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A Specific Chromosome Change and Distinctive Transforming Genes Are Necessary for Malignant Progression of Spontaneous Transformation in Cultured Chinese Hamster Embryo Cells

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Chinese hamster embryo (CHE) cell strains, each initiated from a separate cell stock obtained from different mothers, were transferred successively at intervals of 3 days and the changes in growth properties and karyotypes at various passages were examined. All nine cell strains proliferated at varying growth rates for 60 passages but only 2 (designated CHE A1 and CHE A2) of them expressed malignant phenotypes. The acquisition of tumorigenicity in nude mice was observed in CHE A1 and CHE A2 cells at passages 40 and 10, respectively. After 5 passages, 8 of 9 cell strains contained one or two common additional chromosomes, chromosome 3q and/or chromosome 5, although one cell strain (designated CHE A3) maintained a normal diploid karyotype for 60 passages. Trisomy of chromosome 3q was observed in all tumorigenic CHE A1 and A2 cells. One or two 3q chromosomes were detected in all tumor-derived cell lines established from tumors produced by these tumorigenic cells. DNA from tumorigenic cells and tumor-derived cell lines exhibited a high ability to transform mouse NIH3T3 cells, but we could not detect any activation of Ha-*ras*, Ki-*ras*, *hst*, *erbB-2*, *mos*, *met* or *raf* in any of the transformed NIH3T3 cells. These results suggest that even though cultured CHE cells can transform spontaneously, without any specific chromosome change, to immortal cells, activation of unknown oncogene(s) in addition to a specific chromosome change may be required for their malignant progression. Our results suggest that trisomy of chromosome 3q is this specific chromosome change.

Key words: Transformation — Chinese hamster cell — Trisomy — DNA transfection

From extensive pathological and histological studies of various human tumors, Foulds proposed the concept that cancer arises in a multistep process through qualitatively different stages.[1] Since most malignant tumors in humans contain multiple genetic alterations such as gene amplifications and point mutations, and chromosome deletions and translocations, human cancers have been thought to develop as an accumulation of these genetic changes.[2] For example, sequential alterations in *APC*, *ras*, *DCC*, and *p53* were detected in the progressive stages of human colorectral tumorigenesis.[3]

In vitro transformation systems using primary cultured hamster cells provide useful experimental materials for analyzing the mechanisms of neoplastic development in culture.[4–12] Barrett and Ts'o examined the acquisition of transformed phenotypes in Syrian hamster embryo cells following exposure to a chemical carcinogen, benzo[*a*]-pyrene, and reported that morphological changes appeared at early passages after the treatment, but the ability to grow in 0.3% agar medium only appeared at later passages.[5] Similar progressive phenomena have also been observed in spontaneous neoplastic transformation in Syrian hamster embryo cells transferred successively.[6] Using one normal diploid cell line established from Chinese hamster embryo (CHE) cells, Smith and Sager demonstrated that malignant transformation requires several steps, each induced by different genetic changes.[7] Furthermore, Kraemer *et al.* identified the relationship between multistep progression of spontaneous transformation and stepwise changes in karyotypes in primary cultured CHE cells.[8, 9] A specific chromosome change, the 3q chromosome lacking the short arm of chromosome 3, often appearing in various transformed CHE cells, has been suggested to involve the loss of tumor-suppressor genes.[10, 11] The results obtained from various DNA transfection experiments demonstrated that certain combinations of activated oncogenes could transform primary rodent fibroblasts in culture.[13] Furthermore, Oshimura *et al.* reported a nonrandom chromosome loss in Syrian hamster tumors induced by v-Ha-*ras* and v-*myc* oncogenes.[14] These results suggest that neoplastic transformation of rodent cells *in vitro* arises in a multistep process consisting of more than two steps.

Specific chromosome changes and gene mutations have been studied in various *in vivo* carcinogenesis systems

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using experimental animals. However, little information about these genetic changes is available from *in vitro* transformation experiments using cultured mammalian cells, although the activated oncogene in three tumors derived from 3-methylcholanthrene-transformed C3H/10T1/2 cells was identified as a c-Ki-*ras* gene more than ten years ago.[15] In this study, we report that CHE cells transferred *in vitro* can easily transform to preneoplastic immortal stages and progress toward malignant stages following prolonged passages of cultivation, and that a specific chromosome change, trisomy of chromosome 3q and distinctive transforming genes, which have yet to be identified, are necessary for their malignant progression.

MATERIALS AND METHODS

Cell strains and culture We prepared three Chinese hamster embryo cell strains (CHE A, CHE B, CHE C) from 17- to 18-day-old Chinese hamster embryos removed from three individual mothers, in which four to eight healthy fetuses were growing, as described previously.[16] After preparation, CHE cells were divided into three groups and stored in liquid nitrogen as strains CHE A1, CHE A2, CHE A3, CHE B1, CHE B2, CHE B3, CHE C1, CHE C2, and CHE C3. Aliquots of 10^6 cells from frozen stocks were inoculated into 100-mm plastic dishes containing 15 ml of Dulbecco's modified minimal essential medium (Nissui Seiyaku, Tokyo) supplemented with 10% fetal bovine serum (M. A. Bioproducts, Walksville, MD), 100 units/ml penicillin, and 100 μg/ml kanamycin. The cells were cultured in a CO_2 incubator and transferred successively at intervals of 3 days.

Assay for tumorigenicity Aliquots of 10^7 cells suspended in 0.2 ml of growth medium were injected s.c. into athymic mice (BALB/c, *nu/nu*; Sankyo Lab. Service Corp., Tokyo) for each sample. Tumor appearance was checked weekly after the injection. Tumors bigger than 5 mm in diameter were scored as positive. Tumors bigger than 1.0 cm in diameter were removed from nude mice, trypsinized and returned to subculture for a few passages. These cells were preserved in liquid nitrogen as tumor-derived cells. In this study, we established four tumor-derived cell lines, T40A1 and T60A1 from CHE A1 cells cultured for 40 and 60 passages, and T15A2 and T25A2 from CHE A2 cultured for 15 and 25 passages, respectively.

Karyotype analysis For preparation of metaphase chromosomes, 10^6 cells were seeded into 100-mm dishes. After incubation for 24 h, Colcemid (Gibco, Grand Island, NY) was added at a final concentration of 0.025 μg/ml and incubation was continued for 2 h. Mitotic cells were collected and treated with 0.075 *M* potassium chloride for 20 min at room temperature. The cells were

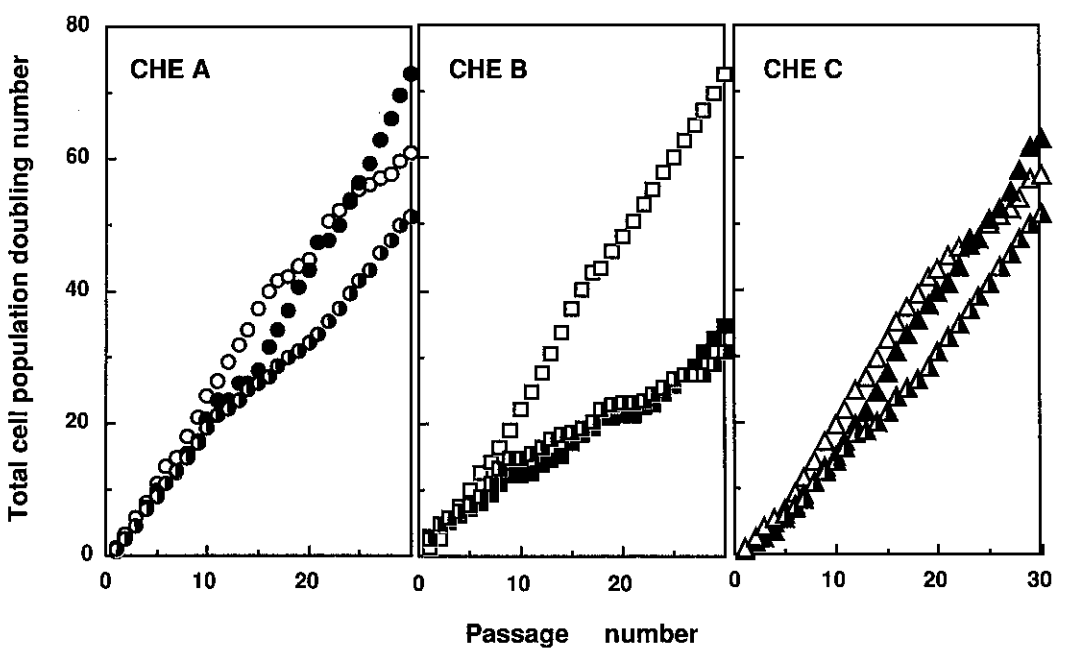

Fig. 1. Growth of CHE cells transferred successively at 3-day intervals as described in "Materials and Methods." ○, CHE A1 cells; ●, CHE A2 cells; ◑, CHE A3 cells; □, CHE B1 cells; ■, CHE B2 cells; ◨, CHE B3 cells; △, CHE C1 cells; ▲, CHE C2 cells; ▲, CHE C3 cells.

fixed in Carnoy's solution (methanol:acetic acid, 3:1) and spread on slide glasses by the air-drying method. The slides were immersed in 0.25% pancreatin (Gibco) solution for 10 to 30 s at room temperature, then stained with 5% Giemsa solution. Twenty or more metaphases per sample were photographed and karyotyped, as described by Kitchin and Sager.[17]

DNA transfection assay High-molecular-weight DNA was prepared as described by Sambrook *et al.*[18] The method for DNA transfection assay was essentially the same as that used previously.[12] NIH3T3 cells (5×10^5) were plated into 100-mm culture dishes and incubated overnight. After incubation, 30 μg of genomic DNA was added to each culture dish. Eight to 12 h after the addition, the cells were treated with 3 ml of 15% glycerol for 1 min at room temperature and refed with fresh medium. Twenty-four hours later, transfected cells were trypsinized and replated into four dishes, and the medium was changed to Dulbecco's modified Eagle's medium containing 5% calf serum. This medium was replaced every 3 days. After 3 weeks of cultivation the dishes were stained and the number of transformed foci was counted. Aliquots of 10^7 cells were injected into nude mice (BALB/c, *nu/nu*) for tumorigenicity assay.

Southern blot analysis High-molecular-weight DNA from NIH3T3 cells transformed by DNA from various CHE transformants was digested with restriction endonucleases. Ten micrograms of digested DNA was subjected to 0.8% agarose gel electrophoresis and blotted onto nitrocellulose filters by the method of Southern.[19] The filters were hybridized with ^{32}P-labeled probes at 42°C for 16–24 h, washed two times for 20 min each at 50°C with 2.0×SSC (300 m*M* sodium chloride, 30 m*M* sodium citrate) plus 0.1% sodium dodecyl sulfate (SDS) and at 50°C with 0.1×SSC plus 0.1% SDS, and placed on Kodak X-ray films at −70°C.

The labeled probes used in this study were provided, directly or through the Japanese Cancer Research Resources Bank, by E. M. Scolnick (pBS-9 for v-Ha-*ras*), T. Senba *et al.* (pKX044 for c-*erbB-2*), M. Oskarsson & G. F. Vande Woude (pMS1 for mouse c-*mos*). C. Cooper *et al.* (P met H for human *met*), and Bonner *et al.* (p627 for human c-*raf*-1). Plasmid p3035 containing human c-Ki-*ras*-2 was obtained from Oncor, Inc., Gaithersburg, MD. *Eco*RI fragment (0.8 kb) of human genomic *hst*-DNA[20] was provided by M. Shibuya (Department of Genetics, Institute of Medical Science, University of Tokyo).

RESULTS

Growth properties of CHE cells cultured for various passages CHE cells were transferred successively using the culture schedule described in "Materials and Methods." CHE cells from frozen cell stock showed heterogeneous morphology and grew at a constant growth rate for 5 passages. At this point most of the cells became fibroblastic but morphologically senescent, and their growth abilities gradually declined. As can be seen in Fig. 1, the growth rates recovered at some point between passages 5 and 10 and the cells proliferated at varying growth rates on successive transfer. Similar results were obtained in colony formation experiments in which the cells were plated at low density. Usually, colony-forming ability of CHE cells gradually declines with increasing passages until passage 5 and recovers after 10 passages (data not shown). Interestingly, there was a wide variation in growth rates between passages 10 and 20 even among different cell strains that originated from the same source of Chinese hamster embryos (CHE A or CHE B), indicating that this variability is not related to inherent diversity of pregnant mothers. CHE B2 and CHE B3 cells that exhibited the lowest growth rate also showed enhanced growth rates after 25 passages.

Table I. Tumorigenicity of CHE Cells Transferred Successively in Culture

Strain	Passage	Tumorigenicity[a]	Latency period[b]
CHE A1	15	0/3	
	40	3/4	81, 90, 105
	60	4/4	15, 23, 23, 23
CHE A2	5	0/3	
	10	2/6	98, 105
	20	3/3	42, 48, 70
	30	3/3	7, 7, 7
CHE A3	40	0/4	
	50	0/4	
	60	0/4	
CHE B1	15	0/3	
	25	0/3	
	40	0/4	
	60	0/4	
CHE B2	15	0/3	
	35	0/3	
	45	0/3	
	60	0/4	
CHE B3	40	0/4	
	60	0/4	
CHE C1	40	0/4	
	60	0/4	
CHE C2	15	0/3	
	50	0/4	
	60	0/4	
CHE C3	15	0/3	
	60	0/4	

a) Number of mice with tumor/number of mice receiving injection.
b) Number of days after which tumors bigger than 5 mm in diameter were detected.

These results indicate that CHE cells have a high probability of escaping from senescence and may proceed to malignant stages only through successive transfer in culture.

Acquisition of tumorigenicity CHE cells cultured for various passages were assayed for tumorigenicity in athymic nude mice, injecting 10^7 cells per mouse (Table I). Only two strains (CHE A1 and CHE A2) exhibited tumorigenicity at different passages between 10 and 40. The latency period for tumor development decreased when the cells were subcultured after 10 or 20 more passages. However, none of the cells derived from the other 7 strains showed any tumorigenic activity, although the testing period lasted for more than 200 days. Since growth recovery was observed during the early period between passages 5 and 10, these tumorigenic cells

Table II. Karyotype Changes in Various CHE Cell Strains and Tumor-derived CHE Cell Lines

Cells	Passage	Modal chromosome number[a)]	Constitution of sex chromosome	Common chromosome change[b)]
CHE cell strain				
CHE A1	5	22	XX or XY	
	15	23	XY	+8
	20	22	XY	
	40	23	XY	+3q
	60	23	XYY	+3q
CHE A2	5	22	XX or XY	
	10	23	XY	+3 or +3q
	20	23	XY	+3q
	30	23	XY	+3q
CHE A3	40	22	XY	
	50	22	XY	
	60	22	XY	
CHE B1	5	22	XX or XY	
	15	23	XY	+3q
	25	22	XY	t(3q;6), −6
	40	22	XY	t(3q;6), −6
	60	22	XY	t(1;5)
CHE B2	15	22	XY	
	35	23	XY	+5
	45	24	XY	+3q, +5
	60	24	XY	+3q, +5
CHE B3	40	23	XY	+5
	50	23	XY	+5, t(3q;3q)
	60	24	XY	+5, t(3q;3q)
CHE C1	5	22	XX or XY	
	20	23	XY	+3q
	40	23	XY	+3q
	60	24	XY	+3q, +5
CHE C2	5	22	XX or XY	
	15	23	XY	+3q
	40	23	XY	+5
	50	23	XY	+5
	60	24	XY	+3q, +5
CHE C3	40	22	XY	
	50	23	XY	+5 or t(1p;4p)
	60	23	XY	+5 or t(1p;4p)
Tumor-derived CHE cell line				
T40A1		24	XYY	+3q, t(3q;5)
T60A1		25	XY or XYY	+3q, +3q, +10
T15A2		23	XY	+3q
T25A2		23	XY	+3q

a) Chromosome number was determined from 100 metaphases.
b) Twenty or more metaphases per sample were used for karyotype analysis.

can be considered to have arisen from premalignant immortal cell lines at different passages.

Changes in karyotypes during successive transfer Normal Chinese hamster cells have 20 autosomal chromosomes and two sex chromosomes. The primary cultures of CHE cells from frozen stocks showed normal diploid karyotype containing a mixture of XX (55%) and XY (45%) chromosomes, indicating a heterogeneous cell population derived from different embryos. However, we found that the proportion of cells having XY chromosome become predominant with increasing passages. The CHE cells cultured for 5 passages contained an unequal mixture of XX (36%) and XY (64%) chromosomes and only one X chromosome was detected in all CHE strains transferred for more than 10 passages.

Table II shows the results of karyotype analysis for all 9 CHE strains cultured for various passages and for four tumor-derived cell lines. After cultivation for 5 passages, the CHE cells showed higher modal chromosome numbers of 23 or 24 and trisomy of chromosome 3q or chromosome 5 was observed in 8 of 9 cell strains. In addition to these numerical changes, 3 of 9 strains contained specific translocated chromosomes, t(3q;6), t(3q;3q) and t(1p;4p) at later passages. Interestingly, the CHE A3 cell strain maintained a normal diploid karyotype for 60 passages and only XY chromosomes were detected in all nine cell strains when the karyotype was analyzed after cultivation for more than 10 passages. In any case, the most common chromosome change in CHE A1 and CHE A2 cells cultured for more than 10 or 40 passages, at which point the cells exhibited tumorigenicity, was trisomy of chromosome 3q (Fig. 2).

We established four tumor-derived cell lines from tumors under the skin of nude mice as described in "Materials and Methods." As shown in Table II, there was a big difference between the two cell groups that originated from CHE A1 and CHE A2 strains. T15A2 and T25A2 cells exhibited only 3q trisomy, but T40A1 and T60A1 cells contained two copies of the long arm of chromosome 3 in addition to various extra chromosomes with or without a translocated chromosome, t(5;3q), between chromosome 3q and chromosome 5.

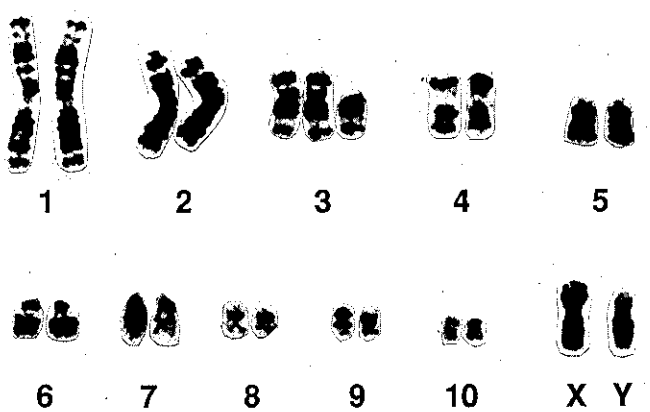

Fig. 2. Giemsa-banded karyotype of CHE A2 cells cultured for 20 passages.

Transforming ability of DNA from various CHE cells Transfection assay using an established mouse NIH3T3 cell line has been used to detect activated oncogenes. Therefore, we examined the transforming ability of NIH3T3 cells transfected with high-molecular-weight DNA extracted from sequential cultures of CHE cells and four tumor-derived cell lines, to determine whether DNA from malignant CHE cells has transforming ability. Table III shows the results of DNA transfection assay. We used DNA from a T24 human bladder carcinoma cell line which contained a mutation in Ha-*ras* gene, as a positive control, for evaluating the transforming activity of sample DNA. As can be seen in Table III, there was a clear tendency for tumorigenic cells derived from strains CHE A1 and CHE A2 to induce foci,

Table III. Transforming Ability of High-molecular-weight DNA from Various CHE Cells

Donor cells used for DNA transfection (Passage number)	No. of foci/No. of dishes	Focus-forming efficiency[a)]
No DNA	0/8	<0.008
Human carcinoma cell line		
T24	12/8	0.15
CHE cell strain		
CHE A1 (20)	0/12	<0.005
CHE A1 (40)	7/12	0.058
CHE A1 (60)	7/11	0.063
CHE A2 (10)	2/8	0.021
CHE A2 (20)	4/12	0.03
CHE A2 (30)	4/8	0.042
CHE A3 (40)	0/8	<0.008
CHE A3 (60)	0/7	<0.01
CHE B1 (60)	0/8	<0.008
CHE B2 (15)	0/8	<0.008
CHE B2 (60)	0/8	<0.008
CHE B3 (60)	0/8	<0.008
CHE C1 (20)	0/12	<0.005
CHE C1 (40)	0/11	<0.007
CHE C1 (60)	1/11	0.007
CHE C2 (60)	0/12	<0.005
CHE C3 (60)	0/12	<0.005
Tumor-derived CHE cell line		
T40A1	7/16	0.053
T60A1	2/16	0.017
T15A2	13/16	0.065
T25A2	4/12	0.027

a) Number of foci per 10^6 transfected cells per μg of DNA.

Table IV. Tumorigenicity of NIH3T3 Cells Transfected with High-molecular-weight DNA from Various Tumor-derived CHE Cell Lines

Donor cells used for DNA transfection	Tumorigenicity[a]	Latency period[b]
No DNA	0/4	
T40A1	4/4	60, 94, 94, 113
T60A1	2/2	80, 80
T15A2	4/4	72, 78, 82, 82
T25A2	2/3	27, 36

a) Number of mice with tumor/number of mice receiving injection.
b) Number of days after which tumors bigger than 5 mm in diameter were detected.

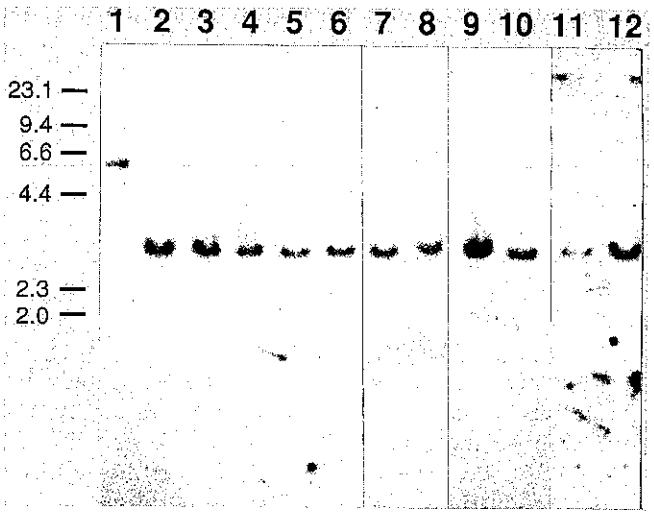

Fig. 3. Southern blot analysis of *Bam*HI-digested DNA from CHE primary culture (lane 1), NIH3T3 cells (lane 2) and various NIH3T3 primary transformants (lanes from 3 to 12). All primary transformants were obtained from tumors (lane 3, T40A1-T1; lane 4, T40A1-T2; lane 5, T40A1-T3; lane 6, T40A1-T4; lane 7, T60A1-T1; lane 8, T60A1-T2; lane 9, T15A2-T1; lane 10, T15A2-T2; lane 11, T25A2-T1; lane 12, T25A2-T2) formed under the skin of nude mice after injection of NIH3T3 cells transfected with DNA from four tumor-derived CHE cell lines. DNA (10 μg) was cleaved and hybridized with ^{32}P-labeled v-Ha-*ras* probe (pBS9).

although their focus-forming efficiencies were much lower than that of the T24 cell line. Similar transforming activity was observed in DNA samples from four tumor-derived cell lines that originated from tumorigenic CHE A1 and CHE A2 cells. Furthermore, we found that these DNA samples also exhibited a high tumor-forming ability when transfected NIH3T3 cells were injected into nude mice, though various latency periods for tumor development were observed among the four tumor-derived cell lines (Table IV).

In order to identify the transforming genes, we isolated DNA from various primary transformed NIH3T3 cells and analyzed whether these transformants contain any known oncogene using the Southern blot technique. Fig. 3 shows the results of the Southern blot analysis of Ha-*ras* gene. When *Bam*HI-cleaved DNA was hybridized with ^{32}P-labeled v-Ha-*ras* probe (pBS9), only one band that migrated to approximately 3.5 kb, the same position as in the case of mouse NIH3T3 cells, was detected in all NIH3T3 primary transformants. This indicates that DNA from tumor-derived CHE cells does not contain any activated Ha-*ras* gene to transform NIH3T3 cells. We performed similar analysis using various oncogene probes, such as Ha-*ras*, Ki-*ras*, *hst*, c-*erbB-2*, *mos*, *met*, and *raf*, which have transforming activity, but we could not detect any DNA fragments specific to Chinese hamster in any of the primary transformed cells. These data suggest that the expression of malignant phenotype in spontaneously transformed CHE cells is associated with the activation of an unknown oncogene(s).

DISCUSSION

It is well known that cancer arises through a complex multistep process. This phenomenon has also been observed in various *in vitro* transformation systems using cultured Chinese hamster[7, 8] and Syrian hamster[5, 6] embryo cells. The ability to identify the genetic changes responsible for the *in vivo* malignant progression is limited, because it is very difficult to detect what cells have transformed and to analyze chromosome changes during the process of tumor development. In this study, therefore, we introduced an *in vitro* transformation system using primary cultured CHE cells to examine the cytogenetic and genetic alterations relating to the expression of malignant phenotypes. We used these cells because they are stable in karyotype during growth in successive transfer,[9, 11, 17] but can spontaneously transform in a multistep manner.[8]

We confirmed that CHE cells can easily transform to immortal cells and proceed towards malignant stages following prolonged passages of cultivation (Fig. 1 and Table I). As reported previously,[9, 11, 17] trisomy of chromosome 3q or 5 was commonly observed (8 out of 9 strains cultured for 10 or more passages), but only 2 strains (CHE A1 and CHE A2) showed tumorigenicity at these passages. Ray *et al.* reported that either total or partial trisomy of chromosome 3 and trisomy of chromosome 5 are observed at an early stage in spontaneous transformation of cultured Chinese hamster cells and postulated that numerical changes of specific chromo-

somes may be associated with the acquisition of immortality.[21] In this study, only trisomy of chromosome 3q was commonly observed in all tumorigenic CHE cells derived from two strains (CHE A1 and CHE A2). Since one strain (CHE A3) maintained a normal diploid karyotype for 60 passages in culture, addition of 3q chromosomes is not essential for the acquisition of immortality at early stages, but might be associated with the genetic change prerequisite for the progression to more malignant stages. This has already been suggested by Sager *et al.* They established an *in vitro* transformation system using a Chinese hamster normal diploid (CHEF/18) cell line, and demonstrated that 3q trisomy is associated with the neoplastic progression of CHEF/18 cells.[7, 11] As described in "Materials and Methods," CHE A1, CHE A2 and CHE A3 cell strains were initiated from different cell stocks, originally derived from the same source of Chinese hamster embryos (CHE A). Nevertheless, CHE A1 and CHE A2 cell strains showed tumorigenicity at quite different passages (40 and 10 passages, respectively), but the CHE A3 cell strain did not express any malignant phenotype until passage 60. These results suggest that the conversion from the preneoplastic immortal stage to the malignant stage is induced by stochastic events such as gene mutations, in addition to a specific chromosome change.

The results of karyotype analysis using primary cultured CHE cells suggest that male CHE cells having XY chromosomes tend to have higher growth abilities than female CHE cells. In fact, the proportions of male and female CHE cells were 64% and 36% at passage 5, when CHE cells showed the slowest growth rate. A similar sex difference was observed in nickel-transformed CHE cells.[22] Conway and Costa proposed that X chromosomal alterations are important to neoplastic transformation in CHE cells because a high frequency of complete or partial deletions of the heterochromatic long arm of the X chromosomes was detected in most of the nickel-transformed cell lines.[22] Klein *et al.* found that microcell transfer of an intact Chinese hamster X chromosome resulted in senescence of these transformed cell lines and propose that the Chinese hamster X chromosome contains one or more senescence-inducing genes.[23] In our experiments, we detected only one set of sex chromosomes (XY) in all nine cell strains cultured for more than 10 passages. These results may indicate that male CHE cells are better able to escape senescence than female CHE cells. This may be because two copies of this gene are present in the female.

The total or partial trisomy of specific chromosomes has often been observed in animal tumorigenesis[24, 25] and in *in vitro* transformation in rodent cells.[12, 26–28] However, the role of these numerical changes in neoplastic cell transformation remains to be resolved. In cell fusion experiments between normal Chinese hamster embryo fibroblasts and their c-Ha-*ras* (EJ)-transformed cells, Craig *et al.* found that neoplastic phenotypes were initially suppressed in the hybrid cells and reexpression of tumorigenicity was observed when there was a loss of the short arm of chromosome 3.[11] Since extensive homology between Chinese hamster chromosome 3 and human chromosome 11p, in which some putative tumor suppressor genes were mapped, has been demonstrated,[10] deletion of the short arm of chromosome 3 may indicate the loss of tumor-suppression activity. As proposed by Klein,[29] it is possible that growth properties of transformed cells are controlled by a balance between genes for expression and suppression of malignant phenotype and that changes in gene dosage by total or partial trisomy of a specific chromosome may result in the expression of transformed phenotypes. In fact, the expression of epidermal growth factor receptor is known to increase with increased dosage of chromosome 7 in human malignant melanoma cells.[30] The fact that all tumor-derived CHE cell lines consistently had one or two copies of chromosome 3q (Table II) suggests that some genes relating to the expression of malignant phenotype may be present in the long arm of chromosome 3. A similar change was observed in X-ray-induced neoplastic transformation in golden hamster embryo (GHE) cells, in which tumor cells formed from X-irradiated GHE cells in nude mice showed an increased dosage of chromosome 9q.[27] Thus, we hypothesize that an imbalance between the long arm in chromosome 3 and the short arm in chromosome 3 is necessary for malignant progression of spontaneous transformation in cultured CHE cells.

We still do not know what genetic changes can be detected in the progression to malignant stages in CHE cells. Since activated transforming genes such as *ras* family have often been identified in a wide variety of human tumors, we examined the transforming activity of DNA from various kinds of CHE cells using a DNA transfection method. As can be seen in Table III, NIH3T3 cells transfected with DNA from tumorigenic cells derived from strains CHE A1 and CHE A2 and their tumor-derived cell lines consistently exhibited a focus-forming ability. Interestingly, all DNA samples from tumor-derived cell lines also showed a high tumorigenic potential (Table IV), indicating that these cell lines contain some dominant transforming genes associated with the acquisition of tumorigenicity. However, we could not detect any Chinese hamster oncogenes, such as Ha-*ras*, Ki-*ras*, *hst*, c-*erbB-2*, *mos*, *met*, and *raf*, in any of the NIH3T3 transformants. In the case of one tumor-derived cell line, T25A2, which was isolated from one tumor produced by the injection of CHE A2 cells cultured for 25 passages, one point mutation at the second position of N-*ras* condon 61 was detected (data not

shown). Because the population of cells having mutant N-*ras* was quite small in 25 passaged CHE A2 cells, and 15 passaged A2 cells and other tumorigenic cells contained no N-*ras* mutation, this oncogene activation may not be directly associated with the malignant progression of CHE cells. Similar negative results have been reported in various transformed rodent cell lines induced by X-rays.[31] Therefore, we conclude that spontaneous *in vitro* transformation of CHE cells involves the activation of some unknown oncogene(s).

In this study, we analyzed the mechanism of *in vitro* neoplastic transformation using primary cultured CHE cells and found that CHE cells transferred *in vitro* can easily transform to permanent stages and progress towards more malignant stages through different chromosomal and genetic changes. Tumor development in human and experimental animals is complex. Recently, multiple changes in several oncogenes and tumor suppressor genes have been detected in various malignant tumors. However, the role of these genetic changes during the tumor development is not well understood. Since Chinese hamster cells are known to be stable in karyotype and have only 22 chromosomes, which can be easily identified, *in vitro* transformation systems using CHE cells are useful models for analyzing the molecular mechanisms of neoplastic development.

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