RAPID COMMUNICATION Jpn. J. Cancer Res. 81, 1095–1100, November 1990

Normal Human Chromosome 1 Carries Suppressor Activity for Various Phenotypes of a Kirsten Murine Sarcoma Virus-transformed NIH/3T3 Cell Line

Hideto Yamada,[1] Izumi Horikawa,[1] Hiroki Hashiba[2] and Mitsuo Oshimura[1,3]

[1]*Department of Molecular and Cell Genetics, School of Life Sciences, Faculty of Medicine, Tottori University, Nishimachi 86, Yonago 683 and* [2]*Laboratory of Cytogenetics, Kanagawa Cancer Center Research Institute, Nakao-cho 54-2, Asahi-ku, Yokohama 241*

In order to identify chromosomes that carry putative tumor-suppressor genes for the various phenotypes of Kirsten sarcoma virus-transformed NIH/3T3 (DT) cells, we performed microcell-mediated chromosome transfer into DT cells. We first isolated mouse A9 clones, containing a single human chromosome 1, 11 or 12 tagged with pSV2-*neo* plasmid DNA. Then, chromosome 1, 11 or 12 was transferred from the A9 clones into DT cells by microcell fusion. The growth rate, colony-forming ability in soft agar and tumorigenicity of the DT cells were controlled by chromosome 1, but not by chromosome 11 or 12, indicating that normal human chromosome 1 carries a putative tumor-suppressor gene(s) that affects various transformed phenotypes of DT cells.

Key words: Human chromosome 1 — Chromosome transfer — Microcell fusion — Tumor suppressor gene — DT cell

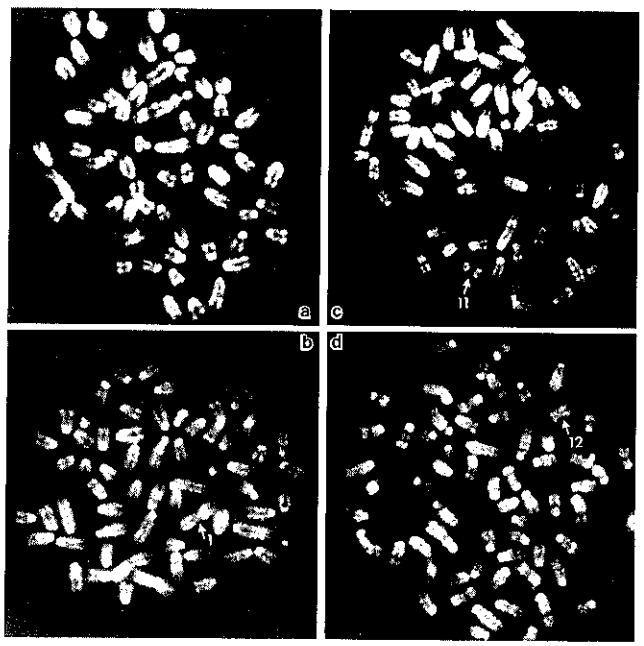
Microcell-mediated chromosome transfer has several advantages as a first step in screening for the chromosomal localization and/or the function of putative tumor-suppressor genes.[1-8] All recipient cells into which a normal chromosome tagged with a dominant selectable gene has been introduced can be isolated by growth under selective conditions for the dominant selectable marker. Since human chromosomes have been suggested to contain a tumor-suppressive function from studies with cell-cell hybrids, restriction-fragment-length polymorphism analyses and chromosomal analyses in various tumors,[9-15] microcell-mediated chromosome transfer to specific tumor cells is a useful technique to confirm the presence of a putative tumor-suppressor gene on the candidate chromosomes.[4-7] Even when a candidate chromosome is not known, this method can be used to search for chromosomes that carry putative tumor-suppressor genes by transfers of various chromosomes.[8] In this study, we applied this method in an attempt to identify the chromosome that carries suppressor activity for a transformed subline of NIH/3T3 (the "DT" cell line) containing Kirsten murine sarcoma virus, a transforming virus carrying the v-Ki-*ras* gene.[16,17]

The method for chromosome transfer via microcell fusion has been described previously.[18,19] Mouse A9 cells containing a single copy of *neo*-tagged chromosome 1, 11 or 12 derived from normal human fibroblasts, termed A9(*neo*1), A9(*neo*11) or A9(*neo*12), respectively, were used as chromosome donors. The purified microcells from these A9 cells were fused with recipient DT cells on collagen-coated dishes. After incubation for 2–3 weeks in selection medium containing 400 μg/ml G418, the surviving DT-microcell hybrids were isolated, and expanded in DMEM supplemented with 10% FCS and 800 μg/ml G418 on collagen-coated dishes.

Chromosome analysis with quinacrine plus Hoechst 33258 stains[20] showed that the microcell hybrids contained a single copy of intact, transferred human chromosome 1, 11 or 12 in the majority of cells in early passage (Fig. 1, Tables I and II). These clones (four DT-#1 clones, four DT-#11 clones and five DT-#12 clones) were used for further studies.

The morphology of microcell hybrids on collagen-coated dishes did not differ remarkably from that of the parental DT cells. Although DT-#1 showed a somewhat flatter morphology compared with those of DT, DT-#11 and DT-#12 on non-collagen-coated dishes, the morphology of these cells was quite different from that of NIH/3T3 cells (Fig. 2). As shown in Table I, parental DT, DT-#11 and DT-#12 cells proliferated with similar population doubling times (11–13 h) on collagen-coated plastic dishes containing 10% FCS, whereas the doubling time for DT-#1 cells was 21–28.1 h, which was similar to that of NIH/3T3 cells (20.6 h). The index of serum-dependent growth indicated that DT-#1 cells as well as NIH/3T3 cells did not grow well under low-serum conditions, compared with DT, DT-#11 and DT-#12 cells. A decrease in the ability to grow in soft agar was observed in DT-#1, but not in DT-#11 or DT-#12. To examine the presence of introduced chromosomes in soft-agar-

[3] To whom reprint requests should be addressed.



positive clones, chromosome analysis was performed (Table II). The majority of cells still contained the intact, introduced human chromosome in the #11 and #12 soft-agar clones (average 84–93% of cells), whereas only a small population of the cells from #1 soft-agar clone contained intact human chromosome 1 (average 2–13% of cells). Thus, cells losing this chromosome may have a selective advantage for growth in soft agar, suggesting that this chromosome carries a specific cellular gene(s) that suppresses the ability to grow in soft agar.

When NIH/3T3, DT and microcell-hybrids (DT-#1, #11 and #12) were inoculated at 5×10^6 cells per site into nude mice, all cells except for NIH/3T3 formed tumors

Fig. 1. Quinacrine plus Hoechst 33258 stained metaphase plates of DT cells (a) and their microcell hybrids with the transfer of chromosome 1 (b), chromosome 11 (c) or chromosome 12 (d). Arrows indicate a normal human chromosome 1, 11 or 12.

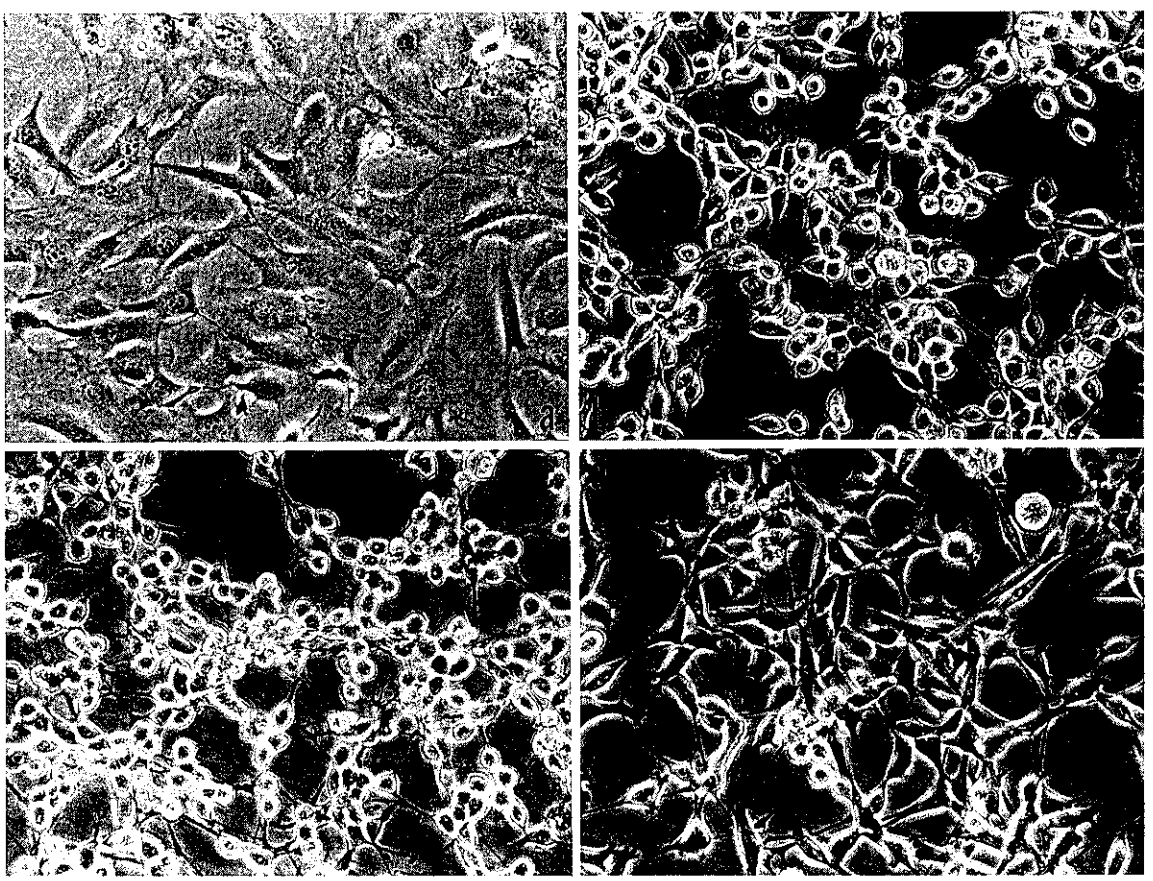


Fig. 2. Phase-contrast photomicrographs of cells on non-collagen-coated dishes (200×magnification). (a) NIH/3T3 cells; (b) DT cells; (c) microcell hybrid with an introduced chromosome 11 (#11-1); (d) microcell hybrid with an introduced chromosome 1 (#1-1).

within 10 day after inoculation. In all cases, the tumors grew progressively until the animals were killed. However, DT, DT-#11 and DT-#12 cells exhibited tumor formation within 7 days, whereas DT-#1 cells showed a latent period of 7–10 days (Table I). Chromosome analysis of tumor cells revealed that 56–76% of tumor cells contained the intact, introduced human chromosome 11 or 12. In contrast, only 0–3% of tumor cells contained the intact human chromosome 1 (Table II). When 5×10^5 cells were inoculated into nude mice, the average latent periods were 7.6 days in DT cells, 7 days in DT-#11 and DT-#12 cells, and 8.8–14 days in DT-#1 cells (Table I). Representative tumor-growth curves of microcell hybrids are shown in Fig. 3.

The present study has shown that the introduction of a single human chromosome 1 derived from normal human fibroblasts into Kirsten murine sarcoma virus-transformed NIH/3T3 (DT) cells controlled *in vitro* transformation properties. The introduction of human chromosome 11 or 12 into the DT cells did not affect the *in vitro* properties of the cells. The loss of an intact *neo*-tagged human chromosome 1 in tumor cells formed from DT-#1 cells strongly suggests that this normal chromosome 1 suppresses the tumorigenicity of the DT cells. Further

Table I. Tumorigenicity in Nude Mice and *in vitro* Properties of Parental DT Cells and Microcell Hybrids Following Transfer of Normal Human Chromosomes

Cells	Doubling time (h)[a]	Index of serum-dependent growth (1%FCS/10%FCS)[b]	Colony-forming ability in soft agar(%) (SAE/PE)[c]	Tumorigenicity[d] (latent period: days)	
				5×10^5 cells	5×10^6 cells
NIH3T3	20.6	0.020	<0.01	nt[e]	0/3 (>30)
DT	11.9	0.26	86.3	5/5 (7.6)	8/8 (<7)
#12 microcell hybrids					
12-1 (92)[f]	12.8	nt	94.0	nt	6/6 (<7)
12-2 (100)	12.2	0.39	94.3	5/5 (7)	4/4 (<7)
12-3 (98)	11.8	0.27	83.6	nt	6/6 (<7)
12-4 (92)	11.0	0.22	77.2	5/5 (7)	6/6 (<7)
12-5 (94)	nt	nt	81.2	nt	6/6 (<7)
#11 microcell hybrids					
11-1 (99)	11.9	nt	82.4	nt	6/6 (<7)
11-2 (94)	11.0	0.16	82.9	5/5 (7)	6/6 (<7)
11-3 (98)	13.0	nt	92.8	nt	6/6 (<7)
11-4 (100)	12.6	nt	80.4	nt	5/5 (<7)
#1 microcell hybrids					
1-1 (75)	28.1	0.020	3.8	6/6 (14)	7/7 (10)
1-2 (96)	21.3	nt	8.1	5/6 (9.6)	8/8 (10)
1-3 (92)	25.1	0.004	9.9	5/6 (8.8)	7/7 (7)
1-4 (88)	21.0	0.025	9.3	5/6 (14)	6/6 (7)

a) Cells were plated on 35 mm collagen-coated dishes at a density of 1×10^5 with DMEM containing 10% FCS. The cells from three dishes were counted daily from the following day to the 5th day. Medium exchanges of all dishes were done daily.

b) Index of serum-dependent growth was determined from the number of cells in media with 1% serum/number of cells in media with 10% serum (1%FCS/10%FCS). Cells were plated on 35 mm collagen-coated dishes at a density of 1×10^4 with DMEM containing 1% and 10% FCS. Medium exchanges of all dishes were done daily. The cells of three dishes were counted after 7 days.

c) To determine colony-forming efficiency on plastic dishes (PE), 2×10^2 cells were plated on three 60 mm collagen-coated dishes with DMEM containing 10% FCS and colonies were counted 8 days later. To determine colony-forming efficiency in soft agar (SAE), cells were plated into 3 dishes with 0.33% soft agar at a density of 2×10^2 cells per dish and macroscopic colonies were counted 2 weeks later.

d) Cells (5×10^6 or 5×10^5 cells per site) were subcutaneously inoculated into 4- to 6-week-old athymic ICR nude mice. No. of tumors formed/No. of injected sites is shown.

e) Not tested.

f) Percentages of metaphases with the introduced, intact chromosome in passage 3 or 4 are shown in parentheses. These data are also shown in Table II.

Table II. Chromosome Analyses of Microcell Hybrids, Soft-agar Clones and Tumor Cells[a]

Cells	Modal chromosome number	Percentage of metaphases with the introduced intact chromosome in passage 3 or 4	Percentage of metaphases with the introduced intact chromosome in soft-agar clone (average)	Percentage of metaphases with the introduced intact chromosome in tumors[b]
#12 microcell hybrids				
12-1	63	92	nt[c]	nt
12-2	63	100	nt	nt
12-3	64	98	80, 82, 96, 100, 100, 100 (93)	nt
12-4	62	92	nt	70
12-5	64	94	nt	56, 75
#11 microcell hybrids				
11-1	62	99	77, 85, 90 (84)	nt
11-2	60	94	83, 83, 95 (87)	nt
11-3	61	98	75, 90, 91, 95, 96, 100 (91)	76
11-4	62	100	nt	nt
#1 microcell hybrids				
1-1	61	75	nt	0
1-2	60	96	0, 14, 20 (11)	3
1-3	58	92	0, 0, 0, 0, 4, 5, 8 (2)	0
1-4	57	88	0, 0, 40 (13)	3

a) At least 20 metaphases were analyzed.
b) Tumors formed by injection of 5×10^6 cells were examined.
c) Not tested.

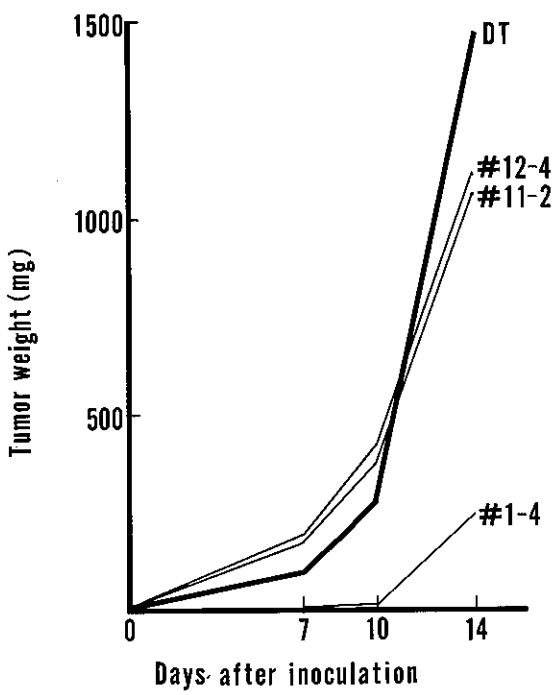


Fig. 3. Tumor-growth curves of parental DT cells and representative microcell hybrids. Each curve shows average tumor weights from 5 or 6 sites where 5×10^5 cells were inoculated.

studies are necessary to ascertain whether other chromosomes are also capable of suppressing the transformed phenotype of this cell line.

In these suppressed #1-microcell hybrids, as well as in #11- and #12-microcell hybrids, RNA complementary to the v-Ki-*ras* oncogene was expressed at a high level, comparable to that observed in parental DT cells (Fig. 4). Thus, the putative suppressor gene(s) on normal human chromosome 1 does not appear to interfere with transcription of the *ras* oncogene. This is consistent with the results from hybrids between normal Syrian hamster embryo (SHE) cells and v-*ras* plus v-*myc*-transformed SHE cells,[12] hybrids between EJ bladder carcinoma cells and normal human fibroblasts[21] and hybrids between flat revertants of Kirsten murine sarcoma virus-transformed cells and other mouse cell lines transformed by *ras* oncogene.[17, 22]

Several lines of evidence support the existence of a putative suppressor gene(s) on human chromosome 1. Deletions of chromosome 1 in human endocrine neoplasia,[23] neuroblastoma[24] and ductal carcinoma of the breast[25] have been frequently observed. In interspecies hybrids between chemically transformed hamster cells and normal human fibroblasts, chromosome 1 was also suggested to carry a suppressor function for anchorage-independent growth.[26] Microcell-mediated transfer of a normal human chromosome 1 into a human endometrial

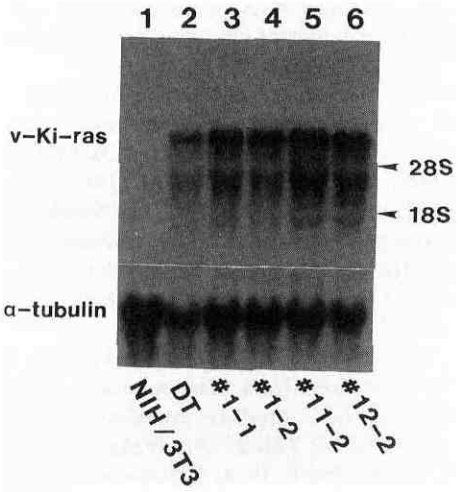


Fig. 4. Northern blot analysis of v-Ki-*ras* mRNA from parental cells and microcell hybrids. Lane 1, NIH/3T3 cells; lane 2, DT cells; lanes 3, 4, #1-microcell hybrid; lane 5, #11-microcell hybrid; lane 6, #12-microcell hybrid. α-Tubulin was used as the control. Total RNA was prepared from subconfluent cells using the guanidium-hot phenol method.[30] The RNA (20 μg per lane) was separated by electrophoresis through a 1.2% agarose gel containing 2.2 *M* formaldehyde, transferred to a nylon membrane (Hybond N, Amersham) and hybridized with ^{32}P-labeled v-Ki-*ras* insert from clone Kis[31] in 50% formamide, 5×SSPE (1×SSPE is 0.18 *M* NaCl, 10 m*M* sodium phosphate, 1 m*M* EDTA, pH 7.7), 1×Denhardt's solution, 200 μg/ml sonicated and denatured salmon testis DNA, and 0.1% sodium dodecyl sulfate (SDS) at 42°C for 24 h. Filters were subsequently washed twice in 2×SSC (1×SSC is 0.15 *M* NaCl, 15 m*M* sodium citrate) containing 0.1% SDS for 5 min at room temperature, and three times in 0.2×SSC containing 0.1% SDS for 1 h at 45°C, and then exposed to an X-ray film at −80°C for 12 h with an intensifying screen. After removal of the v-Ki-*ras* probe, the filters were rehybridized with mouse α-tubulin cDNA probe, Mα1[32] to standardize the RNA content in each lane.

cancer cell line and a human fibrosarcoma cell line also resulted in suppression of tumorigenicity and *in vitro* transformation properties.[2,4,8] Cloning and mapping of the tumor-suppressor genes on chromosome 1 are necessary to clarify the question of whether the same gene(s) is responsible for the suppression of tumorigenicity of these cells.

Recently, Noda *et al.* isolated a 1.8 kb cDNA, designated K*rev*-1, from a human fibroblast cDNA expression library and found that it suppressed transformed phenotypes of the DT cells.[27,28] By chromosomal *in situ* hybridization, the RAP1A gene, whose predicted amino acid sequence is identical to that of K*rev*-1 protein, has been assigned to chromosome bands 1p12-p13.[29] A high level of mRNA expression, irrespective of copy number of the K*rev*-1 plasmid, was required for the remarkable suppression of transformed phenotypes of DT cells, i.e., reversion of morphology, reduction of growth rate and suppression of colony-forming ability in soft agar. In the present study, the introduction of a single copy of normal human chromosome 1 into DT cells also reduced the growth rate and suppressed colony-forming ability in soft agar, although the morphological change was moderate. Thus, the general features of our findings are similar to those of Noda *et al.*[27] However, further studies on the expression level of K*rev*-1 gene in DT-#1-microcell hybrids and the sublocalization of the putative suppressor gene(s) on chromosome 1 are needed to clarify whether or not the K*rev*-1 gene is involved in the suppression observed in our studies.

We thank Dr. M. Noda, The Institute of Physical and Chemical Research, for providing DT cells and Dr. N. Tsuchida, Tokyo Medical and Dental University, and Dr. K. Nose, Tokyo University for providing the recombinant probes, Kis and Mα1, respectively. This study was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan, a Grant-in-Aid from the Ministry of Health and Welfare of Japan and a Grant from the Science and Technology Agency of Japan.

(Received March 28, 1990/Accepted August 28, 1990)

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