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Immortalization of Cells from Brains Derived from a Strain (MSM/MSfB6C3Fl) of Wild Mouse

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Abstract

Escape from cellular aging is the rate-limiting step of multistep carcinogenesis. While normal human cells invariably undergo cellular aging and almost never spontaneously immortalize, cells derived from rodents such as mice are relatively easily immortalized. In this experiment, we studied the immortalization patterns of cells obtained from brain tissues of an inbred strain (MSM/MSfB6C3F1) derived from wild mice. We established 12 cell strains derived from 12 mouse brains in order to investigate whether these cells show cellular aging in the same fashion as human cells or whether these cells are immortalized as easily as rodent cells reported previously. As a result, all cell strains were immortalized up to about 200 days in culture. One strain immortalized very early, in the first 50 days, four strains immortalized in the last 200 days, and the other seven strains became immortal between 150 and 200 days in culture. All immortalized cell strains showed varying amounts of chromosome abnormalities, numerically and structurally, but no specific changes related to immortalization were detected. Before immortalization, three types of cells, glial-like, polygonal flat-thin, and fibroblast-like cells, were observed in culture, but after immortalization most of the cultures became fibroblastic. From these results, we concluded that fibroblast-like cells derived from brains of these mice immortalized in like fashion to fibroblasts of other inbred mice.

KEYWORDS: cells from mouse brains, immortalization, aging, chromosomes

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Immortalization of Cells from Brains Derived from a Strain (MSM/MSfB6C3F1) of Wild Mouse

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Escape from cellular aging is the rate-limiting step of multistep carcinogenesis. While normal human cells invariably undergo cellular aging and almost never spontaneously immortalize, cells derived from rodents such as mice are relatively easily immortalized. In this experiment, we studied the immortalization patterns of cells obtained from brain tissues of an inbred strain (MSM/MSfB6C3F1) derived from wild mice. We established 12 cell strains derived from 12 mouse brains in order to investigate whether these cells show cellular aging in the same fashion as human cells or whether these cells are immortalized as easily as rodent cells reported previously. As a result, all cell strains were immortalized up to about 200 days in culture. One strain immortalized very early, in the first 50 days, four strains immortalized in the last 200 days, and the other seven strains became immortal between 150 and 200 days in culture. All immortalized cell strains showed varying amounts of chromosome abnormalities, numerically and structurally, but no specific changes related to immortalization were detected. Before immortalization, three types of cells, glial-like, polygonal flat-thin, and fibroblast-like cells, were observed in culture, but after immortalization most of the cultures became fibroblastic. From these results, we concluded that fibroblast-like cells derived from brains of these mice immortalized in like fashion to fibroblasts of other inbred mice.

Key words: cells from mouse brains, immortalization, aging, chromosomes

It is well-established that normal diploid cell strains derived from a variety of tissue types and across a large range of mammalian species have a limited proliferative life span and undergo a process of cellular senescence (1–3). Normal human cells are strictly predisposed to cellular aging. In fact, no spontaneous escape from cellular senescence (immortalization) of normal human fibroblasts has been reported since the first report of cellular aging (for review, 4–6). On the other hand, cells from rodents such as mice, rats and hamsters, which are usually used in many laboratories, easily escape the process of cellular aging and immortalize spontaneously. However, this does not mean that these animal cells have no replicative senescence (7). In fact, mouse and rat fibroblasts eventually senesce after about 10 population doublings (8, 9), but they easily escape the process of cellular senescence at a measurable frequency (1 in 10^4 to 10^6), and the immortalized cells rapidly overgrow senescing cultures (for review, 10). Hamster cells also show cellular senescence between passages 5 and 12 (11) and immortalize at a rate of 1 to 2×10^{-6} per cell per generation (12). This difference between animal and human cells is very intriguing and the question arises as to whether this difference is species specific. We describe herein the immortalization process of cells obtained from brains of an inbred strain derived from wild mice.

Materials and Methods

Cells and cultures. Inbred mice (MSM/MsfB6C3F1 strain) derived from wild mice were provided to us by the National Institute of Genetics, Mishima, Japan. These mice are a little different from conventionally used inbred mice. Although they are recently inbred

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from wild mice, their behavior appears wild. Spontaneous tumor development in these mice is very rare (National Institute of Genetics, personal communication). Brains were removed from the newborn mice, separated from meningeal membrane, washed with Ca^{2+} — and Mg^{2+} — free phosphate buffer saline (PBS, pH 7.4), minced into small fragments with a pair of scissors, and incubated in 0.05 % collagenase (Type 1, Sigma Chemical Co., St Louis, MO, USA) solution for 2h at 37°C. Then a single cell suspension was prepared by gently pipetting the collagenase-treated fragments. The cells were seeded into 60-mm plastic dishes with Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) supplemented with 10 % fetal bovine serum (FBS) and 100 μg/ml kanamycin. The cultures were maintained at 37°C in a humidified atmosphere of 5 % CO_2.

Measurement of population doublings of cells. To estimate population doublings (PDs) of the cells in primary culture, initially, the population doubling time (PDT) was determined as follows: when the cells in primary culture became almost confluent (total hours in primary culture are expressed as H), they were detached by trypsin treatment. An aliquot of the cell suspension was stained with 1 % crystal violet in 0.1 M citric acid, and counted with a hemocytometer. Then the total number (TN) of cells in the primary culture was calculated. The remaining cells were seeded into 35-mm culture dishes to obtain growth curves. The cells were counted on days 1, 4 and 7, with the medium renewed on day 4. The PDT of the cells was calculated from the logarithmic phase of the growth curve. Then the initial number (IN) of proliferative cells at the start of primary cultures was calculated using the following equation: $IN \times 2^{H/PDT} = TN$. Then PDs of the cells in primary culture was calculated with the following equation: $PDs = \log_2 TN/IN$. Although the PDT in the primary culture may not be the same as that of the first passaged (secondary) culture, we had to calculate PDs using the above mentioned equation because the presence of many non-proliferative cells and cell debris in primary culture made it impossible to count the number of proliferative cells at the start of culture. After the secondary culture, by subculturing the confluent culture at the split ratio of 1:2 or 1:4, we added 1 or 2 PDs at every passage.

Chromosome analysis. Chromosomes were examined at the earliest stage after immortalization of the cells. Chromosome preparations were made using the conventional trypsin-Giemsa-banding method. Distribution of the chromosome number and G-banded karyotypic analysis were done in at least 25 cells of each culture.

Immunofluorescence. To identify the cultured cells, cells grown on a cover glass were stained using a neural cell typing set (Boehringer Mannheim Biochemica, Germany). This set contains antibodies against neurons (NF 160), astrocytes (GFAP), oligodendrocytes (GalC), fibroblasts (vimentin and fibronectin). The staining procedures were performed according to the manufacturer's instructions.

Tumorigenicity test. The cells tested were trypsinized and suspended in PBS. Then 10^6 cells in 0.1 ml of culture medium were injected into athymic mice (BALB/cAJcl-nu), which were observed for 3 months for the development of tumors.

Results

Normal cells cannot grow indefinitely in culture. They stop cell growth after a certain number of cell divisions. This phenomenon is defined as cellular aging. On the other hand, immortalization of cells means that cells overcome cellular aging and acquire the potential to grow indefinitely in culture. Immortalization of mouse cells was defined by the criteria described by Todaro and Green (1963). They reported that normal mouse embryonal fibroblasts have been shown to undergo immortalization between 14 and 28 population doublings (PDs) in culture.

In the present experiment, three types of immortalization processes were observed. In the first type, WB-3 strain cells grew vigorously immediately after initiation of cultures, and immortalized in the first 50 days, reaching 30 PDs (Fig. 1A). However, only one strain showed this tendency. On the other hand, seven of 12 strains belonged to the second type and immortalized around 150 days (Fig. 1A and 1B). These strains showed no noticeable crisis before immortalization. The other four strains were classified into the third type. These cells showed a retarded proliferation until 100 days in culture, and then began to grow exponentially, resulting in immortalization between 150 and 200 days (Fig. 1C). In these cell strains, growth retardation was especially prominent between 50 and 100 days in culture, indicating that this period was the crisis stage. The immortalization processes of these three types did not correlate to the duration of their primary culture (Table 1).

Figure 2 and Table 1 summarize numerical chromosome aberrations of the 12 immortalized cell strains. The

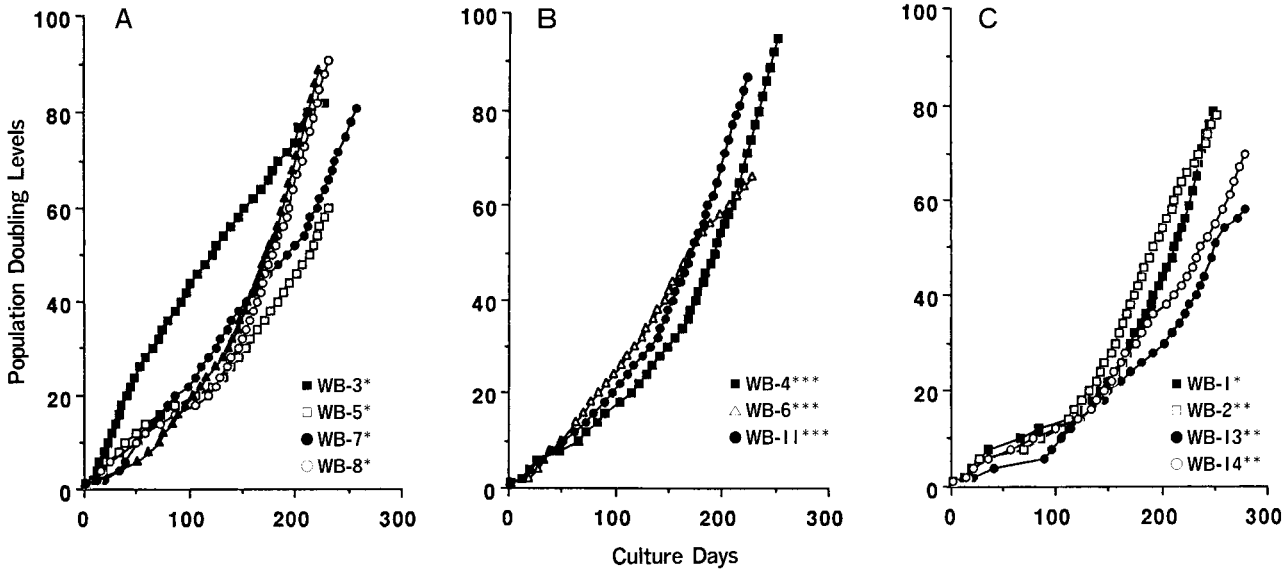

Fig. 1 Time course of immortalization of cells obtained from brains of an inbred strain (MSM/MSfB6C3F1) derived from wild mice. *Diploid, **triploid and ***tetraploid cell strains after immortalization.

Table 1 Characteristics of immortalized cell lines obtained from an inbred strain (MSM/MSfB6C3F1) derived from wild mice

Cell lines[a]	Days in primary culture	Morphology[b]	Modal chromosome number (%)	Tumorigenicity
WB-1	11	Fl→Fib	41 (26.7)	NT[c]
WB-2	11	Fib	47 (25.7)	NT
WB-3	8	Fl	40 (55.9)	+
WB-4	11	Fib	80 (25.9)	+
WB-5	12	Fl→Fib	40 (38.9)	NT
WB-6	19	Fl→Fib	80 (20.0)	NT
WB-7	19	Fl	40 (25.0)	+
WB-8	12	Fib	41 (25.8)	+
WB-11	11	Fib	70 (10.7)	NT
WB-12	12	Fib	40 (47.8)	NT
WB-13	21	Fib	59 (10.0)	NT
WB-14	14	Fl	67 (21.4)	+

a : WB-1 to WB-4, WB-5 to WB-8, and WB-11 to WB-14, newborn mice were obtained from three different mothers, respectively.
b : Fl, flat-thin polygonal cells; Fib, fibroblast-like cells.
c : NT, Not tested.

distribution of their chromosome aberrations was classified into three patterns: diploid (6 strains), triploid (3 strains), and tetraploid (3 strains) distribution of chromosome numbers. None of the six diploid strains were a true diploid cell strain. Actually, the diploid cell strain is defined as that in which at least 75 % of the cells have the same karyotype as normal cells of the species from which the cells were originally obtained (13). In fact, among these diploid cell strains, WB-12 showed the highest peak in diploid chromosome number, but the cells having the diploid karyotype numbered less than 50 %. Interestingly, chromosome distribution of the most rapidly immortalized cells (WB-3) was also in the diploid region. Among the six diploid cell strains, five immortalized without notice-

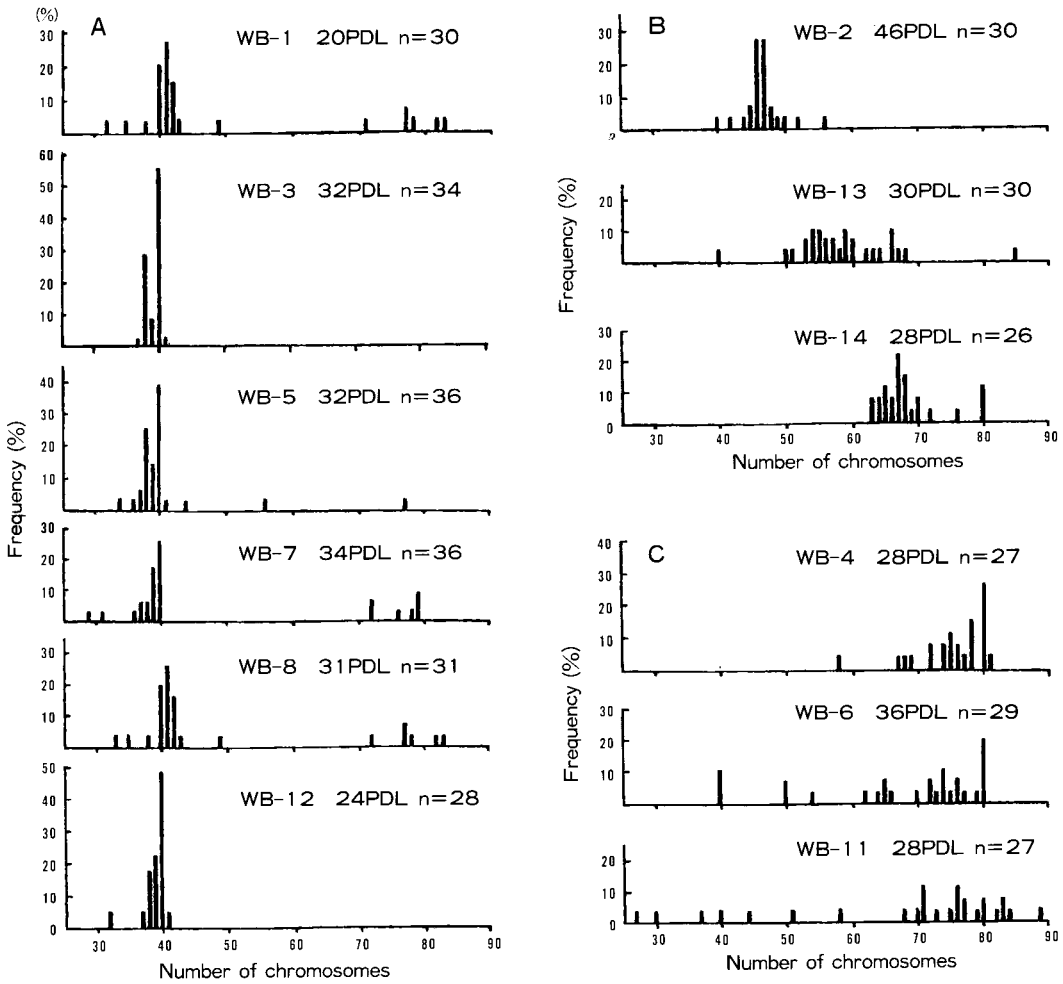

Fig. 2 Distribution of chromosome numbers in 12 immortalized cells obtained from brains of an inbred strain (MSM/MSfB6C3F1) derived from wild mice. PDL: Population doubling level at which chromosomes were examined; n: Number of chromosomes analyzed.

able crisis (Fig. 1A), while the remaining strain showed the crisis (Fig. 1C). Three triploid strains belonged to this crisis group (Fig. 1C). On the other hand, all three tetraploid cell strains immortalized without crisis (Fig. 1B). Taken together, it is difficult to determine what numerical chromosome changes are causally related to immortalization of the cells.

As shown in Table 1, morphologically, six strains were fibroblast-like from the start of culture, while three strains were flat in the early stage of culture, but they became fibroblastic after immortalization. The cells of the other three strains remained flat in morphology even after immortalization and projected long processes. These morphological features are shown in Fig. 3. Immediately after immortalization, there were a few cells (less than 5 %) stained with NF-160 (neuron), GFAP (astrocyte), or GalC (oligodendrocyte) specific antibody in some cultures, indicating that the majority of cells were not brain-specific cells but rather fibroblastic cells. The cells reactive against these specific antibodies disappeared soon after several passages, and all cultures became stained with fibronectin antibody.

Regardless of fibroblast-like or flat cell types, fibroblastic lines, WB-4 and WB-8, and flat cell type line, WB-3, WB-7 and WB-14, developed tumors (about 1 × 1 cm in size) within 14 to 21 days of inoculation, and the

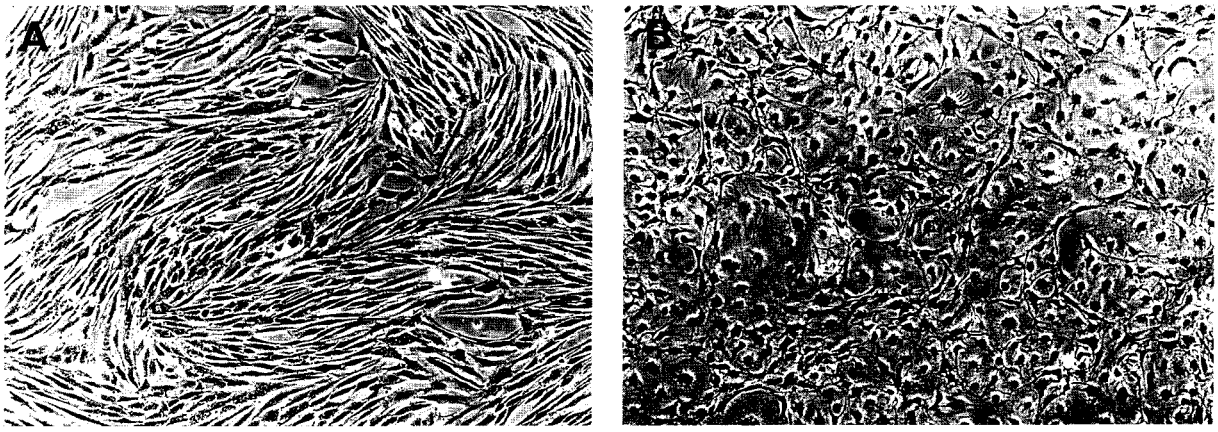

Fig. 3 Representative morphological features of immortalized cells obtained from brains of an inbred strain (MSM/MSfB6C3F1) derived from wild mice, fibroblast-like (**A**) and flat (**B**). Magnification, × 100.

histology of the tumors revealed undifferentiated fibrosarcoma. This tumorigenicity may have nothing to do with the numerical chromosome aberrations, because the distribution of chromosome numbers of WB-3, WB-7 and WB-8 cells was in the diploid region, whereas that of WB-4 and WB-14 cells was in the triploid to tetraploid region. Although we could not test the tumorigenicity of all the immortalized cell strains, most of them seem to be tumorigenic.

Discussion

We studied the immortalization process of cells obtained from brains of an inbred strain derived from wild mice. All 12 of the cultures became spontaneously immortalized. Four of them grew slowly until 100 days, which resembles the crisis of cellular aging, and then began to grow exponentially, resulting in immortalization between 150 and 200 days after initiation of culture. Interestingly, among these four cultures one showed the modal chromosome number in the diploid region, and the distribution of chromosome numbers of the other three cultures was around the triploid region, although a relationship between this chromosomal distribution pattern and the retarded immortalization process remains to be determined. The other 8 strains immortalized without showing a noticeable cellular crisis, and the chromosome distribution of these cultures was in the diploid or tetraploid region. Taken together, it is difficult to definitely determine at present which chromosome losses and/or gains are important for the immortalization process. These results also are consistent with those reported by others (14), who did not detect any specific chromosome changes in the immortalization of rat cells. However, given structural changes of the chromosomes examined, some significant findings may be seen in the immortalized cells.

In this study, we intended to grow brain-specific cells like glial cells and to investigate whether the cells show aging at the cellular level. But we could not culture the brain-specific cells under the present culture conditions. Although we started our cultures with the cells from the brain, we eventually observed the immortalization of fibroblasts. Regarding mouse and human brain-specific cells, there have been no reports on spontaneous immortalization. However, there are many spontaneously immortalized mouse fibroblast cell lines such as 3T3, 3T6 and 10T1/2, but no spontaneously immortalized human fibroblast cell line. Thus, from the viewpoint of fibroblasts, our present results indicate that cellular immortalization or aging is species specific.

It has been reported that the replicative potential of cells varies with many factors such as donor age, cell type, culture conditions, and method of serial cultivation. In the present study, all the above mentioned conditions were identical except for cell type. Actually, it is very difficult to isolate and to collect the same kind of cells from brains for each experiment, because brain tissue consists of various types of cells such as nerve cells, astrocytes, oligodendrocytes, fibroblasts and endothelial cells. Some cultures predominantly contained fibroblastic cells and others flat-thin polygonally spread cells. However, there was no difference in their immortalization patterns. After immortalization, most of the cultures contained fibroblast-

like cells showing undefined cellular characteristics. Although the precise cellular characteristics of these immortalized cells could not be determined, all the immortalized cell lines showed fibronectin. In addition, both flat-type and fibroblast-like cells produced histologically identical tumors which were diagnosed as undifferentiated fibrosarcoma.

These data can be explained in two ways. First, fibroblast-like cells overgrew the brain specific cells, and second, specific cells in the brain lost their differentiated functions over the course of culture. In the future, immortalization of certain types of brain cells should be studied by improving culture methods as much as possible.

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