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Genetic Complementation of the Immortal Phenotype in Group D Cell Lines by Introduction of Chromosome 7

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Human immortal cell lines have been classified into at least four (A–D) genetic complementation groups by cell-cell hybrid analysis, i.e., a hybrid derived from different groups becomes mortal. Recently we have demonstrated that introduction of human chromosome 7 suppresses indefinite division potential in the non-tumorigenic human immortalized fibroblast lines KMST-6 and SUSM-1, both assigned to complementation group D. By extending our microcell-mediated chromosome transfer, we found that chromosome 7 also suppresses division potential in the human hepatoma line HepG2 (again, assigned to group D). Chromosome 7 was thus shown to suppress indefinite growth in the above group D cell lines irrespective of their cell types, or whether they are tumorigenic or not. Since chromosome 7 had no such effect on representative cell lines derived from complementation group A, B or C, these results indicate that the senescence gene(s) commonly mutated in the group D cell lines is located on chromosome 7.

Key words: Cellular senescence — Immortalization — Complementation group — Chromosome transfer

Normal human diploid fibroblasts have a limited proliferative capacity and senesce after 50–80 population doublings in culture.[1)] Transfection of the cells with a DNA tumor virus such as simian virus 40, polyoma virus, adenovirus, and papilloma virus extends their life span, but such cells eventually lose their division potential.[2,3)] However, a population of heavily mutagenized cells[4,5)] or the cells transformed with the above tumor viruses gives rise to mutant clones which have acquired an indefinite division capacity, although at a very low frequency.[6,7)] Therefore, immortalization is now thought to occur by mutational inactivation of multiple genes in normal human cells or a single gene in virally transformed cells.

The immortal trait is indeed genetically recessive since cell-cell hybrids obtained by fusing an immortal line to a normal mortal line or most of those obtained by crossing immortal lines to one another become mortal.[8–12)] Based on these observations, intensive cell-cell hybridization analysis has revealed that human immortalized lines fall into at least four (A, B, C, and D) genetic complementation groups with respect to the phenotype of immortality.[12)] It is therefore suggested that at least four genes are involved in cellular senescence in human cells.

Subsequently, transfer of human metaphase chromosome 1 by microcell-mediated chromosome transfer has made it possible to suppress indefinite division potential in the established Chinese hamster cell line.[13)] In accordance with the presence of the above four genetic complementation groups, human chromosome 4 specifically suppresses the indefinite division potential in HeLa and two other cell lines all belonging to complementation group B.[14)] Recently, we have found that chromosome 7 suppresses the division potential in two non-tumorigenic fibroblast cell lines[15)] that had been immortalized in culture by repeated γ-ray irradiation[4)] or 4-nitroquinoline 1-oxide treatment[5)] and assigned to complementation group D.[12)] Although these cell lines are derived from normal fetal diploid fibroblasts by *in vitro* mutagenesis and are phenotypically similar, it appears that chromosome 7 is generally effective in suppressing the division potential of group D cell lines.[15)]

Here we demonstrate that chromosome 7 also suppressed indefinite division potential in HepG2, which was derived from a human hepatoma and assigned to group D.[16)] We also present evidence that several representative cell lines assigned to complementation group A, B, or C were not affected by introduction of chromosome 7.

MATERIALS AND METHODS

Cell culture HepG2 (hepatoma) cells were cultured in Dulbecco's modified Eagle's medium (Nissui Seiyaku Company Ltd., Tokyo) supplemented with 12.5% fetal

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calf serum (Sigma, St. Louis, MO). HT1080 (fibrosarcoma), HeLa (cervical carcinoma), and TE85 (osteosarcoma) cells were cultured in ES medium (Nissui) supplemented with 10% fetal calf serum as described.[15] The microcell donors A9(NTI8)-2 and A9(3552)-2 contain a single copy of human chromosome 7 and 11, respectively, each tagged with the neomycin-resistance gene (*neo*) in an A9 mouse fibroblast background.[17] The donor cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 800 μg/ml G418 (Geneticin, Sigma). Microcell hybrids were selected in the medium containing 200 μg/ml G418.

Microcell-mediated chromosome transfer Microenucleation, microcell fusion and hybrid isolation were performed as described.[15] The donor cells were cultured in 25 cm^2 flasks (Costar No. 3025, Cambridge, MA) for 2 days. Micronuclei were induced by treatment with colcemid (0.05 μg/ml) for 48 h in the medium containing 20% fetal calf serum and G418. The flasks were filled with serum-free medium containing 10 μg/ml of cytochalasin B (Sigma), and enucleation was performed by centrifugation of the entire flasks placed in acrylic inserts filled with tempered water in a fixed-angle rotor at 10,000*g*. After 1 h of centrifugation, microcells were collected in serum-free medium and the microcell suspension was filtered through 8 μm and 5 μm polycarbonate filters (Nucleopore, Pleasanton, CA) in series. The purified microcells were pelleted by centrifugation at 400*g* for 10 min and resuspended in serum-free medium containing phytohemagglutinin (100 μg/ml). The microcells were attached to the prewashed recipient cell monolayers by incubation for 15 min at 37°C. The cells were fused by treatment with 3 ml of 47% polyethylene glycol (MW 1540, Baker Chemical Co., Phillipsburg, NJ) solution for 1 min followed by extensive washes with serum-free medium. After 1 day of incubation in growth medium, cells were trypsinized and plated onto 90 mm dishes (Nunclon, Denmark) containing G417 at the indicated concentration.

Detection of the *neo* gene on introduced chromosomes Genomic DNA was prepared from the microcell donors, the recipient lines, and microcell hybrids using a DNA extraction kit as described.[15] To detect the *neo* gene tagged on the introduced chromosomes, a sequence in the *neo* gene was amplified from these samples by polymerase chain reaction using a primer set(5′-CATGGTGGAAAATGGCCGCT-3′ and 5′-GAAGAACTCGTCAAGAAGGC-3′) as described previously.[18] After polymerase chain reaction using the reported thermal cycle conditions,[18] amplified products were resolved on 3% agarose gel (NuSieve, FMC BioProducts, Rockland, ME) by electrophoresis and stained with ethidium bromide.

Table I. Division Potential of the Microcell Hybrids Isolated by Introduction of Chromosome 7 in Representative Immortalized Cell Lines Assigned to Each of the Four Genetic Complementation Groups

Cell line	Complementation group	Range of PDs achieved before division cessation	No. of clones with arrested division/No. of clones tested
HepG2	D	10–24	12/17
KMST-6	D	10–30[a]	15/23[a]
SUSM-1	D	10–45[a]	30/44[a]
HT1080	A	>100	0/11
HeLa	B	>100	0/9
TE85	C	>100	0/14

a) Results from ref. 15.

RESULTS

We introduced chromosome 7 and chromosome 11 by microcell-mediated chromosome transfer into HepG2 and control cell lines such as HT1080, HeLa, and TE85 that have been assigned to complementation groups A, B, and C, respectively. G418-resistant microcell hybrids arose at a frequency of 1 to 10×10^{-6} per treated cell for both chromosome 7 and 11 when the above cell lines were used as recipients. Of 17 microcell hybrids of HepG2 containing introduced chromosome 7 examined, 12 clones stopped dividing after 10–24 population doublings (PDs) (exact PD values observed before senescence were: <18 PDs, 5 clones; <20 PDs, 4 clones; <22 PDs, 2 clones; and <24 PDs, 1 clone). In contrast, all of the microcell hybrids of HepG2 containing introduced chromosome 11 tested continued to divide for more than 100 PDs, the criterion for a microcell hybrid to be judged immortal. The proportion of the suppressed to the unsuppressed (~60%) in the microcell hybrids of HepG2 was similar to those obtained in our previous experiments, in which the same donor of chromosome 7, NTI8, was used as in the present study, with KMST-6 and SUSM-1 as recipients.[15] It is therefore suggested that the same senescence gene on chromosome 7 should operate in suppressing indefinite division potential in HepG2, KMST-6 and SUSM-1, since the above value is expected to depend on the position of the putative senescence gene relative to the integration site of the *neo* marker (7q32-33). On the other hand, introduction of chromosome 7 did not affect division potential in any of the microcell hybrids derived from HT1080, HeLa, or TE85 (Table I). All of the microcell hybrids examined continued to divide for more than 100 PDs.

Fig. 1 shows the morphology of two representative microcell hybrids of HepG2 which responded to introduced chromosome 7 as well as one hybrid of HepG2 containing introduced chromosome 11. The affected

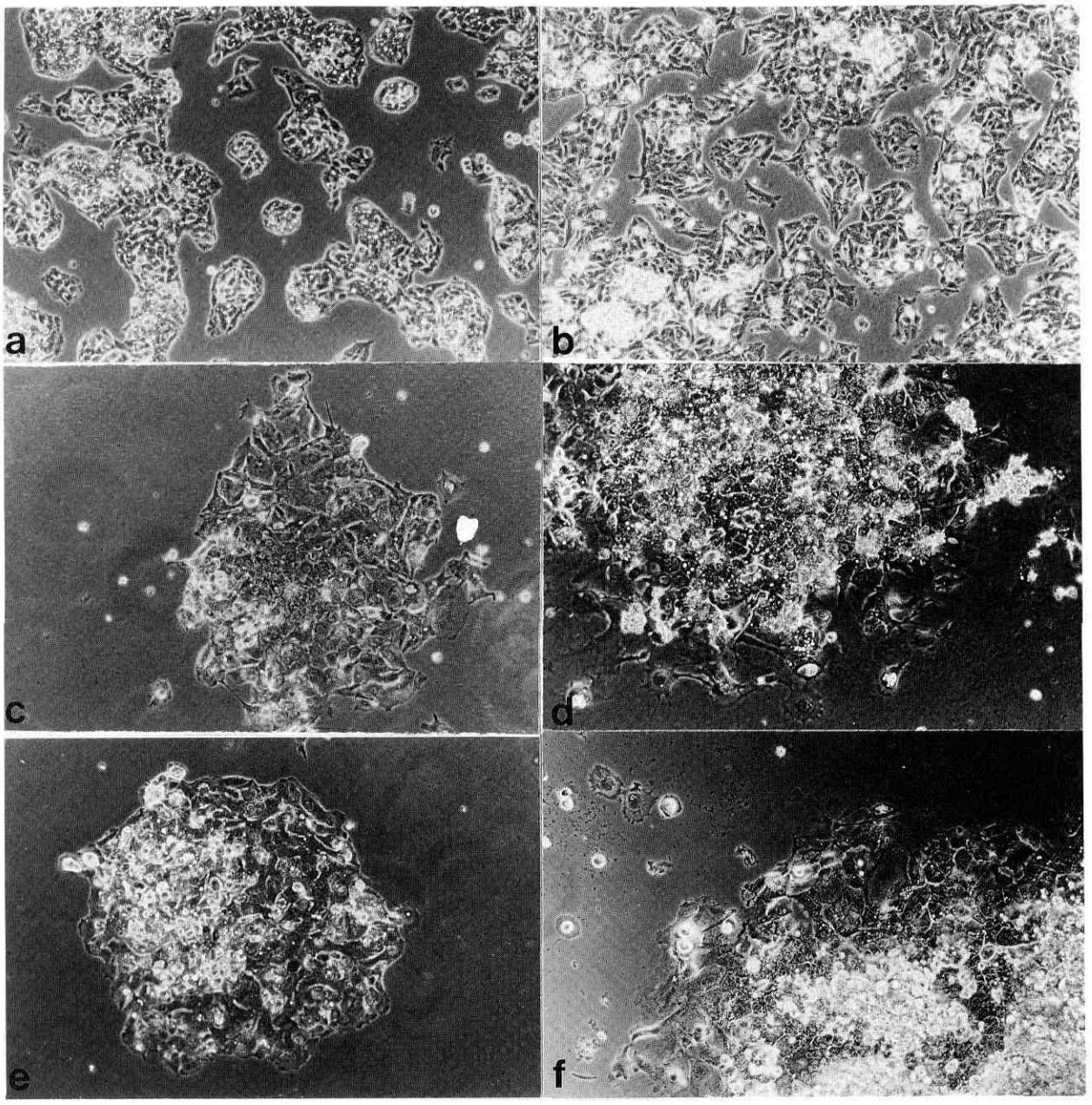

Fig. 1. Phase-contrast micrographs of the microcell hybrids of HepG2. (a) and (b), HepG2 and one microcell hybrid containing introduced chromosome 11; (c) and (d), one microcell hybrid of HepG2 containing introduced chromosome 7 cultured for 4 and 8 weeks post-fusion; and (e) and (f), another microcell hybrid of HepG2 containing introduced chromosome 7 cultured for 4 and 8 weeks post-fusion, respectively. The cells are magnified 100 fold.

hybrids of HepG2 exhibited increase in cell size, heterogeneous cell shapes and orientation, and lower saturation densities, which are all characteristic of normally senesced cells, i.e., the normal diploid cells cultured until their division potential has expired. The unsuppressed microcell hybrids of HepG2 and any of the microcell hybrids of the control cell lines each containing introduced chromosome 7 or 11 did not show any growth retardation or change in cell morphology, as shown in Fig. 2.

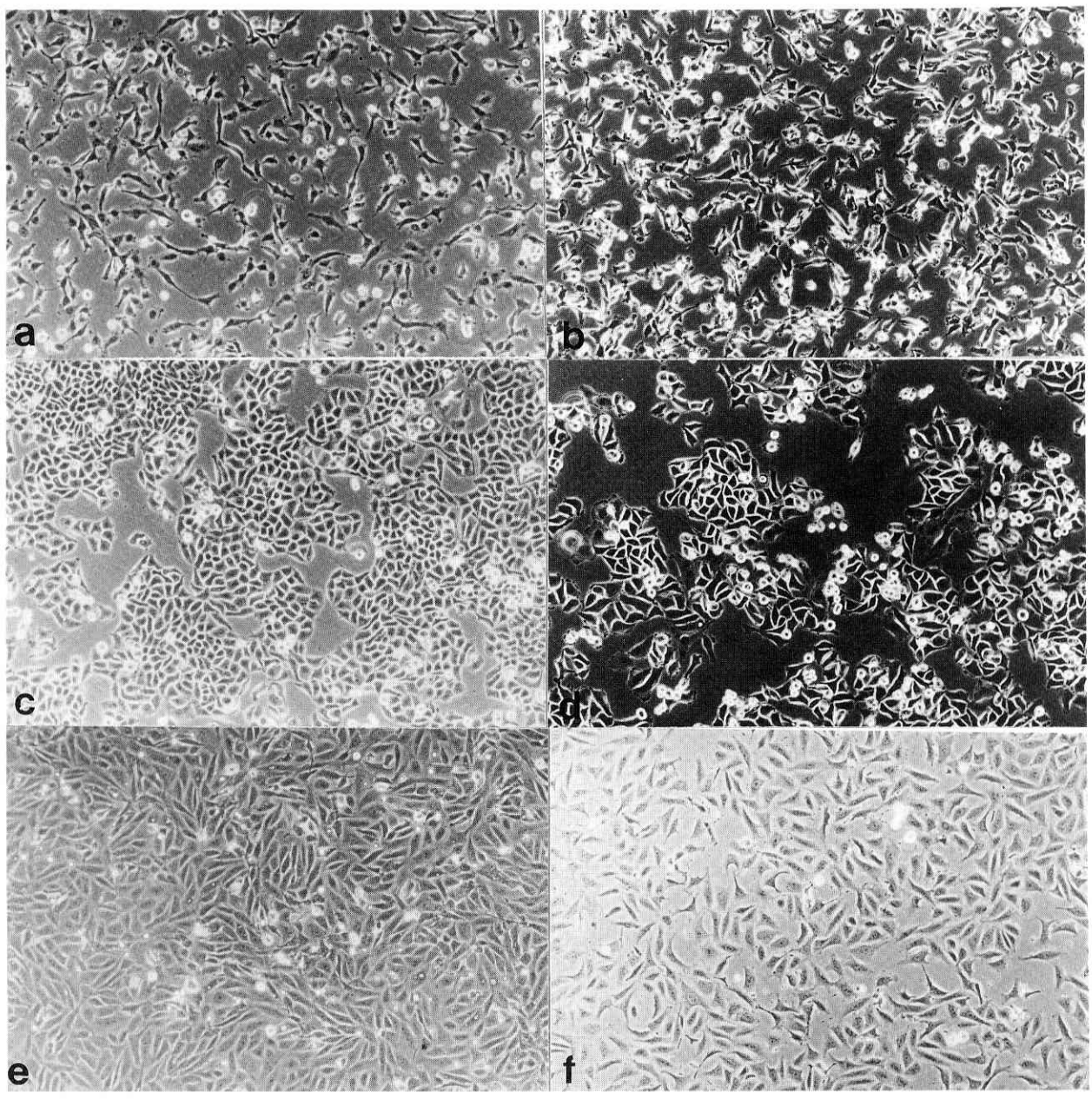

Fig. 2. Phase-contrast micrographs of the microcell hybrids isolated by introduction of human chromosome 7 in HT1080, HeLa, and TE85. (a) and (b), HT1080 and its microcell hybrid; (c) and (d), HeLa and its microcell hybrid; and (e) and (f), TE85 and its microcell hybrid, respectively. The cells are magnified 100 fold.

The presence of introduced chromosome 7 in the suppressed microcell hybrids of HepG2 was confirmed by detecting the *neo* marker integrated on the introduced chromosome after amplification by polymerase chain reaction. Fig. 3 shows clearly the presence of the amplified *neo* sequence in the chromosome donor and the microcell hybrids tested but not in the recipient HepG2.

DISCUSSION

Recently we have demonstrated that introduction of normal chromosome 7 reverses the immortal phenotype of the non-tumorigenic fibroblast lines KMST-6 and SUSM-1 established *in vitro* from normal diploid fibroblasts through physical and chemical treatment, respec-

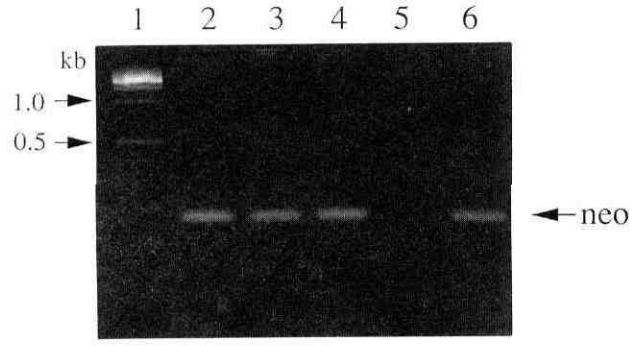

Fig. 3. Detection of the *neo* marker in the microcell hybrids of HepG2 isolated by introduction of human chromosome 7. Lane 1, DNA size marker; lane 2–4, microcell hybrids of HepG2; lane 5, HepG2 (recipient); and lane 6, NTI8 (mouse A9 donor of human chromosome 7). The arrow (right) indicates the 206 bp band derived from the *neo* gene.

tively.[4,5] Since both lines are assigned to complementation group D, it is suggested that the gene(s) responsible for immortalization in the group D cell lines is located on chromosome 7. However, since KMST-6 and SUSM-1 are both derived from fetal diploid fibroblasts and show a similar phenotype, the above conclusion was not considered definitive.[15] In the present study, HepG2 cells derived from a human hepatoma were also shown to stop dividing after introduction of chromosome 7, in much the same way as KMST-6 and SUSM-1. This finding supports the hypothesis that chromosome 7 generally suppresses indefinite division potential in the group D cell lines irrespective of their cell types, or whether they are tumorigenic or not. Although it has yet to be assigned to a complementation group, a chorionic tumor-derived cell line has been shown to respond to chromosome 7 (M. Oshimura, personal communication). To our knowledge, none of the cell lines assigned to complementation group A, B, or C has been shown to be affected by introduction of chromosome 7, as regards division potential. Therefore, it is indicated that the gene(s) mutated commonly in the cell lines assigned to group D is located in chromosome 7.

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REFERENCES

1) Hayflick, L. The limited *in vitro* lifetime of human diploid cell strains. *Exp. Cell Res.*, **7**, 614–636 (1965).
2) Ide, T., Tsuji, Y., Ishibashi, S. and Mitsui, Y. Reinitiation of DNA synthesis in senescent human diploid cells by transfection with simian virus 40. *Exp. Cell Res.*, **143**, 343–349 (1983).
3) Ide, T., Tsuji, Y., Nakashima, T. and Ishibashi, S. Progress of aging in human diploid cells transformed with a tsA mutant of simian virus 40. *Exp. Cell Res.*, **150**, 321–328 (1984).
4) Namba, M., Nishitani, K., Hyodoh, F., Fukushima, F. and Kimoto, T. Neoplastic transformation of human diploid fibroblasts (KMST-6) by treatment with cobalt-60 gamma-rays. *Int. J. Cancer*, **35**, 275–280 (1985).
5) Namba, M., Nishitani, K., Fukushima, F. and Kimoto, T. Multistep carcinogenesis of normal human fibroblasts: human fibroblasts immortalized by repeated treatment with cobalt-60 gamma rays were transformed into tumorigenic cells with Ha-ras oncogene. *Anticancer Res.*, **8**, 947–958 (1988).
6) Neufeld, D. S., Ripley, S., Henderson, A. and Ozer, H. Immortalization of human fibroblasts transformed by origin-defective simian virus 40. *Mol. Cell. Biol.*, **7**, 2794–2802 (1987).
7) Shay, J. W. and Wright, W. E. Quantitation of the frequency of immortalization of normal human diploid fibroblasts by SV40 T-antigen. *Exp. Cell Res.*, **184**, 109–119 (1989).
8) Bunn, C. L. and Tarrant, M. L. Limited lifespan in somatic cell hybrids and cybrids. *Exp. Cell Res.*, **127**, 385–396 (1980).
9) Muggleton-Harris, A. L. and DeSimone, D. W. Replicative potentials of various fusion products between WI-38 and SV40 transformed WI-38 cells and their components. *Somatic Cell Genet.*, **6**, 689–698 (1980).
10) Pereira-Smith, O. M. and Smith, J. R. Expression of SV40 T antigen in finite life-span hybrids of normal and SV40-transformed fibroblasts. *Somatic Cell Genet.*, **7**, 411–421 (1981).
11) Pereira-Smith, O. M. and Smith, J. R. Evidence for the recessive nature of cellular immortality. *Science*, **221**, 946–966 (1983).
12) Pereira-Smith, O. M. and Smith, J. R. Genetic analysis of indefinite division in human cells: identification of four complementation groups. *Proc. Natl. Acad. Sci. USA*, **88**, 6042–6046 (1988).
13) Sugawara, O., Oshimura, M., Koi, M., Annab, L. A. and Barrett, J. C. Induction of cellular senescence in immortalized cells by human chromosome 1. *Science*, **247**, 707–710 (1990).
14) Ning, Y., Weber, J. L., Killary, A. M., Ledbetter, D. H., Smith, J. R. and Pereira-Smith, O. M. Genetic analysis of

indefinite division in human cells: evidence for a cell senescence-related gene(s) on human chromosome 4. *Proc. Natl. Acad. Sci. USA,* **88,** 5635–5639 (1991).

15) Ogata, T., Ayusawa, D., Namba, M., Takahashi, E., Oshimura, M. and Oishi, M. Chromosome 7 induces cellular senescence of non-tumorigenic immortalized human fibroblast cell lines. *Mol. Cell. Biol.,* **13,** 6036–6043 (1993).

16) Spiering, A. L., Pereira-Smith, O. L. and Smith, J. R. Correlation between complementation groups for immortality and DNA synthesis inhibitors. *Exp. Cell Res.,* **195,** 541–545 (1991).

17) Koi, M., Shimizu, M., Morita, H., Yamada, H. and Oshima, M. Construction of mouse A9 clones containing a single human chromosome tagged with neomycin-resistance gene via microcell fusion. *Jpn. J. Cancer Res.,* **80,** 413–418 (1989).

18) Sasaki, Y. F., Ayusawa, D. and Oishi, M. Construction of a normalized cDNA library by introduction of a semi-solid mRNA-cDNA hybridization system. *Nucleic Acids Res.,* **22,** 987–992 (1994).