 weeks. The number of transformed foci per plate served as malignancy of transformants.

DNA microarray analysis

At least 10^7 cells were harvested and frozen in liquid nitrogen. Total RNA was extracted, purified, assessed for yield and purity, and cDNA probes were synthesized with the Atlas™ Pure Total RNA Labeling System (Clontech) according to the manufacturer's instructions. Hybridization of the ^{33}P-labeled probes to the Atlas Array of Mouse Cancer 1.2 k Array (Clontec 7858-1), on which 1176 cDNAs of cancer-related genes were spotted, were performed with Atlas™ cDNA Expression Arrays according to the manufacturer's instructions. The phosphor images of hybridized arrays were analyzed with AtlasImage™ (Clontech). Genes that were up- or down-regulated more than fivefold relative to the negative controls are discussed.

RESULTS

The number of foci per dish produced by the selected transformants increased in the order A5 < A6 < L21 < L11 (Table I). The transformants isolated from PLLA were more malignant than those isolated from PU. A31-1-1 cells treated with MC induced 30 foci per plate, as expected. Figure 1 shows the actual foci features. The A5 and A6 foci resembled those on the MC-treated dishes. The extracellular matrix appeared lyzed in transformants L11 and L21.

Fourteen genes increased expression more than fivefold in at least one transformant (Fig. 2, Table II). The three most markedly up-regulated genes were c-*fos* protooncogene, FBJ osteosarcoma oncogene B, and Jun oncogene; all increased most in L11. The only

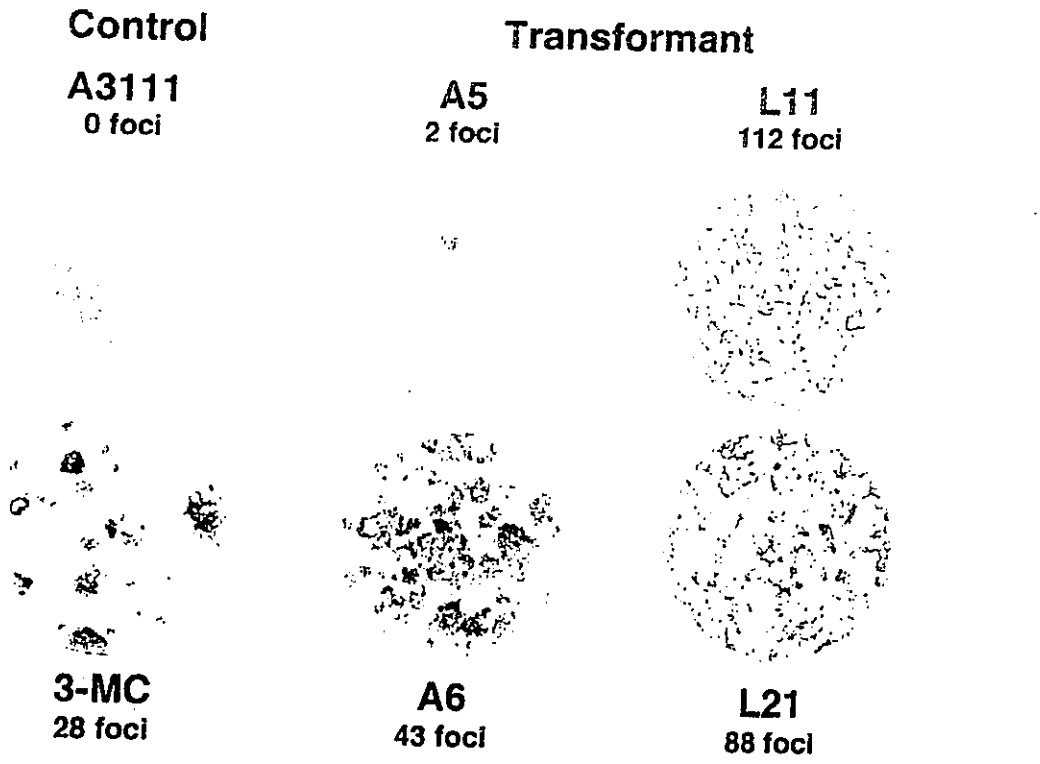

Figure 1. Photographs of dishes (6 cm in diameter) with Giemsa-stained foci in the confirmative transformation assay. The number of foci is shown in the representative plate of the control and each transformant. The control cells grew in monolayers and stained pink. The transformed cells stained blue were observed in the other plates, and the extracellular matrix of L11 and L21 plates appeared lyzed.

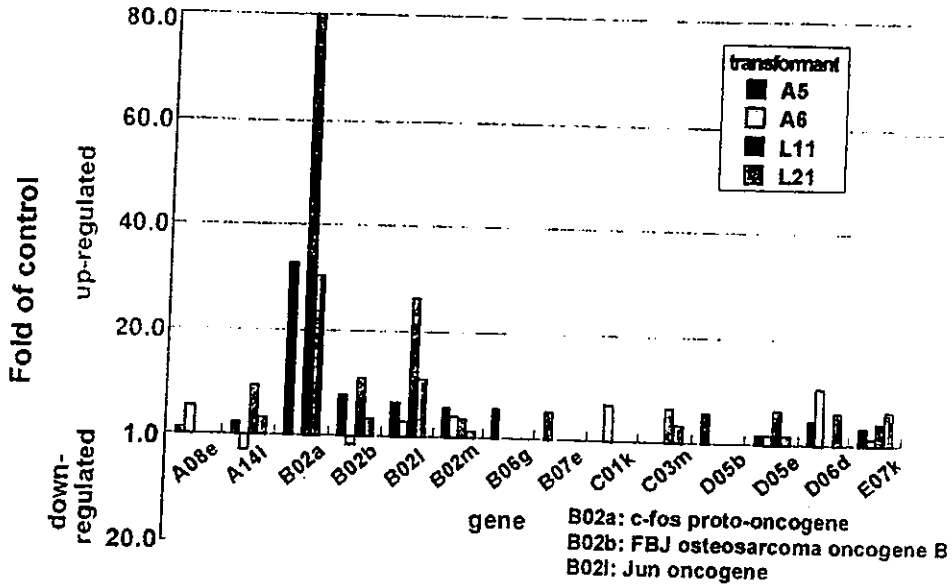

Figure 2. Expression profile of transformant genes whose expression increased more than fivefold in at least one transformant (see Table II for name key).

transformant that did not show any appreciable change in gene expression was A6.

Twenty-five genes decreased expression more than fivefold in at least one transformant (Fig. 3, Table III). The four most markedly down-regulated genes were pleiotrophin (PTN), histidine triad nucleotide-binding protein, protein kinase C iota, and large multifunctional protease 7; all except for large multifunctional protease 7, decreased most in L11. Transformant A6 showed a 20-fold decrease in the expression of large multifunctional protease 7

Figure 4 shows the expression profiles of 30 oncogenes and tumor suppressor genes. *c-fos* Protooncogene, FBJ osteosarcoma oncogene B, and Jun oncogene were up-regulated markedly in transformants A5, L11, and L21 The expression levels of ras, src, raf, mitogen-activated protein kinases, MEK, and p53 were similar in parental cells and transformants within an approximate twofold increase or decrease.

Among the extracellular matrix-related genes, HSP60, HSP65, HSPD1, mitochondrial matrix protein P1 precursor, 60-kDa chaperonin, GroEL protein, and matrix metalloproteinase 9 were markedly down-regulated, especially in transformant L11 (Fig. 5).

Expression of transforming growth factor (TGF) β1 and 2 and 8 connexin-related genes did not change significantly in any transformants (data not shown).

Table IV lists the genes that were up- or down-regulated more than fivefold relative to parental controls. Large multifunctional protease 7 was down-regulated more than fivefold, in all transformants. The gene expression profile of A6 was unique among the

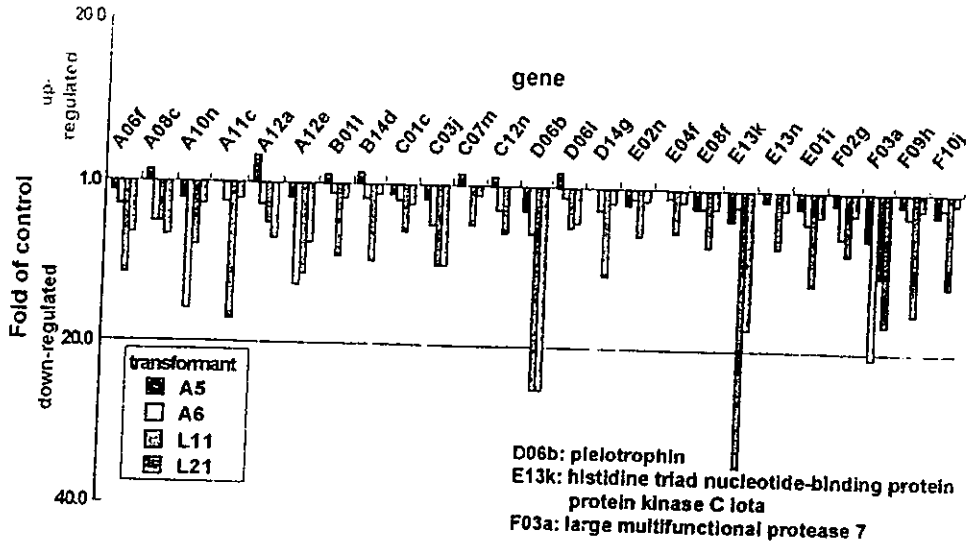

Figure 3. Expression profile of transformant genes whose expression decreased more than fivefold in at least one transformant (see Table III for name key).

TABLE II
Transformant Genes Whose Expression Increased More Than Fivefold

Code	Gene
A08e	Integrin β 7
A14l	Insulin-like growth factor binding protein 10
B02a	*c-fos* protooncogene
B02b	FBJ osteosarcoma oncogene B
B02l	Jun oncogene
B02m	*junB* protooncogene
B06g	HSP27, HSP25, HSPB1, growth-related 25-kDa protein
B07e	N-oxide forming dimethylaniline monooxygenase 1, hepatic flavin-containing monooxygenase 1, dimethylaniline oxidase 1
C01k	Retinoic acid receptor β, nuclear receptor subfamily 1 group B member 2
C03m	Caspase-activated DNase, DNase inhibited by DNA fragmentation factor
D05b	Insulin-like growth factor II precursor, multiplication-stimulating polypeptide
D05e	Leukemia inhibitory factor, cholinergic differentiation factor
D06d	Proliferin
E07k	Nonreceptor type 16 protein tyrosine phosphatase

four transformants in that proliferin was up-regulated more than 10-fold, thrombospondin 1 (TS-1) was down-regulated more than 10-fold, and large multifunctional protease 7 was down-regulated more than 20-fold. L11 showed the most appreciable changes in expression intensity and in the number of genes down-regulated. In that transformant, *c-fos* protooncogene and Jun oncogene were markedly up-regulated whereas PTN, histidine triad nucleotide-binding protein, and protein kinase C iota were markedly down-regulated. L21 showed marked up-regulation in the expression of *c-fos* protooncogene and Jun oncogene and marked down-regulation in the expression of PTN, histidine triad nucleotide-binding protein, protein kinase C iota, and large multifunctional protease 7.

The only significant association we observed between changes in gene expression and malignancy were for cellular tumor antigen p53, procollagen VI alpha 3 subunit, and connexin 43. The relationship was inverse and was observed when the decrease in expression was less than fivefold (data not shown).

DISCUSSION

DNA microarray analysis of two transformants (A5 and A6) induced on PU film and two (L11 and L21) induced on PLLA film showed L11 to be the most malignant and the one that underwent the most appreciable changes in gene expression levels.

Both *c-fos* and Jun were up-regulated in all transformants except A6. *c-fos*, a protooncogene, is the cellular homolog of *v-fos*, which was originally isolated from a murine osteosarcoma. Fos protein is a major component of the AP-1 transcription factor complex, which includes the jun family. In the present study, the genes involved in bone formation, namely *c-fos*, FBJ osteosarcoma oncogene B, Jun, PTN, ADAM-TS, and MMP9, were among those that changed expression levels (Table IV). They were up- or down-regulated markedly in L21 and even more so in L11. Wang et al.[3] demonstrated in transgenic and chimeric mice that overexpression of *c-fos* affects bone, cartilage, and hematopoietic cell development. Wang et al.[4] also showed that mice lacking *c-fos* are growth retarded, develop osteopetrosis with deficiencies in bone remodeling and tooth eruption, and have altered hematopoiesis. Onyia et al.,[5] investigating gene expression in rat osteoblast-like osteosarcoma cells (ROS 17/2.8) cultured *in vivo*, demonstrated that at 56 days, *c-fos* expression increased up to fivefold, *c-jun* expression increased 2.1-fold, and MMP-9 expression decreased to undetectable levels. Those findings are consistent with the present finding in L11, that is, that *jun*, *fos*,

TABLE III
Transformant Genes Whose Expression Decreased More Than Fivefold

Code	Gene
A06f	Cdk 6 inhibitor, Cdk 4 inhibitor C, Cdk inhibitor 2C
A08c	Fat tumor suppressor homolog (*Drosophila*)
A10n	Thrombospondin 1
A11c	VCAM-1 precursor
A12a	Cysteine-rich intestinal protein
A12e	Delta-like homolog 1, preadipocyte factor 1, SCP 1, FA1, ZOG
B01l	EB1 APC-binding protein
B14d	HSP60, HSP65, HSPD1, mitochondrial matrix protein P1 precursor, 60-kDa chaperonin, GroEL protein
C01c	Apoptosis inhibitor 1
C03j	Clusterin precursor, clustrin, apolipoprotein J, sulfated glycoprotein 2
C07m	Platelet-derived growth factor receptor α precursor
C12n	Hek2 murine homolog, Mdk5 mouse developmental kinase, Eph-related tyrosine-protein kinase receptor
D06b	PTN
D06l	Small inducible cytokine A9
D14g	Avian sarcoma virus CT10 (v-*crk*) oncogene homolog
E02n	Nonreceptor type 11 protein tyrosine phosphatase, phosphotyrosine phosphatase
E04f	Cdk7, CDC2-related kinase 4, Cdk-activating kinase, 39-kDa protein kinase, MO15, MPK7
E08f	Serum-inducible kinase
E13k	Histidine triad nucelotide-binding protein, protein kinase C iota
E13n	Menage a trois 1
F01i	A disintegrin-like and metalloprotease with thrombospondin type 1 motif protein 1
F02g	Matrix metalloproteinase 9
F03a	Large multifunctional protease 7
F09h	Developmentally d neural precursor cell expressed
F10j	Tubulin cofactor a

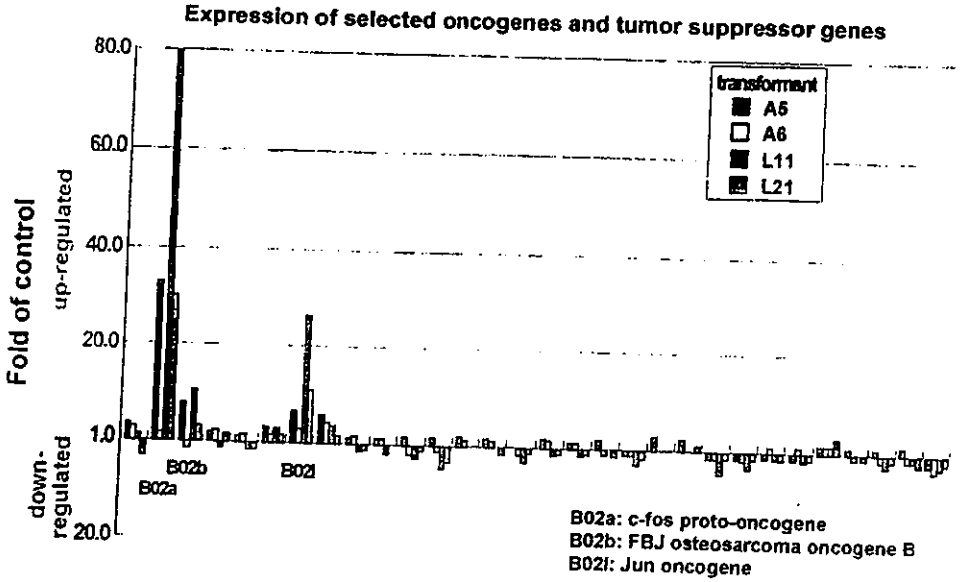

Figure 4. Expression profile of transformant oncogenes and tumor suppressor genes. Thirty-nine spots of oncogene- and tumor suppressor gene-related genes were on the DNA tip and 30 of them were analyzable, but not in all transformants. Only those genes whose expression level changed at least fivefold are named.

and osteosarcoma genes were up-regulated whereas MMP9 was down-regulated, suggesting that PLLA stimulated BALB/3T3 cells to express genes related to osteogenesis.

Nakamura et al.[6] observed bone formation in 6 of 22 tumors induced in rats by a 2-year PLLA subcutaneous implantation. Tumor incidence was 44% (22/50) with PLLA[6] and 38% (11/29) with PU,[1] which correlates well with the *in vitro* malignancy incidence data in the present study. Isama and Tsuchiya,[7] and Ikarashi et al.[8] reported that low-molecular-weight PLLA increases alkaline phosphatase activity and stimulates calcification of mouse osteoblast-like MC3T3-E1 cells.

PTN, a heparin-binding protein that can function as a neurite-promoting factor[9] or a mitogenic factor for fibroblasts,[10] contains two β-sheet domains that correspond to TS-1 repeats.[11] The expression of PTN is increased in various human tumors, suggesting it as a tumor marker and a target for tumor therapy. PTN was shown to regulate bone morphogenetic protein-induced ectopic osteogenesis in rats.[12]

A disintegrin-like and metalloprotease with TS-1 motif protein 1 (ADAM-TS) is a family of zinc-dependent proteases that has an important role in a variety of normal and pathological conditions such as arthritis and cancer. They consist of a signal sequence, a propeptide, a metalloproteinase domain, a disintegrin-like domain, a cysteine-rich region, and a variable number of TS-1 repeats. High levels of their tran-

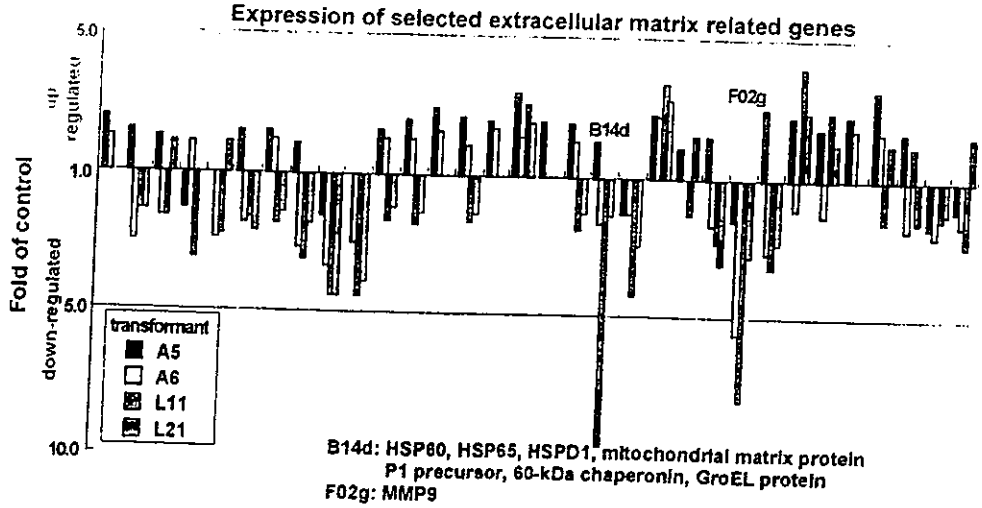

Figure 5. Gene expression profile of extracellular matrix-related genes. Thirty-two extracellular matrix-related genes were analyzable but data were not available in some transformants even in them. The code and name of genes whose expression changed fivefold or less are not shown. L11 showed clear down-regulation in expression of genes of codes B14d and F02g.

TABLE IV
Transformant Genes Whose Expression Increased or Decreased More Than Fivefold of Controls[a]

Transformant	Up-Regulated	Down-Regulated
A5	**c-*fos* protooncogene (33.0)** FBJ osteosarcoma oncogene B Jun oncogene *jun*B protooncogene HSP27, HSP25, HSPB1, growth-related 25-kDa protein N-oxide forming dimethylaniline monooxygenase 1, hepatic flavin-containing monooxygenase 1, dimethylaniline oxidase 1 Insulin-like growth factor II precursor, multiplication-stimulating polypeptide	Large multifunctional protease 7
A6	Integrin β 7 Retinoic acid receptor β, nuclear receptor subfamily 1 group B member 2 **Proliferin (10.7)**	Fat tumor suppressor homolog (*Drosophila*) **Thrombospondin 1 (15.8)** **Delta-like homolog 1, preadipocyte factor 1, SCP 1, FA1, ZOG (12.7)** Clusterin precursor, clustrin, apolipoprotein J, sulfated glycoprotein 2 PTN Matrix metalloproteinase 9
C11	Insulin-like growth factor binding protein 10 **c-*fos* protooncogene (79.8)** **FBJ osteosarcoma oncogene B (10.9)** **Jun oncogene (26.3)** Caspase-activated DNase, DNase inhibited by DNA fragmentation factor Leukemia inhibitory factor, cholinergic differentiation factor	**Large multifunctional protease 7 (20.7)** **Cdk 6 inhibitor, Cdk 4 inhibitor C, Cdk inhibitor 2C (11.5)** Fat tumor suppressor homolog (*Drosophila*) Thrombospondin 1 **VCAM-1 precursor (17.0)** **Delta-like homolog 1, preadipocyte factor 1, SCP 1, FA1, ZOG (11.2)** EB1 APC-binding protein HSP60, HSP65, HSPD1, mitochondrial matrix protein P1 precursor, 60-kDa chaperonin, GroEL protein Apoptosis inhibitor 1 **Clusterin precursor, clustrin, apolipoprotein J, sulfated glycoprotein 2 (10.0)** Platelet-derived growth factor receptor α precursor Hek2 murine homolog, Mdk5 mouse developmental kinase, Eph-related tyrosine-protein kinase receptor **PTN (25.2)** Small inducible cytokine A9 **Avian sarcoma virus CT10 (v-*crk*) oncogene homolog (11.0)** Nonreceptor type 11 protein tyrosine phosphatase, phosphotyrosine phosphatase Cdk7, CDC2-related kinase 4, Cdk-activating kinase, 39-kDa protein kinase, MO15, MPK7 Serum-inducible kinase **Histidine triad nucelotide-binding protein, protein kinase C iota (34.4)** Menage a trois 1 **A disintegrin-like and metalloprotease with thrombospondin type 1 motif protein 1 (11.8)** Matrix metalloproteinase 9 **Large multifunctional protease 7 (10.4)** **Developmentally d neural precursor cell expressed (15.0)** **Tubulin cofactor a (11.8)**
L21	**c-*fos* protooncogene (30.3)** **Jun oncogene (11.1)** Proliferin Nonreceptor type 16 protein tyrosine phosphatase	Cdk 6 inhibitor, Cdk 4 inhibitor C, Cdk inhibitor 2C Fat tumor suppressor homolog (*Drosophila*) Cysteine-rich intestinal protein Delta-like homolog 1, preadipocyte factor 1, SCP 1, FA1, ZOG **Clusterin precursor, clustrin, apolipoprotein J, sulfated glycoprotein 2 (10.0)** **PTN (25.2)** **Histidine triad nucleotide-binding protein, protein kinase C iota (17.2)** **Large multifunctional protease 7 (16.6)**

[a]Genes in regular and bold text were up- or down-regulated between 5- and 10-fold, and more than 10-fold, respectively. Figures in parentheses indicate fold-increase or -decrease in gene expression compared with parental cells.

scripts are observed in some tumor biopsies and cell lines, including osteosarcomas, melanoma, and colon carcinoma cells.[13,14]

Vascular cell adhesion molecule 1 (VCAM-1) is inducible by inflammatory cytokines and lipopolysaccharides such as interleukin 1, tumor necrosis factor α, interferon γ, and interleukin 4. It functions by binding with integrin $\alpha_4\beta_1$. Kawaguchi and Ueda[15] reported that VCAM-1 was not expressed in the seven osteosarcoma specimens tested in a study on the distribution of integrins and their matrix ligands in osteogenic sarcomas. Those results agree with the finding in the present study that osteosarcoma-like gene expression was down-regulated in L11.

BALB/3T3 cells are sensitive to transformation and must be handled carefully. Repeated subculture and overgrowth are not advised. We cultured the isolated foci under constant conditions to investigate the difference in gene expression. Because DNA microarray analysis was done only once, we discussed only genes that showed clear differences in expression from the controls. Based on these preliminary data, further studies are needed to confirm bone formation by PU and PLLA

CONCLUSIONS

The gene that showed the greatest change in expression after cell culture on PU was *c-fos* protooncogene. Osteogenesis was a common function of proteins encoded by genes that underwent a marked change in expression. Although the changes in gene expression induced by PU and PLLA differed in intensity, the results were consistent with previously reported findings of *in vivo* tumor formation. PLLA had a greater effect than PU on the expression levels of genes related to bone formation. In the transformants, both up-regulation of oncogenes and down-regulation of other kinds of genes were induced, and the latter appeared to be more related to the malignancy of transformants than the former.

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TISSUE ENGINEERING

再生医療工学

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工業調査会

1 再生医療をとりまく規制とその現状・今後

医療機器・細胞組織医療機器関連の薬事法の改正が2003年からスタートしている。

その内容は，（1）多様性に富んだ医療機器のリスクに応じた新クラス分類とその承認制度の見直し，（2）細胞組織医療機器が含まれる生物由来製品の感染リスクに応じた安全対策の充実，（3）市販後安全対策の抜本的見直し，が急ピッチで進められている。また，第三者認証制度の導入において必要な規格・基準の整備も行われつつある。本稿では，細胞組織医療機器や新たな制度である生物由来製品の薬事法の改正内容を中心に記載した。

細胞組織医療機器などの薬事法改正について

2000年12月26日付けでヒトまたは動物由来成分を原料として製造される医薬品などの品質および安全性確保についての医薬安全局長通知（医薬発第1314号）において，別添1「細胞組織利用医薬品などの取り扱いおよび使用に関する基本的考え方」と別添2「ヒト由来細胞・組織加工医薬品などの品質および安全性の確保に関する指針」の二つの文書が示された。

別添1の基本的考え方は，ヒトや動物の細胞・組織から構成される医薬品および医療機器（2005年度から医療用具は，医療機器に名称変更される予定）について，品質および安全性の確保ならびに細胞・組織の取扱いに関する科学的および倫理的妥当性を確保するための方策をまとめている。

別添2の指針は，「基本的考え方」に基づき，ヒトの細胞・組織に培養処理などの加工を施して製造される医薬品および医療機器について，品質および安全性の確保のために必要な基本的技術要件を定めており，治験前に厚生労働省に提出する品質および安全性の確認申請時に，必要な添付資料の内容を示している。

平成13（2001）年3月28日付けで「薬事法施行規則の一部を改正する省令等の施工について（細胞組織医薬品及び細胞組織医療用具に関する取扱いについて）」（医薬発第266号）省令及び告示が公布され，平成13年

4月1日より施行されている。

ヒトまたは動物の細胞または組織より構成された医療機器および医薬品に関して科学技術の進歩に伴う感染症への対策が急務となり，ドナースクリーニング，感染因子の不活化など，ドナーに由来する感染症への対策，培養などの処理により細胞または組織が有害な性質のものとならないことの確認など，品質および安全性を確保するために特別の対策が必要とされ，改正された。

細胞組織医療機器などの適用範囲

ヒトまたは動物の細胞または組織より構成される医療機器および医薬品であり，動物由来の組織を利用して承認を取得している生体弁や心のう膜も含んでいる。

改正概要

承認申請書の記載方法の変更(詳細は，最新の薬事法で確認のこと)や，GMP関係省令についても一部改正された。その概要は，細胞組織医療機器などを製造または輸入するにあたり，細胞もしくは組織由来または製造工程中の感染症などの伝播による危険性を排除し，不適切な製造や取扱いによる品質および安全性上の問題の発生を防止するために，製造管理と品質管理に必要な要求事項を定めた。

具体的には，

1) 細胞組織医療機器などの製造所の構造設備の基準への適合，原料の受け入れ，加工処理，製品の保管などを行う区域について，他区域からの区分，必要な構造および設備の要求。
2) 加工処理の「加工」とは，疾病の治療や組織の修復または再建を目的として，細胞または組織の人為的増殖，細胞または組織の活性化を目的とした薬剤処理，生物学的特性改変，遺伝子工学的改変，非細胞または非組織成分とのハイブリッド化，カプセル化などを施すこと。
3) 細胞組織医療機器などは，生物学的製剤などと同様に原料として使用する人，動物，植物または微生物から得られたものにかかる事項や使用動物の規格に関する事項について，製品標準書を作成。
4) 細胞組織医療機器などの製造，保管および出納ならびに衛生管理に関する記録については，遅発性感染症の危険性を否定し得ないことから，安全性の確保上必要な情報を得るために，少なくとも所定の期間記録を保存す

ることとなっている。すなわち，特定生物由来製品の場合，医療機関での患者使用記録の保管期間は 20 年間とし，製造業者などでの提供者・製造記録の保管期間は 30 年間と規定。生物由来製品では，製造業者などでの提供者・製造記録の保管が求められ，人血液成分以外の成分に関する記録は 10 年間，人血液成分を含む場合の人血液成分に関する記録は 30 年間保管となっている（生物由来製品の特性に応じて保管期間は異なる。最新版の薬務公報に目を通し，現時点での正確な情報を入手し，確認すること）。

5) 細胞または組織の取り違えや細菌，真菌，ウイルスなどの伝播の危険性を避けるために，製造工程において複数のドナーからの細胞，または組織を同一室内で同時期に取り扱ったり，交叉汚染を引き起こすような保管方法をとらないこと。さらに，ドナーまたはドナー動物ごとに細胞や組織，中間製品および製品を管理する必要がある。

6) 原料となる細胞または組織について，後述した内容において，適格なものであることを確認し，その結果に関する記録を作成すること。

（ｉ） 当該細胞または組織を採取した施設，

（ii） 当該細胞または組織を採取した年月日，

（iii） 当該細胞または組織が人に係るものである場合には，ドナースクリーニング（ドナーについて，問診，検査などによる診断を行い，細胞組織医薬品の原料となる細胞または組織を提供する適格性を有するかどうかを判定することをいう）のためのドナーの問診，検査などによる診断の状況，

（iv） 当該細胞または組織が動物に係るものである場合には，ドナー動物の受入の状況ならびにドナースクリーニング（ドナー動物について，試験検査および飼育管理を行い，細胞組織医薬品の原料となる細胞または組織を提供する適格性を有するかどうかを判定することをいう）のためのドナー動物の試験検査および飼育管理の状況，

（ｖ） 当該細胞または組織を採取する作業の経過（採取する作業経過に関する記録と採取作業において微生物などに汚染されていない旨が確認できるものであること。），

（vi） （ｉ）から（ｖ）までに掲げるもののほか，細胞組織医療機器などの品質の確保に関し必要な事項（製造に使用する試薬に関する試験検査結果を指す。），

7) 「施設」は組織を採取した医療施設もしくは動物の細胞または組織を採取した施設を指す。

8) 「適格性を有する」とは，「細胞組織医薬品および細胞組織医療用具に関する基準」の以下のいずれにも該当し，原料となる条件を満たしているもの。

細胞組織医療機器について，薬事第42条の規定に基づき，品質および安全性確保の観点から，原料または材料となる細胞または組織に関する基準を定めている（2001年3月厚生労働省告示第101号関係）。この基準を満たさない細胞または組織は，品質および安全性についての情報が十分でないことから，製造業者は，これら細胞または組織を原料または材料として医薬品または医療機器として製造すべきでない。

「基準の概要」

医薬品または医療機器の原料または材料となる細胞または組織については，

- 必要な衛生管理と人員を持つ施設で採取されていること，
- ドナースクリーニングが適切に行われていることが確認できること，
- 採取作業が適切に行われていることが確認できること，
- 必要な記録を確認できること，

が必要である。

ドナースクリーニングの項目など具体的な内容については，個別の製品ごとに異なることから，具体的事項については承認申請書に記録する。

生物由来製品に関する制度の概要

① 生物由来製品に関する制度の創設について

人または動物の細胞，組織などに由来する原材料を用いて製造される生物由来製品は，その特性として，原材料の汚染に由来する感染リスクなどについて，注意を払う必要がある。生物由来というこの共通特性に着目し，原材料採取・製造から市販後に至る，一貫した安全性確保体制を導入し，製品の安全性を図るために創設された。

② 生物由来製品に関する制度の主な内容

（a） 生物由来製品および特定生物由来製品の指定

製品の感染症リスクを考慮した科学的評価に基づき，指定を行い，生物由来製品は約700製品，特定生物由来製品は約280製品について指定し，公表した。

平成 15（2003）年厚生労働省告示 209 号「厚生労働大臣が指定する生物由来製品及び特定生物由来製品を定める件」

（b） 生物由来原料基準

生物由来原材料を用いるすべての医薬品などの原材料について，品質・安全性の確保のために，適格性の基準を制定している。

平成 15 年度厚生労働大臣告示 210 号「生物由来原料基準を定める件」及び平成 15 年 5 月 20 日医薬発第 0520001 号「生物由来製品及び特定生物由来製品の指定並びに生物由来原料基準の制定等について」

（c） 血液製剤などの使用記録などの保管期間

（前述した。）

（d） 表示

特定生物由来製品，生物由来製品それぞれの容器・包装に識別表示を行う。血液成分を含む特定生物由来製品については，採血国，献血，非献血の別を記載。

（e） 添付文書記載要領

2003 年 5 月 15 日付け医薬発第 0515005 号医薬局長通知「生物由来製品の添付文書に記載すべき事項について」および 2003 年 5 月 20 日付け医薬安発第 0520004 号医薬局安全対策課長通知「生物由来製品の添付文書の記載要領」において，生物由来製品に係る添付文書の具体的な記載要領などを定めている。

（e） 感染症定期報告

生物由来製品については，その原料が細胞組織などであり，未知の感染因子を含有している可能性を否定できない。1996 年度の薬事法改正において，製造業者に対して，感染症に対して製品との関連が否定できない症例の報告・研究報告義務を明確化した。2002 年度の改正薬事法においては，感染症対策をより綿密に行うために，感染症定期報告制度を導入し，2003 年 7 月 30 日より施行している。

2003 年 5 月 15 日付け医薬発第 0515008 号医薬局長通知「生物由来製品に関する感染症定期報告制度について」において，生物由来製品に係る感染症定期報告について示し，2003 年 10 月 24 日付け薬食安発第 1024006 号厚生労働省医薬食品局安全対策課長通知，「生物由来製品の感染症定期報告に係る調査内

容および記載方法について」を発出した。

その調査内容は，（1）研究報告について，（2）調査方針について，（3）種類別発生状況および発生症例一覧について，（4）論文などにより発表された感染症症例に関する報告の取扱いについて，（5）調査対象外成分について，（6）調査対象文献など，から構成されている。

局長通知別紙様式の記載方法については，（1）生物由来製品の概要について，（2）生物由来成分の概要について，（3）出荷数量などについて，（4）安全性に関する見解などについて，（5）同一生物種などから人に感染すると認められる疾病についての研究報告について，（6）当該生物由来製品などによるものと疑われる感染症について，など，記載方法が詳細に示されている。

これらの通知などは，新たな発出や廃止が常に行われているので，細心の注意を払い正確な情報を入手することが肝要である。

（f）使用対象者への説明並びに記録および保存

医療関係者に対し，特定生物由来製品の適正な使用のための必要な事項についての使用対象者への説明を義務づけている。また，特定生物由来製品の遡及調査などを可能とするために，使用の対象者の氏名などの記録およびその保存を義務付けた。

製造業者に対しては，生物由来製品の遡及調査などを可能とするため，販売などを行った生物由来製品に関する記録およびその保存を義務付けている。

（g）製造業者などの生物由来製品製造管理者の設置要件の規定

生物由来製品の製造業者などは，生物製品製造管理者を設置しなければならない。その承認の対象者は，以下のように規定されている。

（i） 医師，医学の学位を持つ者

（ii） 歯科医師であって細菌学を専攻した者

（iii） 細菌学を専攻し，修士課程を修めた者

（iv） 大学などで微生物学の講義および実習を受講し，修得した後，3年以上の生物由来製品もしくはそれと同等の保険衛生上の注意を要する医薬品，医療用具などの製造等（治療薬として製造する場合も含む。）に関する経験を有する者

医療機関からの副作用等報告制度（医師・薬剤師などの医薬関係者から直接厚生労働省に報告される副作用・不具合または感染症報告の報告事項を規定）

2003年5月15日付け医薬発第0515014号医薬局長通知「医療機関等からの