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Source: Zoological Science, 18(4) : 483-496

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.18.483

ZOOLOGICAL SCIENCE **18**: 483–496 (2001)

Establishment of Three Cell Lines Derived from Frog Melanophores

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ABSTRACT—Three cell lines have been established from melanotic and amelanotic melanophores of wild and albino *Rana nigromaculata* and albino *Rana porosa brevipoda* after experiencing three culture crises. The population doubling rate increased with culture time. Cells from both wild and albino *R. nigromaculata* changed shape twice in ten years. They began as large pigmented or non-pigmented epithelial-like cells at the beginning of the cultures. The melanophores of albino *R. nigromaculata* unexpectedly became pigmented shortly after the beginning of the culture. Over time, they transiently aquired a small, non-pigmented, bipolar morphology and finally became large non-pigmented epithelial-like cells after about 8 to 10 years. On the contrary, cells from albino *R. porosa brevipoda* remained large, non-pigmented, epithelial-like cells over the entire course of the culture. Pigmented cells contained many mature melanosomes, while non-pigmented epithelial like cells contained premelanosomes with characteristic internal structures. Non-pigmented, fibroblast-like cells contained numerous small vesicles in place of typical premelanosomes. The cells of established cell lines, including those from wild *R. nigromaculata*, were unpigmented and showed no tyrosinase activity in histochemical dopa reactions. The number of chromosomes deviated from the normal pattern in the three cell lines. Each of the three cell lines had a different modal number than the other two, and the chromosomal compositions of the cells within the same lines differed from one another. Even within the same cell lines, the mode changed as the culture progressed without any consistent trends.

INTRODUCTION

A considerable number of studies have been reported hitherto on the isolation and cultivation of amphibian cells from embryos, tadpoles, and adults of various species, such as *Rana pipiens* (Auclair, 1961; Malamud, 1967; Rafferty, 1969; Freed *et al*., 1969; Freed and Mezger-Freed, 1970; Wong and Tweedell, 1974), *Rana sylvatica, Rana clamitans, Hyla cruci-fer* (Rafferty, 1969), *Rana catesbeiana* (Wolf and Quimby, 1964; Ide,1973,1974; Kondo and Ide, 1983; Nishikawa and Yosizato, 1985, 1986; Yoshizato and Nishikawa, 1985), *Bufo marinus* (Handler *et al*., 1979), *Bufo americanus* (Freed *et al*., 1969), *Bombina orientalis* (Ellinger *et al*., 1983), *Xenopus laevis* (Pudney *et al*., 1973; Godsell, 1974; Miller and Daniel, 1977; Anizet *et al*., 1981; Asashima *et al*., 1986; Nishikawa *et al*., 1990; Fukui *et al*., 1992) and *Notophthalmus viridescens* (Ferretti and Brockes, 1988). However, most of these studies were concerned with relatively short-term cultures. With the exception of the study by Fukui *et al*. (1992), which led to the establishment of a cell line derived from a *Xenopus laevis* tumor, none of these studies sought to establish cell lines by culturing cells continuously more than 4 years.

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In general, the characteristics of cells of established lines are known to differ from those of cells before the establishment. The cells have to adapt to a new environment during the course of culture, but there seem to be no reports that clearly demonstrate the changes of cellular characteristics along with the passage during the long-term culture. Studies on the nature of cells during long-term cultivation should help to elucidate the plasticity of cells in coping with changes in their environments, and unravel some aspect of the mechanism of cell survival under artificial conditions at the cellular level.

In the present study, we performed serial subcultures of melanotic and amelanotic melanophores of ranid frogs for more than 10 years and established 3 cell lines from them. We also characterized proliferative activity, cell morphology and chromosome constituents of these cell lines.

MATERIALS AND METHODS

Frogs

We used three strains of experimental animals in the present study: froglets of wild type *Rana nigromaculata* and albino *Rana nigromaculata* (KM stock), both of which had tyrosinase activity, and albino *Rana porosa brevipoda* (BR stock), which had no tyrosinase activity. Both KM and BR stocks were established by Nishioka and Ueda (1985), and all the specimens had been raised in the Laboratory for Amphibian Biology of Hiroshima University.

Culture methods

A method used by Ide (1974) for culture of melanophores of *Rana catesbeiana* tadpoles was employed with a slight modification. For dissociating cells, pieces of dorsal skin were taken from pithed froglets (2 ~ 4 individuals) with a pair of scissors, sterilized in 70% ethanol for 15 sec, washed twice in distilled water, and then cut into small pieces in 70% Ca^{2+},Mg^{2+}-free Hanks'solution (Hanks'CMF). The skin pieces were incubated at 4°C for 30 min in 0.5% trypsin (1:250, DIFCO, USA) dissolved in 70% Hanks'CMF. Then, the dermis was separated manually from the epidermis under a microscope with forceps and incubated at 37°C for 1.5 to 3 hr in 70% Hanks'CMF containing 0.5% collagenase (Type 1A, Sigma, USA) and 0.35% trypsin. Culture medium containing 10% fetal bovine serum (GIBCO, USA) was added to the resultant cell suspension to weaken the enzyme activity. The cell suspension was filtered through a platinum mesh (150 mesh) to remove undissociated pieces of tissues. The filtrate was layered on the top of a discontinuous Ficoll density gradient (Ficoll 400, Pharmacia, USA) (5%, 10% and 20%) and centrifuged at 750×g for 20 min. The sedimented cells were suspended in culture medium and plated on Falcon plastic dishes (#3001). Culture medium was a mixture of 6 parts of conditioned L-15 (GIBCO), 3 parts of double-distilled water, 1 part of fetal bovine serum, 100 units/ml of penicillin (Meiji; Tokyo), 100 μg/ml of streptomycin (Meiji), and 2.5 μg/ml of Fungizone (GIBCO BRL). Cells were cultured at 25°C and media were exchanged every 4 or 5 days. To prepare conditioned medium, neuroretina of 11-day-old chick embryos was digested with 0.5% trypsin in Hanks'BSS solution at 37°C for 1 hr, centrifuged, resuspended in Eagle's MEM (Nissui Pharmaceutical, Tokyo), plated on Falcon dishes (#3003), and cultured at 37°C. When the cultures became confluent, the spent medium was replaced with L-15 medium containing 10% fetal bovine serum. The L-15 medium was withdrawn after 24 hr and stored in a freezer until use.

Electron microscopy

Cultured cells were pre-fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 at room temperature for 1 hr and postfixed with 1% osmic acid in the same buffer at room temperature for 30 min. After dehydration in an alcohol series, the cells were embedded in Epok 812. Sections were cut on a MT6000 Sorvall ultramicrotome with a diamond knife, stained with uranyl acetate and lead citrate, and observed with a Hitachi H-300 transmission electron microscope at 80 Kv.

Tyrosinase activity was examined by histochemical dopa reaction according to the method of Wakamatsu *et al.* (1984). Briefly, cultured cells were prefixed with 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) at room temperature for 1 hr, incubated with 5 mM L-DOPA (L-dihydroxyphenylalanine, Wako Chem. Tokyo) in the same buffer at 37°C for 5 hr, and postfixed with 1% osmic acid at room temperature for 30 min. As a negative control, cells were incubated with a mixed solution of 5 mM L-DOPA and 5 mM DEDTC (sodium diethyldithiocarbomate, Wako Chem.), a specific inhibitor of tyrosinase. The subsequent procedure for electron microscopy was performed as described above.

Chromosome preparation

Chromosome preparations were prepared by the following air dry method: 10 μg/ml colchicin (Merck, USA) was added to the culture medium 4 hr before the cells were harvested. Cells dissociated from the plastic surface by trypsinization were collected by centrifugation, suspended in 0.075 M KCl solution, and left undisturbed for 30 min. After the fixative (ethanol 3/acetic acid 1) was added to the suspension, the cells were collected by centrifugation and resuspended in fresh fixative. This fixation process was repeated twice. Finally, the cells were suspended in a small amount of fixative, one drop of which was placed on a clean slide and allowed to dry spontaneously. Chromosomes were stained in Giemsa's solution.

RESULTS

Of the three established cell lines, LAH1 was from wild-type *R. nigromaculat*a, LAH2 from albino *R. nigromaculata* (KM stock) and LAH3 from albino *R. porosa brevipoda* (BR stock). In the first week of culture of these cell lines, melanophores, xanthophores, iridophores and nonpigmented dermal cells transiently adhered to the surface of the plastic dishes. Some of these cells exhibited mitotic activity, but almost all of them but the melanophores were shed from the plastic surface within a month or so. Eventually, only melanophores survived and proliferated, showing some changes in cell shape, degree of pigmentation, proliferation rates, and chromosomal constitution. Each cell line exhibited characteristic behavior but all three commonly experienced crises three times when the number of cells did not increase as a whole. The cells became immortal and proliferated continuously after the last crisis. For the sake of cenvenience, in this report we refer to the period up to the last crisis as the "pre-immortalization phase", and after the last crisis as the "post-immortalization phase".

I. Cell line LAH1

This cell line derived from melanotic melanophores of wild-type *R. nigromaculata* was in the 118th passage and the cumulative population doubling number was 446 at 12 years after the beginning of the culture.

1) Proliferation of cells in culture

a) Pre-immortalization phase

The cells of the LAH1 cell line began to proliferate about 20 days after plating, but the initial proliferation rate was very low. The first passage culture was made 97 days after the beginning of the culture. Then 436 days elapsed towards the end of the 10th passage (Table 1). The cumulative cell population doubling number during this period was 12.8 and the mean population doubling time was calculated to be 34.1 days. A crisis took place during the 14 days of the 11th passage and the number of cells did not increase. A cumulative cell population doubling number of 2.7 was counted during the 103 days between the 12th and 15th passages, and mean population doubling time during this period was 38.1 days. The second crisis came at the 16th passage, which lasted 126 days. The cumulative population doubling number was 7.0 and the mean population doubling time was 46.3 days during the 324 days between the 17th and 22nd passages. The third crisis took place during the 33 days of the 23rd passage, and again the number of cells did not increase. The cumulative population doubling number was 22.5 for a period of 3 years up to the third crisis (Table 1).

b) Post-immortalization phase

The mean population doubling time for each year was calculated during the course of the culture. It was longest in the 4th year from the 24th to 26th passages (383 days). The cumulative population doubling number was 2.2 and the mean population doubling time was 174.1 days. After this period, the population doubling rate increased gradually. The popu-

lation doubling number was 8.1 in the 5th year (27~32 passages, 343 days), 10.7 in the 6th year (33~36 passages, 308 days), 14.5 in the 7th year (37~41 passages, 406 days), and 25.2 in the 8th year(42~48 passages, 380 days). The mean population doubling times in the 5th to 8th years were 42.3, 28.8, 28.0 and 15.1 days, respectively. The population doubling rate further increased in the following years, as shown by the cumulative population doubling numbers of 75.5 in the 9th year (49~67 passages, 338 days), 98.4 in the 10th year (68~87 passages, 362 days), and 79.0 in the 11th year (88 ~100 passages, 359 days). The mean population doubling times during these periods were calculated to be 4.5, 3.7 and 4.5 days, respectively (Table 1).

2) Cell morphology

Cells of this line generally changed shape twice. At the beginning of culture, the melanophores adhering to the plastic surface were large, epithelial-like in shape, and dark brown. A small non-pigmented area was located near the center of the cell and the nucleus was located near this area or in the peripheral region (Fig. 1A). Cells often protruded several dendritic processes when they were not closely adjacent to each other. Non-pigmented cells first began appearing 2 years and 4 months after the beginning of culture, i.e.,during the period between the second and the third crises, and they gradually increased in number after their initial appearance (Fig. 1B). This type of cell and the dark-colored epithelial-like cells coexisted for a while, and by the end of 5 years from the beginning of the culture all of the pigmented cells were replaced with non-pigmented fibroblast-like cells (Fig. 1C). These cells proliferated in tandem to form networks or spiral aggregates with several centers, but there was no contact between the cells at any parts along their margins even at confluence. Non-pigmented epithelial-like cells appeared at about 8 years and 4 months after the beginning of the culture, and eventually all of the fibroblast-like cells were replaced by this type of cell (Fig. 1D). The cells were in close contact with each other in a pavement arrangement, and no free space was found on the surface of the culture dish at confluence. At the transitional stage from fibroblast to epithelial cell types, a small number of large, lightly pigmented cells appeared transiently.

3) Pigmentary organelles

The ultrastructure of cells in the 6th passage (1 year and 2 months after the beginning of culture) that maintained the original pigmented, large epithelial-like morphology was observed. The cells contained many fully pigmented melanosomes, a small number of partly pigmented melanosomes (immature melanosomes), and unmelanized premelanosomes (Fig. 2A). In non-pigmented fibroblast-like cells of the 45th passage (7 years and 7 months after the beginning of culture), small spherical bodies with fuzzy fibrous inclusion were found in place of premelanosomes (Fig. 2B). The cytochemical localization of tyrosinase activity in these non-pigmented cells of the 46th passage was examined by dopa reaction, but no reaction product was observed throughout the cytoplasm. After the second transformation, premelanosomes with typical internal structure reapeared in non-pigmented epithelial-like cells of the 62nd passage (8 years and 9 months after the beginning of culture) (Fig. 2C).

4) Constitution of chromosomes

R. nigromaculata has 26 chromosomes, including 5 pairs of larger chromosomes and 8 pairs of smaller ones (Nishioka, 1972; Nishioka *et al*., 1987). In an examination of chromosomes from a total of 134 cells in the 49th passage (8 years and 2 months after the beginning of culture), it was found that almost all cells (96.3%) had 26 chromosomes (Table 4). The karyotype was normal and chromosomes with structural aberrations were not found. The only mitotic spread detected had 52 chromosomes, including 20 large chromosomes and 32 small ones. However, at the 54th passage, only about 3 months after the preceding examination, chromosomal constitution of cells was aberrant, exhibiting 9 different chromosome counts ranging from 20 to 56 in a total of 160 mitotic spreads examined. Cells with 28 chromosomes were the most frequent and numbered 103 (64.4%). The second most frequent were those with 27 chromosomes, and they numbered 34 (21.3%). There were only 9 (5.6%) cells with 26 chromosomes and their karyotypes were all aberrant. There were 2 mitotic spreads with hypertetraploid chromosomes (Table 4). When the structural aberrations of chromosomes were classified into 8 types as exemplified in Fig. 3, there were 7 types (1st to 7th types) of aberrations in these mitotic spreads (Table 5). Dicentric chromosomes of the 6th type were most frequent, that is, 28 chromosomes of this type were found in 26 cells. The 5th and 7th types of chromosomes with remarkable aberration occurred in 3 and 2 spreads, respectively (Table 5).

Nine groups of cells ranging in chromosome count from 21 to 54 were observed in a total of 339 mitotic spreads examined at the 98th passage (10 years and 10 months after the beginning of culture). The mode of the chromosome number, 27, was found in 236 (69.6%) spreads. The second and third most frequent spreads were 56 cells having 26 chromosomes and 16 cells having 25 chromosomes. There were only 8 (2.4%) spreads having 28 chromosomes, the most frequent chromosome number in the preceding examination. Three cells had hypertetraploid chromosomes (Table 4). There were 6 types with structurally aberrant chromosomes, among which dicentric chromosomes of the 6th type were most frequent. In total, 61 aberrant chromosomes of this type were found in 51 cells. Nine chromosomes of the 5th type and 2 of the 7th type appeared in 8 and 2 spreads, respectively (Table 5).

II. Cell line LAH2

The cell line LAH2 derived from tyrosinase-positive amelanotic melanophores of albino *R. nigromaculata* was in the 127th passage and the cumulative population doubling number was 465 at 13 years after the beginning of culture.

1) Proliferation of cells in culture

a) Pre-immortalization phase

In early culture, amelanotic melanophores could hardly be distinguished in color from all cells but the chromatophores. Some cells began to proliferate slowly at about 10 days after

plating and small cell colonies arising from one or a few cells were seen scattered over the plate. There were differences in doubling rates among these colonies. Cells in some colonies unexpectedly became pigmented from about 30 days after plating, and the pigmentation gradually increased thereafter. Finally, all of the cells became more or less pigmented and the well-pigmented cells could not be distinguished from pigmented melanophores derived from wild-type frogs in coloration. The 1st passage culture was made on 68 days after the beginning of culture, but proliferation of cells was very slow and the cumulative population doubling number was 1.7 during the 284 days of this passage. Eventually, the cumulative population doubling number was increased to 6.9 during the 482 days from the 1st to 5th passages, and the mean population doubling time was 69.9 days (Table 2). The first crisis came during the 56 days of the 6th passage, and the number of cells did not increase during this time. The cumulative population doubling number was 2.0 for the 130 days of the 7th passage. The second crisis occurred during the 8th passage. The cumulative population doubling number for the 306 days (9~12 passages) was 4.5 and the mean population doubling time was 68.0 days. The number of cells did not increase

Table 1. Serial subcultivation of cell line LAH1

Date	'86 8/8 ~11/13	'86 11/13 ~'88 1/23	'88 1/23 ~2/6	'88 2/6 ~5/19	'88 5/19 ~9/22	'88 9/22 ~'89 8/12	'89 8/12 ~9/14	'89 9/14 ~'90 10/2	'90 10/2 ~'91 9/10	'91 9/10 ~'92 7/14
Passage		p1 ~p10	p11	p12~p15	p16	p17~p22	p23	p24~p26	p27 ~p32	p33~p36
Duration (days) (cumulative)[1]	97	436 (533)	14 (547)	103 (650)	126 (776)	324 (1100)	33 (1133)	383 (1516)	343 (1859)	308 (2167)
P. D. N.[2] (c. p. d. n.)[3]		12.8	0	2.7 (15.5)	0	7.0 (22.5)	0	2.2 (24.7)	8.1 (32.7)	10.7 (43.4)
P. D. T. (days)[4]		34.1		38.1		46.3		174.1	42.3	28.8

[1] (cumulative), cumulative days from the beginning of culture.
[2] P. D. N., cumulative population doubling number in concerned period.
[3] (c. p. d. n.), cumulative population doubling number from the beginning of cultur.
[4] P. D. T., mean population doubling time in concerned period.

Table 2. Serial subcultivation of cell line LAH2

Date	'85 9/6 ~11/13	'85 11/13 ~ '87 3/10	'87 3/10 ~5/5	'87 5/5 ~9/12	'87 9/12 ~11/7	'87 11/7 ~ '88 9/8	'88 9/8 ~10/20	'88 10/20 ~'89 10/3	'89 10/3 ~'90 9/18	'90 9/18 ~'91 9/23	'91 9/23 ~'92 9/16
Passage		p1 ~p5	p6	p7	p8	p9~p12	p13~p14	p15~p22	p23~p28	p29~p38	p39~p51
Duration (days) (cumulative)[1]	68	482 (550)	56 (606)	130 (736)	56 (792)	306 (1098)	42 (1140)	348 (1488)	350 (1838)	370 (2208)	359 (2567)
P. D. N.[2] (c. p. d. n.)[3]		6.9	0	2.0 (8.9)	0	4.5 (13.4)	0	11.4 (24.8)	16.1 (40.9)	27.4 (68.3)	46.5 (114.8)
P. D. T. (days)[4]		69.9		65.0		68.0		30.5	21.7	13.5	7.7

[1] (cumulative), cumulative days from the beginning of culture.
[2] P. D. N., cumulative population doubling number in concerned period.
[3] (c. p. d. n.), cumulative population doubling number from the beginning of culture.
[4] P. D. T., mean population doubling time in concerned period.

Table 3. Serial subcultivation of cell line LAH3

Date	'85 5/28 ~7/5	'85 7/5 ~ '86 1/14	'86 1/14 ~2/6	'86 2/6 ~5/10	'86 5/10 ~5/29	'86 5/29 ~ 10/25	'86 10/25 ~11/1	'86 11/1 ~'87 5/28	'87 5/28 ~'88 5/24	'88 5/24 ~'89 5/25
Passage		p1 ~p26	p27	p28~p37	p38	p39~p48	p49	p50~p71	p72~p112	p113~p147
Duration (days) (cumulative)[1]	38	193 (231)	23 (254)	93 (347)	19 (366)	149 (515)	7 (522)	208 (730)	362 (1092)	366 (1458)
P. D. N.[2] (c. p. d. n.)[3]		49.9	0	21.5 (71.4)	0	10.9 (82.3)	0	40.3 (122.6)	86.0 (208.6)	99.1 (307.7)
P. D. T. (days)[4]		3.9		4.3		13.7		5.2	4.2	3.7

[1] (cumulative), cumulative days from the beginning of culture,
[2] P. D. N., cumulative population doubling number in concerned period.
[3] (c. p. d. n.), cumulative population doubling number from the beginning of culture.
[4] P. D. T., mean population doubling time in concerned period.

during the 42 days of the 13th and 14th passages, and this was the third crisis. The cumulative population doubling number was 13.4 during the 3-year period up to the last crisis and the mean population doubling number per year was 4.5. This mean population doubling number was somewhat smaller than that of the LAH1 cell line (Table 2).

b) Post-immortalization phase

The population doubling rate increased after immortalization, as seen in the cumulative population doubling number of 11.4 in the 4th year (15~22 passages, 348 days), 16.1 in the 5th year (23~28 passages, 350 days), and 27.4 in the 6th year (29~38 passages, 370 days). The mean population doubling times in these years were 30.5, 21.7 and 13.5 days, respectively. The cumulative population doubling numbers were 46.5 in the 7th year (39~51 passages, 359 days), 38.2 in the 8th year (52~61 passages, 359 days), and 50.1 in the 9th year (62~75 passages, 371 days). The mean population doubling times during these periods were 7.7, 9.4, and 7.4 days (average 8.1 days). The cumulative population doubling numbers were 69.2 in the 10th year (76~93 passages, 367 days), 65.8 in the 11th year (94~106 passages, 366 days) and 65.4 in the 12th year (107~117 passages, 336 days). The mean population doubling times were 5.3, 5.6 and 5.1 days, respectively (average 5.3 days). (Table 2)

2) Cell morphology

As stated above, the cells were non-pigmented and epithelial-like in appearance at the beginning of culture (Fig. 1E), and in the following 1 month they gradually aquired color. The pigmented cells could hardly be distinguished in coloration from melanophores in early culture of wild-type frogs (Fig. 1F). Non-pigmented fibroblast-like cells appeared about 1 year after the beginning of culture (before the first crisis), and almost all cells were replaced by this type of cell in the following year (Fig. 1G). Their proliferation pattern closely resembled that of non-pigmented fibroblast-like cells of the LAH1 cell line. They proliferated in a tandem arrangement, but they did not exhibit a pavement arrangement even at confluence. Subsequently, non-pigmented epithelial-like cells appeared in less than 10 years after the beginning of culture, and these cells finally replaced the fibroblast-like cells (Fig. 1I). They exhibited a pavement arrangement at confluence, leaving no free space on the surface of the culture dishes. During the transitional stage of substitution, a small number of large, slightly pigmented cells similar to those found in the transitional stage from fibroblast-like to epithelial-like cells in the LAH1 cell line appeared transiently (Fig. 1H).

3) Pigmentary organelles

As stated above, amelanotic melanophores brought into culture started to produce dark brown pigment (Fig. 1F). Electron microscopic observation showed a large number of mature melanosomes within the cell bodies, and these cells could hardly be distinguished from the mature melanophores of wild-type frogs. After the transformation of these melano-

’92 7/14 ~’93 8/24	’93 8/24 ~’94 9/8	’94 9/8 ~’95 8/12	’95 8/12 ~’96 8/8	’96 8/8 ~’97 8/2
p37~p41	p42~p48	p49~p67	p68~p87	p88~p100
406 (2573)	380 (2953)	338 (3291)	362 (3653)	359 (4012)
14.5 (57.9)	25.2 (83.1)	75.5 (158.6)	98.4 (257.0)	79.0 (336.0)
28.0	15.1	4.5	3.7	4.5

’92 9/16 ~’93 9/10	’93 9/10 ~’94 9/16	’94 9/16 ~’95 9/18	’95 9/18 ~’96 9/18	’96 9/18 ~’97 8/20
p52~p61	p62~p75	p76~p93	p94~p106	p107~p117
359 (2926)	371 (3297)	367 (3664)	366 (4030)	336 (4366)
38.2 (153.0)	50.1 (203.1)	69.2 (272.3)	65.8 (338.1)	65.4 (403.5)
9.4	7.4	5.3	5.6	5.1

’89 5/25 ~’90 5/29	’90 5/29 ~’91 5/25	’91 5/25 ~’92 5/28	’92 5/28 ~’93 5/27	’93 5/27 ~’94 5/21	’94 5/21 ~’95 5/25	’95 5/25 ~’96 5/25	’96 5/25 ~’97 5/29
p148~p181	p182~p214	p215~p245	p246~p276	p277~p307	p308~p342	p343~p371	p372 ~p390
369 (1827)	361 (2188)	369 (2557)	364 (2921)	359 (3280)	369 (3649)	366 (4015)	369 (4384)
101.1 (408.8)	101.2 (509.9)	95.0 (605.0)	102.5 (707.5)	119.1 (826.6)	138.5 (965.1)	122.7 (1087.7)	119.0 (1206.8)
3.6	3.6	3.9	3.6	3.0	2.7	3.0	3.1

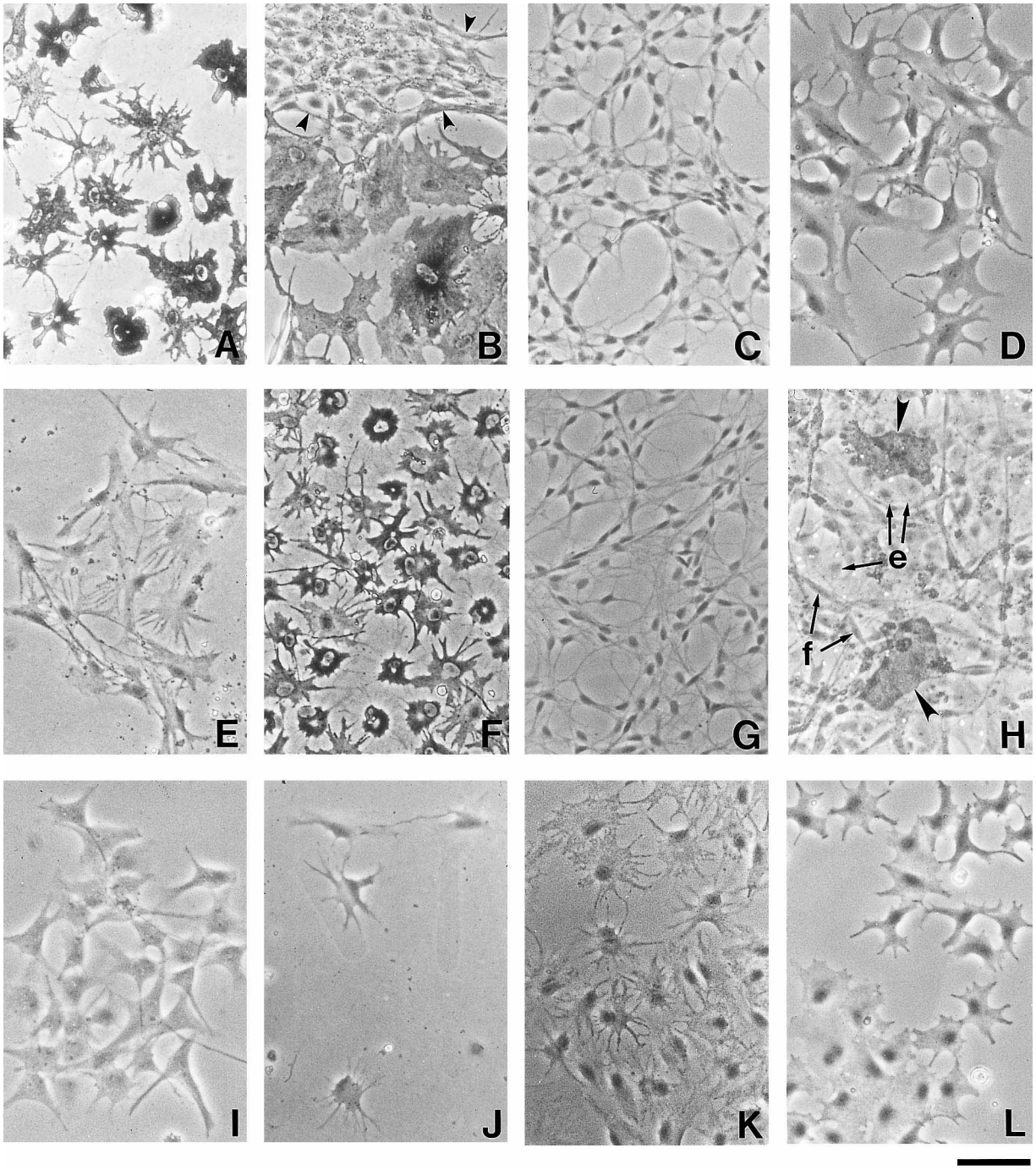

Fig. 1. Photomicrographs of melanophores derived from *Rana nigromaculata*. (cell line LAH1), albino *Rana nigromaculata*. (cell line LAH2), and albino *Rana porosa brevipoda*. (cell line LAH3). Photographs A~D were taken from cell line LAH1. A) 30 days after plating. Original dark-colored melanophores. B) 2 years and 5 months after plating. Pigmented melanophores and non-pigmented cells (arrowheads) are coexisting. C) 7 years and 7 months after plating. Only non-pigmented fibroblast-like cells are present. This photograph was taken from a resin-embedded preparation. D) 10 years and 9 months after plating. Only non-pigmented epithelial-like cells are present. Photographs E~I were from cell line LAH2. E) 60 days after plating. Original amelanotic melanophores. F) Five months after plating. Note the presence of pigmented melanophores that were originally amelanotic. G) 3 years and 8 months after plating. Only non-pigmented fibroblast-like cells are present. This photograph was taken from a resin-embedded preparation. H) 10 years and 3 months after plating. Non-pigmented fibroblast-like cells, non-pigmented epithelial-like cells and large, slightly pigmented cells (arrowheads) are coexisting. This photograph was taken from a resin-embedded preparation. I) 11 years and 8 months after plating. Only non-pigmented epithelial-like cells are present. Photographs J~L were from cell line LAH3. J) 7 days after plating. Original amelanotic melanophores. K) and L) Non-pigmented epithelial-like cells, 6 months after plating and 12 years after plating, respectively. e, non-pigmented epithelial-like cell; f, non-pigmented fibroblast-like cell. bar, 50 μm.

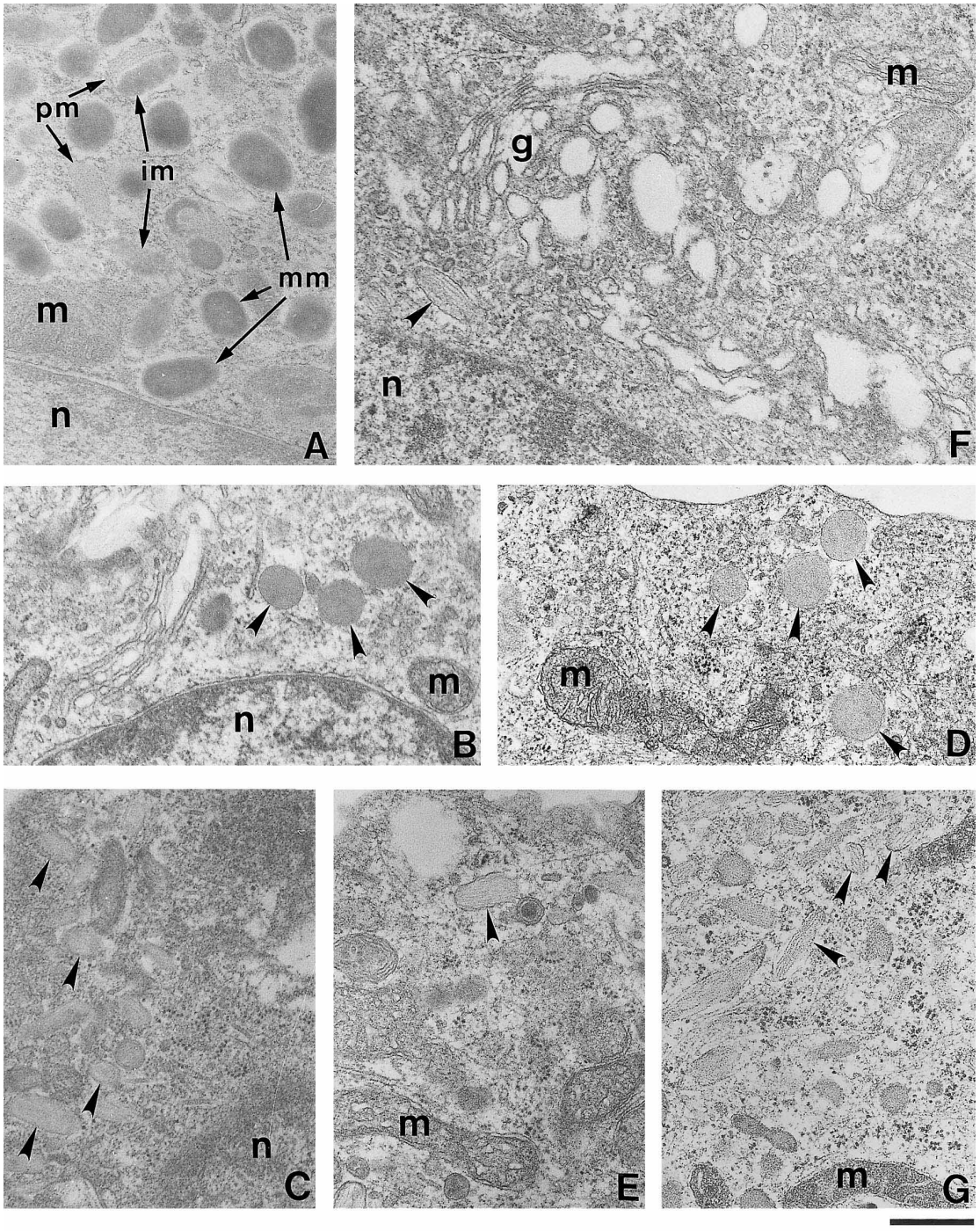

Fig. 2. Electron micrographs of cultured cells derived from *Rana nigromaculata*. (cell line LAH1), albino *Rana nigromaculata*. (cell line LAH2), and albino *Rana porosa brevipoda*. (cell line LAH3). A) an originally pigmented cell in cell line LAH1 in the sixth passage, 1 year and 2 months after the beginning of culture. Mature melanosomes, immature melanosomes and premelanosomes are seen. B) a fibroblast-like cell in cell line LAH1 in the 45th passage, 7 years and 7 months after the beginning of culture. Spherical bodies (arrowheads) with fuzzy fibrous inclusion are seen. C) an epithelial-like cell in cell line LAH1 in the 62nd passage, 8 years and 9 months after the beginning of culture. Premelanosomes (arrowheads) ellipsoidal in shape are seen. D) a fibroblast-like cell in cell line LAH2 in the 19th passage, 3 years and 8 months after the beginning of culture. Spherical bodies (arrowheads) with the same fuzzy fibrous inclusion as that found in the fibroblast-like cell in cell line LAH1 are seen. E) an epithelial-like cell of cell line LAH2 in the 96th passage, 11 years and 3 months after the beginning of culture. A premelanosome (arrowhead) with the same ellipsoidal shape as that found in the epithelial-like cells in cell line LAH1 is seen. F) a dopa-treated cell of cell line LAH2 in the 97th passage, 10 years and 4 months after the beginning of culture. No dopa-reaction product is found anywhere in spite of the occurrence of long ellipsoid-shaped premelanosome (arrowhead). G) an epithelial-like cell of cell line LAH3 in the 282nd passage, 8 years and 2 months after the beginning of culture. Premelanosomes (arrowheads) with the same ellipsoidal shape as that found in the non-pigmented epithelial-like cells in cell lines LAH1 and LAH2 are seen. g, Golgi apparatus; m, mitochondrion; n, nucleus; im, immature melanosome; mm, mature melanosome; pm, premelanosome. bar, 0.5 μm.

Table 4. Frequency of mitotic spreads by chromosome number in cell lines LAH1, LAH2 and LAH3

Cell line	Passage No.	Total mitotic spreads	Kind of mitotic spreads by chromosome number											
			20≧	21	22	23	24	25	26	27	28	29	32~34	48~58
LAH1	p49	134	0	0	0	1 (0. 7%)	1 (0.7%)	2 (1.5%)	129 (96.3%)	0	0	0	0	1 (0.7%)
	p54	160	1 (0.6%)	0	0	1 (0.6%)	2 (1.3%)	2 (1.3%)	9 (5.6%)	34 (21.3%)	103 (64.4%)	6 (3.8%)	0	2 (1.3%)
	p98	339	0	2 (0.6%)	6 (1.8%)	7 (2.1%)	3 (0.9%)	16 (4.7%)	56 (16.6%)	236 (69.8%)	8 (2.4%)	1 (0.3%)	0	3 (0.9%)
LAH2	p14	893	0	1 (0.1%)	1 (0.1%)	7 (0.8%)	52 (5.8%)	258 (28.9%)	568 (63.6%)	5 (0.6%)	1 (0.1%)	0	0	0
	p51	318	0	1 (0.3%)	1 (0.3%)	2 (0.6%)	5 (1.6%)	21 (6.6%)	284 (89.3%)	1 (0.3%)	0	0	0	3 (0.9%)
	p115	209	7 (3.3%)	8 (3.8%)	7 (3.3%)	19 (9.1%)	75 (35.9%)	82 (39.2%)	9 (4.3%)	1 (0.5%)	0	0	1 (0.5%)	0
LAH3	p63	128	0	0	0	0	1 (0.8%)	5 (3.9%)	37 (28.9%)	85 (66.4%)	0	0	0	0
	p312	174	0	0	0	1 (0.6%)	6 (3.4%)	6 (3.4%)	23 (13.2%)	53 (30.5%)	78 (44.8%)	6 (3.4%)	1 (0.6%)	0
	p391	400	0	0	0	7 (1.8%)	10 (2.5%)	23 (5.8%)	96 (24.0%)	249 (62.3%)	9 (2.3%)	0	0	6 (1.5%)

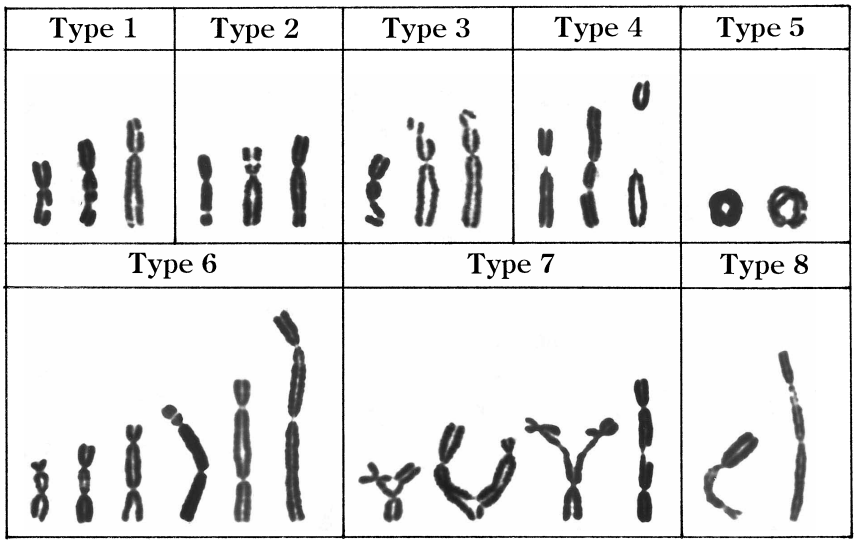

Fig. 3. Types of structurally aberrant chromosomes. Type 1, chromatid gap; Type 2, chromosome gap; Type 3, chromatid break; Type 4, chromosome break; Type 5, chromosome exchange (intrachange, ring chromosome); Type 6, chromosome exchange (interchange, dicentric chromosome); Type 7, chromatid exchange (interchange, asymmetric); Type 8, attenuation (a kind of ill-defined chromosomal aberration)

phores into non-pigmented fibroblast-like cells, numerous spherical bodies similar to those found in the fibroblast-like cells of LAH1 were observed (Fig. 2D). After the second transformation into epithelial-like cells, typical premelanosomes reappeared in the cytoplasm (Fig. 2E).

Tyrosinase activity was not detected either in non-pigmented fibroblast-like cells in the 70th passage (8 years and 8 months after the beginning of culture) or in non-pigmented epithelial-like cells in the 97th passage (10 years and 4 months after the beginning of culture) (Fig. 2F).

4) Constitution of chromosomes

In a total of 893 cells of the 15th passage immediately after the third crisis (3 years one month after the beginnig of culture), 8 groups of cells ranging in chromosome count from 21 to 28 were observed. The modal number, 26, was found in 568 cells (63.6%), and cells containing 25 chromosomes (258; 28.9%) and 24 chromosomes (52; 5.8%) were the second and third most frequent (Table 4). The majority of the cells with 26 chromosomes had 5 pairs of larger chromosomes and 8 pairs of smaller ones, and the karyotypes of these cells were almost normal. However, most of the 258 cells with 25 chromosomes had abnormal karyotypes. Each of the 219 spreads out of the 258 examined had a dicentric chromosome, suggesting that a fusion of 2 chromosomes had occurred in these

Table 5. Frequency of structurally aberrant chromosomes

Cell line	Passage No.	Total mitotic spreads	Types of structurally aberrant chromosomes							
			Type 1	Type 2	Type 3	Type 4	Type 5	Type 6	Type 7	Type 8
LAH1	p49	134	0	0	0	0	0	0	0	0
	p54	160	8 (6)	6 (5)	5 (5)	3 (3)	3 (3)	28 (26)	2 (2)	0
	p98	339	9 (6)	13 (12)	0	4 (4)	9 (8)	61 (51)	2 (2)	0
LAH2	p14	893	–	–	–	–	9 (9)	296 (265)	1 (1)	–
	p51	318	–	–	–	–	1 (1)	1 (1)	–	–
	p115	209	7 (6)	5 (5)	3 (3)	0	2 (2)	66 (58)	4 (4)	3 (3)
LAH3	p63	128	–	–	–	–	5 (5)	47 (43)	0	–
	p312	174	50 (43)	6 (4)	6 (6)	0	13 (12)	47 (43)	0	2 (2)
	p391	400	27 (24)	40 (33)	9 (8)	9 (9)	20 (19)	141 (116)	5 (5)	2 (2)

Types 1~8 are corresponding with those in Fig. 5.
Type 1, chromatid gap; Type 2, chromosome gap; Type 3, chromatid break;
Type 4, chromosome break; Type 5, chromosome exchange (intrachange, ring chromosome);
Type 6, chromosome exchange (interchange, dicentric chromosome); Type 7, chromatid exchange
Type 8, attenuation; Figures in parentheses show the number of spreads;

cells. Three types of chromosomes with marked structural abnormalities were found, i.e., the 6th type (296 chromosomes in 265 spreads), 5th type (9 in 9 spreads), and 7th type (1) were found (Table 5). When cells were sorted by the number of centromeres instead of chromosomes, 819 (91.7%) contained 26 centromeres.

Nine groups of cells ranging in chromosome count from 21 to 52 were observed in a total of 318 mitotic spreads examined at the 51st passage (about 4 years after the first examination). Cells containing 26 chromosomes were most abundant (284 cells, 89.3%), and their karyotypes were normal, having 5 pairs of larger cromosomes and 8 pairs of smaller ones. Cells containing 25 (21; 6.6%) and 24 chromosomes (5; 1.6%) were the second and the third most frequent (Table 4). Chromosomes with remarkable structural aberrations were scarce (Table 5).

Chromosome analysis of 209 cells in the 115th passage (a little less than 5 years after the second examination) revealed that the cells varied widely in chromosome number. There were 11 different patterns in the chromosome count which ranged from 18 to 34. The most frequent type, which numbered 82 (39.2%), contained 25 chromosomes. Cells containing 24 (75; 35.9%) and 23 (19; 9.1%) chromosomes were the second and third most frequent whereas those containing 26 chromosomes, the most numerous cell type in the previous examination, numbered only 9 (4.3%), and their karyotypes were aberrant (Table 4). The numbers of centromeres were 25 in 118 cells (56.5%), 24 in 41 (19.6%), 26 in 15 (7.2%), and 23 in 13 (6.2%). Of the structurally aberrant chromosomes, 66 of the 6th type, 4 of the 7th type, and 2 of the 5th type appeared in 58, 4, and 2 spreads, respectively (Table 5).

III. Cell line LAH3

This cell line derived from tyrosinase-negative amelanotic melanophores of *R. porosa brevipoda* was in the 413th passage and the cumulative population doubling number was 1335 at 13 years after the beginning of culture.

1) Proliferation of cells in culture

a) Pre-immortalization phase

Cells began to proliferate about one week after plating. Although they subsequently proliferated actively, many of them exfoliated from the surface of culture dishes at the same time. During the period from the 1st to 26th passage (193 days), the cumulative population doubling number was 49.9, and the mean population doubling time was 3.9 days (Table 3). The first crisis took place in the 27th passage, and the number of cells did not increase for 23 days. Cumulative population doubling number during the 28th to the 37th passages (93 days) was 21.5 and the mean population doubling time was 4.3 days. The second crisis came at the 38th passage. After this period, cells proliferated and exfoliated repeatedly during the 39th to the 48th passages (149 days). The cumulative population doubling number was 10.9, and the mean population doubling time was 13.7 days. Then the third crisis took place during the 49th passage (Table 3).

b) Post-immortalization phase

The cumulative population doubling number in the period of the 50th to the 71st passages (208 days) from immediately after the third crisis to the end of the second year was 40.3, and the mean population doubling time was 5.2 days during this period. In the third year (72~112 passages, 362 days), the cumulative population doubling number was 86.0 and the mean population doubling time was 4.2 days. In

the following 5 years (113~276 passages, 4~8 years), the cumulative population doubling numbers per year were almost constant, i.e., 99.1, 101.1, 101.2, 95.0 and 102.5, respectively, and the mean population doubling time was calculated to be 3.7 days. Cumulative population doubling numbers in each of the 4 years from the 9th to the 12th year (277~390 passages) were 119.1, 138.5, 122.7 and 119.0, and the mean population doubling time for these 4 years was 2.9 days (Table 3).

2) Cell morphology

Unlike the other two cell lines, the LAH3 cells were non-pigmented and epithelial-like in shape from the beginning of the culture, and the shape remained almost unchanged during the entire course of the culture (Fig. 1 J~L). The cells exhibited a pavement arrangement at confluence, leaving little free space on the surface of the culture dishes.

3) Pigmentary organelles

Cells in the 282nd passage (8 years and 2 months in culture) contained typical premelanosomes with a characteristic inner structure. The profile of premelanosomes remained unchanged in the cells up to the 305th passage (a little less than 9 years in culture) (Fig. 2G). Tyrosinase activity was not detected in the cells in the 305th passage.

4) Constitution of chromosomes

R. porosa brevipoda also has 26 chromosomes including 5 pairs of larger chromosomes and 8 pairs of smaller ones (Nishioka, 1972; Nishioka *et al.*, 1987). The cells in culture had variable chromosome counts and the mode deviated from the normal mode of 26 in every examination. In the first examination carried out at the 63rd passage (1 year and 9 months after the beginning of culture), 4 different groups of chromosome counts ranging from 24 to 27 were observed in a total of 128 mitotic spreads. The two most frequent group, consisting 85 cells (66.4%) and 37 (28.9%) cells contained 27 and 26 chromosomes, respectively (Table 4). Though the chromosome counts in the latter group, 26, is the same as that in normal diploid cells, 36 cells out of 37 contained an aberrant dicentric chromosome. Four out of the 5 cells containing 25 chromosomes had 2 dicentric chromosomes and the fifth had 1 dicentric chromosome. Therefore, when the spreads were classified by the number of centromeres, almost all cells (98.4%) fell into a single group having 27 centromeres. Of the marked structural abnormality, 47 chromosomes of the 6th type and 5 of the 5th type were found in 43 and 5 cells, respectively (Table 5).

In 174 mitotic spreads examined at the 312th passage (9 years after the beginning of culture), 8 groups of mitotic spreads ranging in chromosome count from 23 to 32 were recognized. The three most numerous spreads contained 28, 27 and 26 chromosomes, and had total cell counts of 78 (44.8%), 53 (30.5%) and 23 (13.2%), respectively (Table 4). Cells containing 28, 27 and 26 centromeres numbered 99 (56.9%), 46 (26.4%) and 14 (8.0%). Forty-seven aberrant chromosomes of the 6th type and 13 of the 5th type appeared in 43 and 12 spreads (Table 5).

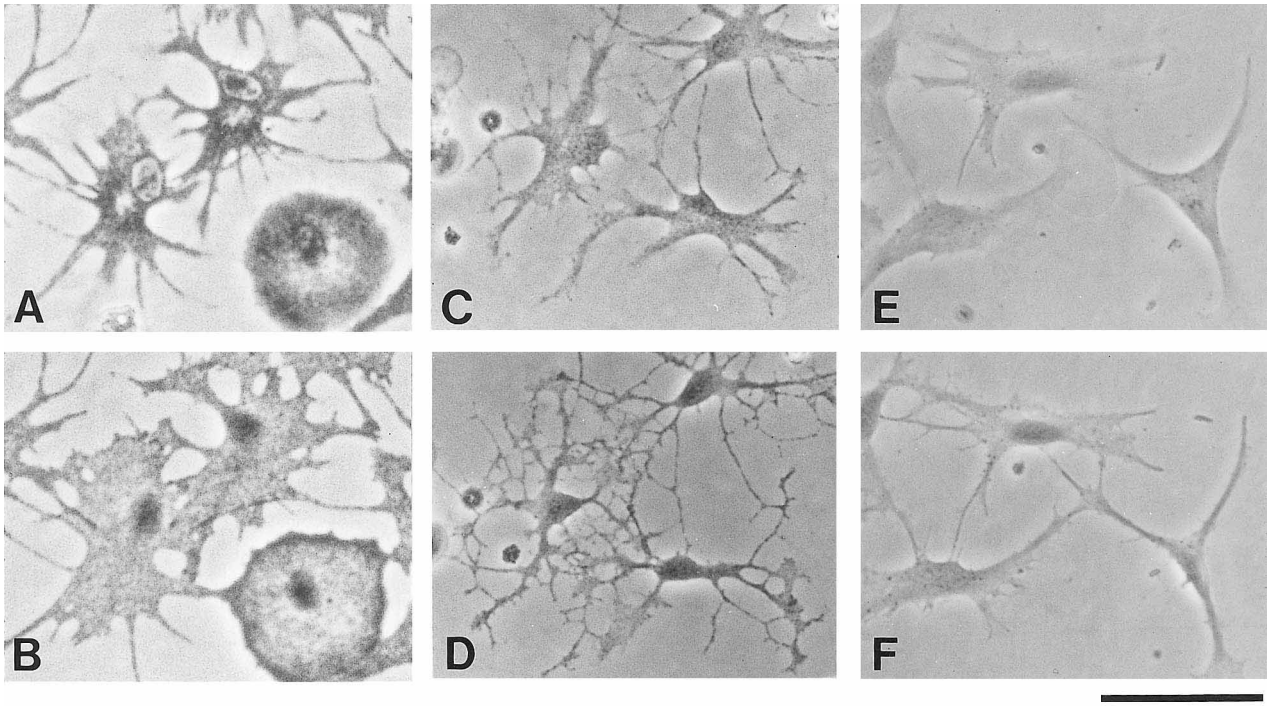

Fig. 4. Responses of cultured melanophores to melanophore stimulation hormon (α-MSH; 0.1 μg/ml). A and B: cell line LAH1. A) pigmented melanophores 281 days after the beginning of culture just before MSH treatment. B) 90 minutes after the MSH treatment. Pigment dispersion are clearly seen. C and D: cell line LAH2. C) amelanotic melanophores 15 days after plating just before MSH treatment. D) 90 minutes after the MSH treatment. The area of cell body decrease, but local dilation of dendrites and excess branching of thin processes are observed. E and F: cell line LAH3. E) amelanotic melanophores 10 days after plating just before MSH treatment. F) 90 minutes after the MSH treatment. The area of cell body decrease and processes become partially dilated. bar, 50 μm.

In a total of 400 mitotic spreads examined at the 391st passage (12 years after the beginning of culture), 10 different chromosome counts ranging from 23 to 58 were observed. Cells containing 27, 26, and 25 chromosomes were found in 249 (62.3%), 96 (24.0%) and 23 (5.8%) spreads, respectively, whereas cells with 28 chromosomes, the most numerous cell type in the last examination, numbered only 9 (2.3%) (Table 4). The cells having 28, 27, 26 and 25 centromeres numbered 19 (4.8%), 325 (81.3%), 30 (7.5%) and 13 (3.3%), respectively. When we looked for chromosomes with marked structural aberrations, found 141 chromosomes of the 6th type, 20 of the 5th type, and 5 of the 7th type in 116, 19 and 5 cells, respectively (Table 5).

Responses of cells of the three cell lines to melanophore stimulating hormon (MSH)

Pigmented cells of LAH1 281 days after the beginning of culture responded to α-MSH (Sigma) at a concentration of 0.1 μg/ml with melanosome dispersion, and increase in the area of cell body (Fig. 4A and B). Amelanotic cells of LAH2 15 days after plating responded to α-MSH with the decrease of the area of cell body, and partial dilation and excess branching of the dendrites (Fig. 4C and D). Amelanotic cells of LAH3 10 days after plating also responded to α-MSH in a similar means as LAH2 (Fig. 4E and F). The magnitude of the response of these cells to this hormon seemed to decrease gradually as the generation of passages proceeded, becoming (totally) insensitive after the transformation into non-pigmented fibroblast-like cells.

DISCUSSION

Crisis of culture

Of the amphibian cell lines that transformed or became spontaneously immortal in earlier investigations, only a few described by Kondo and Ide (1983) were reported to encounter crises. Each of their 4 cell lines met with a crisis at the 67th passage on the average. On the contrary, all of the 3 cell lines in the present study were spontaneously established through 3 crises. In general, culture cells have an inherent division age, crisis develops when their division age is exhausted, and they die soon thereafter (Hayflick and Moorhead, 1961). There are occasionally some cells that escape from the crises and proliferate continuously, ultimately becoming immortalized cell lines. Although cultured human diploid fibroblasts rarely become immortalized, after they are infected with DNA oncogenic virus they can double in population more than 20 to 30 times before their division age is exhausted (Moyer *et al*., 1964; Girardi *et al*., 1965; Ide *et al*., 1984). However, crisis develops in culture after this life extension and all cells die except for rare cases (Moyer *et al*., 1964; Girardi *et al*., 1965; Gotoh *et al*., 1979; Shay *et al*., 1993). Shay *et al*.(1991) distinguished the crisis induced by exhaustion of the division age from that occurring after life extension, denominating them as mortality stage 1 (M1) and mortality stage 2 (M2), respectively. The factors involved in M1 mechanism are regarded to be Rb and p53 proteins (Stein *et al*., 1990; Hara *et al*., 1991; Kulju and Lehman, 1995; Atadja *et al*., 1995), or some unidentified negative regulators against cell proliferation. Among factors participating in the crisis after life extension induced by infection with DNA oncogenic virus (M2), an instability of chromosomes due to a shortening of telomeres may be a possible cause of crisis (Counter *et al*., 1992, 1994). When this interpretaion of M1 and M2 mechanisms is applied to the 3 crises in each of the 3 cultures in the present study, 2 of the 3 crises may be conformable to M1 and M2, but there is no known interpretation of the mechanisms involved in the third crisis. The mechanisms of the crises that took place in the LAH3 cell line may differ from those in the cell lines LAH1 and LAH2, since population doubling rate near crises and average period of crises in the former greatly differed from those in the latter two cell lines. It is unclear whether the 3 crises encountered in this study are reproducible in other amphibnian cell lines, so far, there has been no report describing more than a single crisis in any cell line.

Population doubling time

Population doubling times of 4 cell lines derived from haploid and diploid embryos of *Rana pipiens* (Freed *et al*., 1969) were reported to be 30 to 70 hr. According to Rafferty (1969), heteroploid cells proliferated more rapidly than diploid cells. While the population doubling time of diploid kidney cells of embryonic *Rana sylvatica* was 30 hr, that of heteroploid cells from kidney of adult *Xenopus laevis* was 22 hr. In a cell line derived from a carcass of metamorphosing *X. laevis*, the cell count increased 7 or 8 times in 5 days (Pudney *et al*.., 1973). Other population doubling times reported were 46 hr for cells derived from *R. pipiens* kidneys (Malamud, 1967), 17 ~45 hr for cells from *X. laevis* kidneys (Godsell, 1974), 90 hr for cells from *R. pipiens* pronephroi (Wong and Tweedell, 1974), 39 hr for cells from *X. laevis* tadpoles (Anizet *et al*., 1981), 68 hr at 25°C for cells from *Bombina orientalis* embryos (Ellinger *et al*., 1983), and 75.5 hr at 25°C for cells from a tumor of *X. laevis* (Fukui *et al*., 1992).

In the LAH1 and LAH2 cell lines, mean population doubling times in the present study differed largely from those reported earlier. In early stages of the culture in this study, the mean population doubling times ranged from 34 to 174 days, greatly exceeding the 17 to 90 hr range stated above. However, population doubling rates of LAH1 and LAH2 became gradually higher in later stages of culture. The population doubling time of LAH1 became much shorter (89 hr) in the 10th year and that of LAH2 was 122 hr in the 12th year. One of the causes for the change in the mean population doubling time during the cultures may have been a process of selection that took place at every passage culture, whereby cells dividing faster than the other cells in the same population were prone to survive in further generations. In the LAH3 cell line, the mean population doubling time did not change largely from the beginning to the later stages of culture. Outside of a period of 5 months between the second and the third crisis, when the doubling time was 14 days, the mean population

doubling time was 124 hr in the 200 days just after the third crisis, and 65 hr in the 10th year. The latter population doubling time was comparable to those reported in other amphibian cell lines.

Cell morphology

Looking at the morphology of amphibian cells in culture, Yoshizato and Nishikawa (1985) recognized two cell types, macrophage-like cells and fibroblastic cells, in culture of mesenchymal cells derived from tail fins of *Rana catesbeiana* tadpoles. Asashima *et al.* (1986) distinguished four morphologically different cell types in cells derived from a tumor of *Xenopus laevis* two and a half years after the beginnig of culture. Subsequently, one of those cell types, stellate type, continued to proliferate for more than 4 years (Fukui *et al.*, 1992). In some other amphibian cell cultures, cells have been reported to be either epithelial-like or fibroblast-like. However, cells has been rarely witnessed to change in shape in the course of the culture. It is reported that predominant cell types change during cultivation in some cell lines (Pudney *et al.*,1973; Anizet *et al.*,1981; Ellinger *et al.*,1983), but whether the changes are a reflection of an actual disguise of cells or merely a result of changes in proliferation rates in different cell types is not known. In the present LAH1 and LAH2 cell lines, large pigmented epithelial-like cells at the early stages of culture changed into small, non- pigmented, fibroblast-like cells and finally into large, non-pigmented, epithelial-like cells. It seems reasonable to conjecture that the observed changes in cell shape may be related to two events. Firstly, the changes may directly related to changes in the quantity and three-dimensional positioning of cytoskeletons and adherence molecules. Secondly they may be indirectly related to changes in the quantity and quality of other cytoplasmic constituents, including pigmentary organelles. It is noteworthy that the occurrence of small spherical bodies (possible precursors of premelanosomes) in place of premelanosomes were commonly observed in fibroblast-like cells of LAH1 and LAH2, and that a reappearance of premelanosomes came with the next transformation from fibroblast to epithelial-like morphology. Consistently with this observation, cells of LAH3 that did not change their epithelial-like shape throughout the culture always contained premelanosomes in the cytoplasm.

The second change in cell morphology occurred when the cell population doubling rates reached a plateau. It is presumed that the change is a function of expendable surplus energy, part of which is used in rising population doubling rates, and that the growth of cells induced by the increase of cellular materials and organelles may be achieved by the consumption of surplus energy.

Tyrosinase activity was not detected by the dopa reaction in non-pigmented cells in the three cell lines. However, in our earlier study (Okumoto *et al.*, 1995) transfection of tyrosinase cDNA of wild type *R. nigromaculata* into cells of LAH3 cell line at the 326th passage (9 years and 6 months after the beginning of culture) has been shown to transform tyrosinase-negative amelanotic melanophores into melanotic melanophores inducing a definite increase in the number of pigmentary organelles and melanin diposition in premelanosomes. During the transition of cell shape from fibroblast to epithelial-like cells in LAH1 and LAH2 lines, a small number of large, slightly pigmented cells appeared transiently, but the background of this phenomenon remains unclear. The mechanism involved here seems to be different from the temporary enhancement of tyrosinase expression observed in the early culture of LAH2, where the increased melanization may have been due to the decrease in endogenous tyrosinase inhibitor(s) from the environment, or due to the augmentation of the enzyme activity by factors involved in the conditioned culture medium.

Chromosomal constitution

Aberrations in the chromosomal constitution are known to occurs in cultured cells, especially in immortalized cells, regardless of tissues or species from which they are derived. In amphibian cell cultures, there are reports of cells with normal chromosomal constitutions and those with aberrant ones (Wolf and Quimby, 1964; Rafferty, 1969; Freed and Mezger-Freed, 1970; Pudney *et al.*, 1973; Wong and Tweedell, 1974; Handler *et al.*, 1979; Anizet *et al.*, 1981; Ellinger *et al.*, 1983; Kondo and Ide, 1983; Fukui *et al.*, 1992).

Chromosomal constitutions in each of the 3 cell lines in the present study were examined three times at arbitrary points, and it was found that they differed at every examination. The cell morphology of the LAH1 cell line changed from a fibroblast-like type to an epithelial-like type at about 8 years and 4 months after the beginnig of culture, and chromosomal constitutions changed greatly in this apporoximately 8-year period. The majority of the cells (96%) contained diploid number of 26 chromosomes at 8 years and 2 months and karyotypes were normal, but 3 months later the modal number of chromosomes was 28 and structurally aberrant chromosomes were often found in mitotic spreads. It is interesting that a large change in the mean population doubling time occurred concurrently with the change in chromosome constitutions before and after the transformation of cell morphology, though this was the only linked change noted to have occurred throughout the present study. The modal number of chromosomes further changed to 27 at 11 years after the beginning of culture.

The pattern of changes in the chromosomal constitution in the LAH2 cell line was noteworthy. The first examination was carried out immediately after the 3rd crisis. Cells containing 26 chromosomes were most numerous (63.5%) and normal in karyotype, while those containing 25 chromosomes accounted for about 30% of cells and frequently had a small number of structurally aberrant chromosomes. However, 4 years after the first examination, about 90% of cells had diploid number of 26 chromosomes and were normal in karyotype. No structurally aberrant chromosomes were found in almost all cells examined at this time point. This observation showed that an aberrant chromosomal constitution occurring once in a population of cells does not always diversify into

several populations, while cells with normal karyotype survive selectively on some occasions. Chromosomal constitutions were again diversified greatly in 5 years after the second examination. In the LAH3 cell line, the modal number of chromosomes also varied at every examination, and a large number of structually aberrant chromosomes were observed in these cells.

As described above, chromosomal constitutions of the 3 cell lines differed at every examination performed in the course of the culture, but no trend in the pattern of change could be found.

Though the three cell lines show some deviation in chromesome constitution, whether malignant alterations has occurred is unknown, since the injection of the cells into susceptible live animals has not been tried. Also studies on the expression of p53 or telomerase are yet to be carried out. Nevertheless, the behavior of these cells in culture is more like normal, exhibiting a moderate growth to make monolayer, maintaining flattened epithelial cell-like morphology adhering strongly to the substratum, and producing premelanosomes throughout the entire course of the culture.

ACKNOWLEDGEMENTS

The author is especially indebted to Professor Emeritus Midori Nishioka, Hiroshima University, for her encouragement and guidance during the course of this work. He also wishes to express his heartfelt gratitude to Professor Emeritus Masataka Obika, Keio University, and Professor Katsutoshi Yoshizato, Hiroshima University, for their valuable suggestions and critical review of the manuscript. He is also profoundly grateful to Professor Hiroyuki Ide, Tohoku University, for his kindness of giving instruction in the culture method.

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(Received November 6, 2000 / Accepted February 9, 2001)