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Restoration of the Cellular Senescence Program and Repression of Telomerase by Human Chromosome 3

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Telomeres, at the end of chromosomes, shorten with each cell division, resulting in cellular senescence. Tumor cells, unlike normal somatic cells, express a telomerase that maintains the telomere length. Deletion of a gene(s) on chromosome 3 is common in human renal cell carcinoma (RCC) and reintroduction of a normal chromosome 3 into an RCC immortal cell line restored the program of cellular senescence. The loss of indefinite growth potential was associated with the loss of telomerase activity and shortening of telomeres in the RCC cells with a normal chromosome 3. However, microcell hybrids that escaped from senescence and microcell hybrids with an introduced chromosome 7 or 11 maintained telomere lengths and telomerase activity similar to those of the parental RCC23. Thus, restoration of the cellular senescence program by chromosome 3 is associated with repression of telomerase function in RCC cells.

Key words: Cellular senescence — Telomere — Telomerase — Chromosome 3

Telomeres are repeated sequences located on both ends of individual chromosomes in eukaryotes.[1)] The sequence of human telomere DNA consists of guanine-rich tandem repeats of $(TTAGGG)_n$, which can be 10–15 kb pairs long.[2, 3)] Telomeres progressively shorten with age in somatic cells in culture and *in vivo*,[4, 5)] because DNA replication results in the loss of sequences at the 5′ ends of double-stranded DNA.[5, 6)] Somatic cells do not express the enzyme, telomerase, that adds repeated telomere sequences to chromosome ends.[5, 7)] Telomerase activity has been detected in immortalized and tumor cells *in vitro* and in primary tumor tissues and represents an important difference between normal cells and cancer cells.[8)] It has been proposed that telomere shortening causes destabilization of chromosomes, growth arrest, and possibly cellular senescence.[5, 9)] Thus, while telomerase activity and the maintenance of telomere length might be an obligatory step in progression and immortalization in most human tumor cells, information is lacking about the regulation of telomerase activity in tumor cells.

Hybrids between immortal cells and normal cells senesce, indicating that immortal cells have lost, mutated or inactivated genes that are required for the program of senescence in normal cells. Some hybrids between different immortal cells can result in senescent hybrids, indicating that different complementation groups for immortal cell lines exist.[10)] Genes involved in the senescence program have been mapped to over 10 different loci by introduction of human chromosomes via microcell fusion and restoration of the senescence program.[11–19)] Multiple pathways of cellular senescence have also been demonstrated by chromosome transfer,[19)] indicating that the functions of the senescence genes mapped to different chromosomes are probably variable. One possibility is that one or more of the senescence genes defined by chromosome transfer may suppress telomerase activity in tumor cells, resulting in telomere shortening and cellular senescence. To test this hypothesis, we examined telomerase activity and the length of terminal restriction fragments (TRFs) in microcell hybrids by introducing single human chromosomes into a renal cell carcinoma cell line.[11)] We found that chromosome 3, but not chromosome 7 or 11, resulted in suppression of telomerase, TRF shortening and restoration of cellular senescence.

RCC23 is a non-tumorigenic human renal cell carcinoma cell line, established from the primary tumor tissue

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of a 56-year-old female with non-papillary carcinoma stage III. Karyotype analysis showed loss of the short arm of chromosome 3 due to an unbalanced translocation between chromosomes 3 (breakpoint at p11) and 8 (breakpoint at q11). Restriction fragment length polymorphism (RFLP) analysis also revealed that the renal cell carcinoma cell line RCC23 exhibited allele loss on the short arm of chromosome 3.[11] Mouse A9 cells (10^6), which contain a pSV2neo-tagged chromosome 3 derived from normal human fibroblasts, were plated into 25 cm^2 flasks (Costar, Cambridge, MA) 2 days before micronucleation. Micronuclei were induced by Colcemid treatment (0.05 μg/ml) in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum. After 48 h, the flasks were filled with prewarmed serum-free DMEM containing 10 μg/ml of cytochalasin B (Sigma Chemical Co., St. Louis, MO), placed in acrylic inserts designed to hold the flasks, and filled with water at 30°C. The flasks in the inserts were centrifuged in a fixed-angle rotor at 10,000g for 1 h at 34°C. The pelleted microcells were resuspended in 10 ml of serum-free DMEM and filtered in series through 8-, 5-, and 3-μm polycarbonate filters (Nucleopore, Pleasanton, CA). The purified microcells were pelleted by centrifugation at 400g for 10 min and resuspended in 2 ml of serum-free DMEM containing 100 μg/ml of phytohemagglutinin (PHA) (Difco Laboratories, Detroit, MI). The microcells were attached to prewashed renal cell carcinoma recipient cell monolayers by incubating for 15 min at 37°C. The cells were fused by treatment with 3 ml of 47% PEG solution (MW 1540; Wako Chemical Industries, Ltd., Osaka) for 1 min followed by extensive washing in serum-free DMEM. After 24 h, the cells were trypsinized and split into three plates (100 mm) with growth medium containing 500 μg/ml of G418. G418-resistant microcell-hybrid clones were isolated and further expanded for investigation of *in vitro* growth properties, TRF and telomerase activity.

In 4 microcell hybrids with an introduced chromosome 3 (MC#3-1, MC#3-2, MC#3-4 and MC#3-8), saturation densities were reduced, and their population doubling times (growth rates) were prolonged compared with the parental cell line.[11] The microcell hybrids were flat in shape and clearly different from the recipient RCC23 cells. Cells from 3 clones (MC#3-1, MC#3-2 and MC#3-4) senesced after 23–43 population doublings. One of the microcell hybrid clones regained a similar morphology, growth rate and saturation density to those of the immortal RCC23 cells (MC#3-8R). Chromosome and RFLP analyses revealed that MC#3-8R had lost the introduced chromosome 3. Chromosome 7 or 11 was also introduced into RCC23 cells (MC#7-3, MC#7-8 and MC#11-3): all of these clones showed similar biological and morphological features to the parental RCC23 cells.[11]

TRF lengths were determined by hybridization of the $(TTAGGG)_4$ probe to *Hin*f I-digested DNAs from cultured normal kidney cells (NKC), RCC23, RCC23

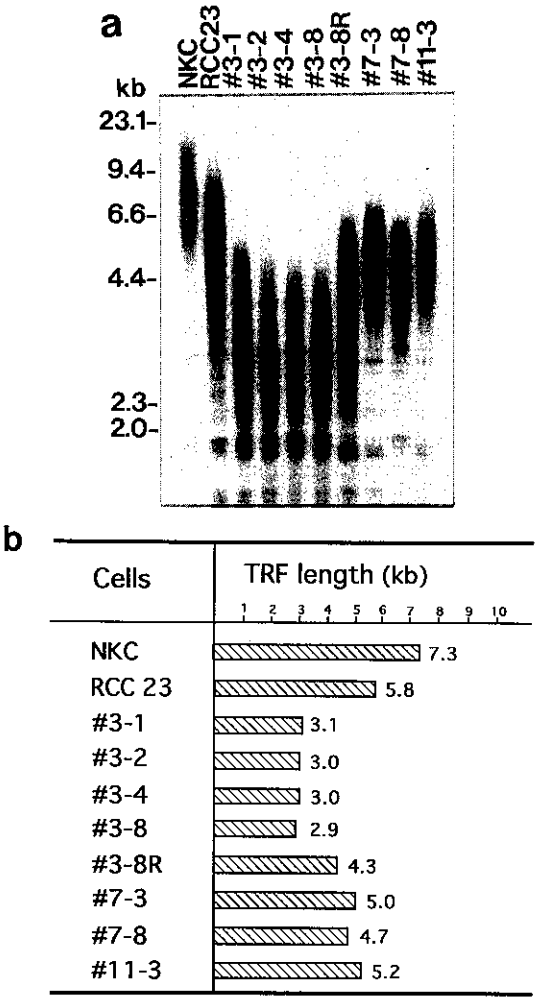

Fig. 1. (a) Southern blot analysis of telomere length of normal kidney cells, RCC23 cells and microcell-hybrid clones with chromosome 3. NKC, normal kidney cell; RCC23, parental cell; #3-1, #3-2, #3-4 and #3-8, microcell-hybrids with chromosome 3; #3-8R, revertant clone; #7-3 and #7-8, microcell-hybrids with chromosome 7; #11-3, microcell-hybrids with chromosome 11. *Hin*dIII-digested λ DNA was used as a marker. (b) Mean telomere length from southern blot analysis in Fig. 1 (a). DNA was extracted from primary cultured normal kidney cells, RCC23 cells and microcell hybrids at 23–43 population doublings.[4] *Hin*fI-digested DNA (5 μg) was separated by electrophoresis on a 0.7% agarose gel, transferred to a nylon membrane, and hybridized to the ^{32}P-labeled telomeric probe $(TTAGGG)_4$ in 6×SSPE-1% SDS at 50°C. The membrane was washed in 6×SSC-0.1% SDS at 50°C, then autoradiographed at −80°C. The mean length of TRF (terminal restriction fragment) was determined from the densitometric peak.

microcell hybrids with the introduction of chromosome 3 (MC#3), chromosome 7 (MC#7), or chromosome 11 (MC#11) and from the revertant clone, MC#3-8R (Fig. 1a). The peak TRF values were 7.3 kb and 5.8 kb for cultured NKC and RCC23 cells, respectively (Fig. 1b). Although the NKC and RCC23 DNAs were not obtained from the same individual, their TRF lengths were compatible with the results previously reported by others,[20] indicating that the telomere shortening is associated with malignant transformation of RCC23 cells. Four of the MC#3 clones showed a significant reduction of *in vitro* growth rate with morphological alterations, and 3 of them senesced after several months of culture (23–43 population doublings). All 4 of these clones showed a reduction in the TRF lengths, when compared with parental RCC23 cells: 2.9–3.1 kb vs. 5.8 kb (Fig. 1b). The difference of TRF lengths between RCC23 cells and MC#3 clones was 2.7–2.9 kb. If the mean reduction rate in MC#3 clones is assumed to be the rate of telomere reduction of normal cells *in vitro* (65 bp/generation),[5] the calculated number of generations required for the observed telomere shortening (2.8 kb) is 43, which is consistent with the population doublings of the microcell hybrids when TRFs were examined just prior to senescence. After several passages in culture, one clone MC#3-8 lost the introduced chromosome 3, regained similar growth properties and morphology to those of the parental RCC23 cells,[11] and continued to proliferate even beyond 100 population doublings (MC#3-8R). Interestingly, the telomere length in the MC#3-8R cells was longer than that in the original MC#3-8 cells: 2.9 kb vs. 4.3 kb. The TRF lengths in MC#7 and MC#11, which did not senesce, were similar to that of the parental RCC23 cells (Fig. 1b).

To examine whether or not telomere reduction is due to loss of telomerase activity in cells, we measured its

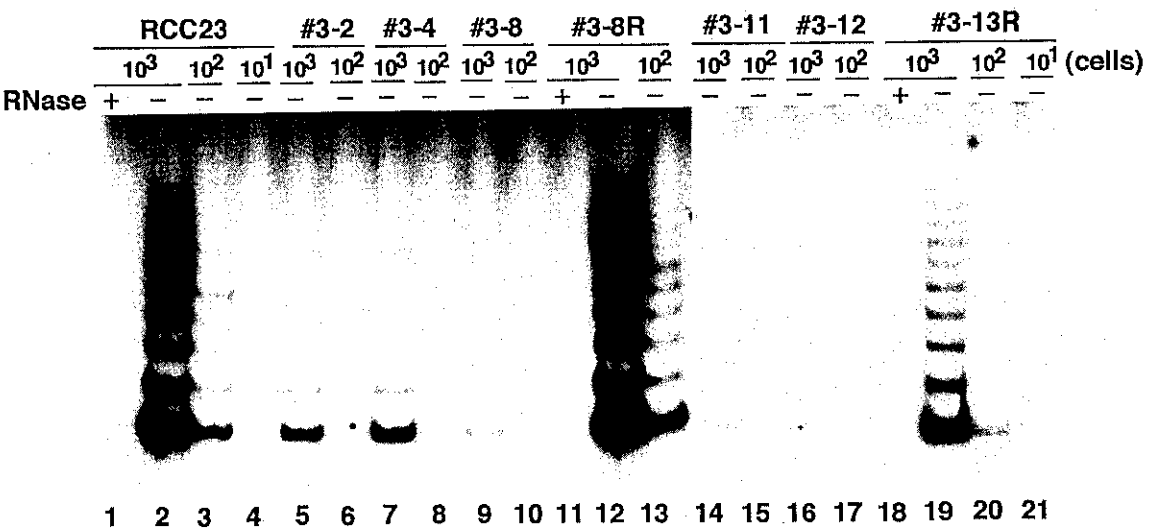

Fig. 2. Telomerase activity in RCC23 and microcell hybrid cells with chromosome 3. RCC23 (lanes 1 to 4), parental cell line; #3-2 (lanes 5 and 6), #3-4 (lanes 7 and 8) and #3-8 (lanes 9 and 10), microcell hybrids with chromosome 3; #3-8R (lanes 11 to 13), revertant clone from #3-8; #3-11 (lanes 14 and 15), #3-12 (lanes 16 and 17) and #3-13R (lanes 18 to 21), microcell hybrids derived from RCC23-5 (a subclone of RCC23). Cells (1×10^5) were washed once in ice-cold phosphate-buffered saline, and pelleted at 2,000*g* for 6 min at 4°C. The pellets were suspended with 200 μl of ice-cold lysis buffer [10 m*M* Tris-HCl (pH 7.5), 1 m*M* $MgCl_2$, 1 m*M* EGTA, 0.1 m*M* AEBSF, 5 m*M* β-mercaptoethanol, 0.5% CHAPS, 10% glycerol], and incubated for 30 min on ice. After centrifugation at 16,000*g* for 20 min at 4°C, the supernatant was collected, quickly frozen in liquid N_2 and stored at −80°C. Extract equivalent to approximately 10^3, 10^2 or 10^1 cells was used for each telomerase assay.[8] Telomerase activity was assayed by the one-tube PCR-based telomerase assay as previously described.[8] Assay tubes contained 0.1 μg of CX primer (5′-CCCTTACCCTTACCCTTACCCTAA-3′) at the bottom sequestered by a wax barrier. Fifty μl of reaction mixture above the wax barrier consisted of 20 m*M* Tris-HCl (pH 8.3), 1 m*M* $MgCl_2$, 63 m*M* KCl, 0.005% Tween-20, 1 m*M* EGTA, 50 μ*M* dNTPs, 4 μCi of [α-^{32}P]deoxycytidine triphosphate (10 mCi/ml, 3000 Ci/mmol), 1 μg of T4g32protein (Amersham), 5 μg of bovine serum albumin, 2 units of Taq DNA polymerase (AmpliTaq, Perkin-Elmer), 0.1 μg of TS primer (5′-AATCCGTCGAGCAGAGTT-3′) and the extract. After 30 min at 20°C for extension of the TS primer by telomerase, the reaction mixture was heated at 90°C for 3 min to inactivate the telomerase activity and processed through 31 cycles of 95°C for 45 s, 50°C for 45 s, and 72°C for 1 min. The extract pretreated with RNase (0.1 μg per sample, for 20 min at 37°C) was incubated to determine if the processive ladder was RNase-sensitive as detected by autoradiography. Half the volume of the reaction products was electrophoresed on 10% polyacrylamide non-denaturing gel.

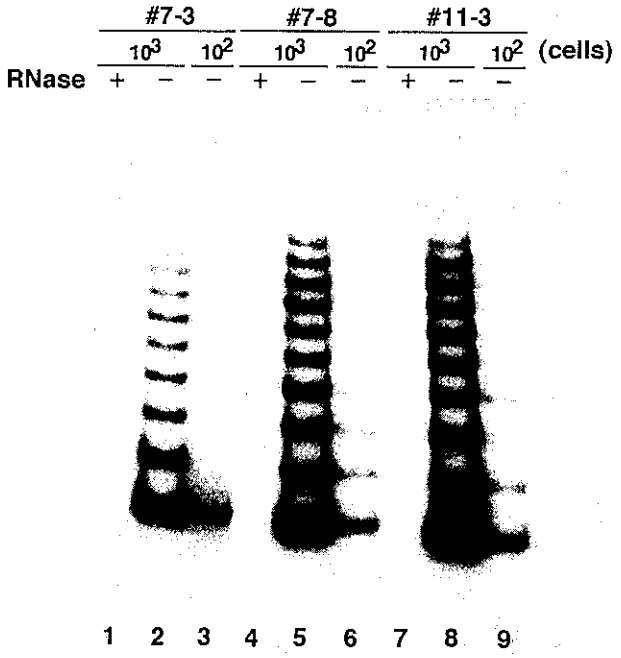

Fig. 3. Telomerase activity in microcell hybrids with chromosome 7 or 11. #7-3 (lanes 1 to 3) and #7-8 (lanes 4 to 6), microcell hybrids with chromosome 7; #11-3 (lanes 7 to 9), microcell hybrid with chromosome 11.

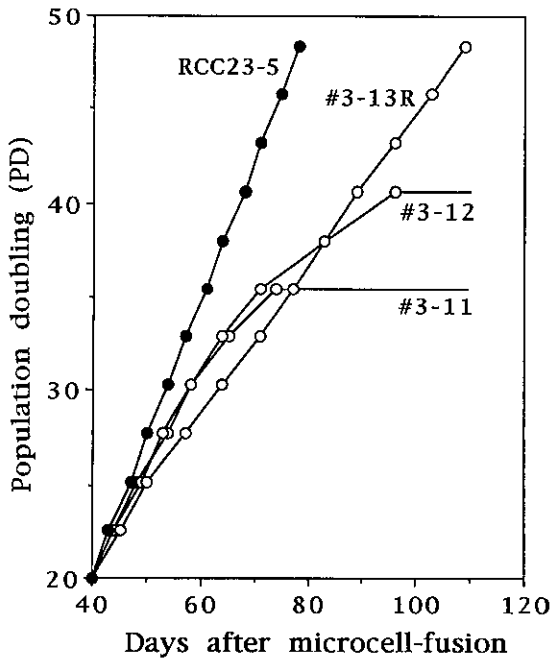

Fig. 4. Growth curve of RCC23-5 subclone and 3 microcell hybrid clones with the introduction of chromosome 3, i.e. #3-11, -12, and -13R. To determine the population doubling, cells were plated in 100 mm dishes at the density of 2.5×10^5 cells for each passage, when the cell density reached 80% confluency.

activity by the primer extention telomere repeat amplification protocol (TRAP) assay,[8] in which telomerase synthesizes telomeric repeats onto oligonucleotide primers.[5] All 3 MC#3 clones examined, i.e., MC#3-2, -4 and -8, showed markedly reduced telomerase activity (less than 1/10 of that of RCC23 cells), whereas MC#3-8R cells and other microcell hybrids (MC#7-3, MC#7-8 and MC#11-3) maintained a similar activity level to that in the parental RCC23 cells (Figs. 2 and 3). It is a formal possibility that telomerase-negative cells preexisted in the original RCC23 cell population and were selected. However, all 10 subclones isolated from RCC23 cells used in the first experiment were positive for telomerase activity. Furthermore, similar effects of chromosome 3 were observed in an additional experiment using a freshly isolated subclone (RCC23-5) which was positive for the telomerase activity. The 3 microcell hybrid clones that were isolated (MC#3-11, MC#3-12 and MC#3-13), proliferated more slowly than the parental RCC23-5 cells. Two clones (MC#3-11 and -12) stopped dividing at 36 and 42 population doublings after the introduction of chromosome 3, whereas the remaining clone (MC#3-13) continued growing like the parental cells even after 70 or more population doublings (Fig. 4). Chromosome analyses revealed that an intact, transferred chromosome 3 was present in MC#3-11 and -12 cells, whereas chromosome 3 had been lost in MC#3-13 cells. Therefore, this clone is likely to be a revertant (hereafter MC#3-13R). Telomerase activities of the clones were examined at 25 and 35 population doublings: the parental RCC23-5 cells and clone MC#3-13R cells were positive for telomerase activity, whereas clones MC#3-11 and MC#3-12 were negative at both passages (Fig. 2). While the TRF lengths were maintained in the parental RCC23-5 and clone MC#3-13R cells, the TRF lengths were reduced as a function of number of passages in clones MC#3-11 and -12. Mixing of telomerase-positive and -negative extracts from RCC23 and MC#3-2, or MC#3-4 cells (data not shown) excluded the possibility of the presence of some inhibitory factors for the telomere extension in the telomerase-negative clones.

The link between replicative senescence, immortalization and the shortening of telomeres was documented by Harley and coworkers.[20] In somatic cells, telomerase activity is repressed and telomeres progressively shorten with each cell division.[5] Cells may stop dividing when the telomere reaches a critical length in a proliferative cell, although little is known about the mechanisms linking telomere loss to cessation of cell proliferation at the M1 (mortality 1) stage.[9,21] Immortalization of cells is

associated with activation of telomerase activity at or near crisis of the M2 (mortality 2) stage, and immortal tumor cells can maintain telomere length.[9,21] In the present study, we observed a strong association of telomere loss with the restoration of the cellular senescence program in tumor cells. Because senescence was induced by the introduction of chromosome 3, but not chromosome 7 or 11, the findings may indicate the presence of a gene(s) on human chromosome 3 repressing telomerase activity. However, the gene for telomerase repression may or may not be the putative tumor suppressor gene on chromosome 3 thought to be important for renal cell carcinomas.[11]

Normal somatic cells in culture generally senesce, whereas tumor-derived cells are often, but not without exception, immortal and grow indefinitely.[22,23] As shown by somatic genetic studies, for cells to escape the senescence program, normal genes must be lost or inactivated.[10-19] For example, the introduction of specific chromosome(s) by microcell-mediated chromosome transfer has been shown to induce senescence of human and rodent tumor and transformed cells.[11-19] We showed that two different normal chromosomes induce senescence in the same endometrial carcinoma cell line, which suggests that multiple pathways to senescence are inactivated in this cell line.[19] Thus, this hypothesis has implications for the mechanisms of cellular senescence and its role in carcinogenesis. Because chromosome 3 did not induce cellular senescence in a human cervical carcinoma cell line, which is strongly positive for telomerase activity (data not shown), not all tumor cells may be defective for the same gene controlling telomerase activity. According to the multiple pathways model, the senescence program is activated by multiple independent pathways and immortal cells arise due to defects or mutations in genes in each of the pathways.[19] The present findings are consistent with the hypothesis that the telomerase function is one of the pathways involved in immortalization and that each pathway involves multiple genes.

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