培養されたチャイニーズ・ハムスターhai細胞からレプリカ培養法による栄養要求性細胞の分離とそれらの特性について

誌名	The Japanese journal of genetics
ISSN	0021504X
著者	鈴木, 文男 堀川, 正克
巻/号	50巻3号
掲載ページ	p. 247-256
発行年月	1975年6月

JAPAN. J. GENETICS Vol. 50, No. 3: 247-256 (1975)

ISOLATION AND CHARACTERIZATION OF NUTRITIONALLY DEFICIENT CELLS FROM CULTURED CHINESE HAMSTER *HAI* CELLS BY A REPLICA PLATING METHOD

FUMIO SUZUKI AND MASAKATSU HORIKAWA

Division of Radiation Biology, Faculty of Pharmaceutical Sciences,
Kanazawa University, Kanazawa 920

Received September 5, 1974

During about two decades since genetic and biochemical studies of single somatic mammalian cells *in vitro* were developed by Puck *et al.* (Puck and Marcus 1955; Puck *et al.* 1956a; Puck and Fisher 1956b), a number of tools and techniques necessary for the genetic analysis of cultured somatic mammalian cells have been developed. Many kinds of mutant cells such as drug-resistant cells (Gartler and Pious 1966; Chu and Malling 1968), nutritional auxotrophs (Kao and Puck 1968, 1969), and temperature-sensitive mutants (Naha 1969; Thompson *et al.* 1970) have been isolated from various mammalian cell lines, and techniques for genetic analysis with somatic cell hybrids have been developed (Barski *et al.* 1960, 1961; Littlefield 1964; Ephrussi and Weiss 1965; Weiss and Ephrussi 1966).

However, the lack of a replica plating method suitable for cultured mammalian cells has delayed more detailed genetic analysis in somatic mammalian cells. In the previous papers (Suzuki *et al.* 1971; Suzuki and Horikawa 1973) we described a technique of a replica plating method for cultured mammalian cells which is a modification of the technique descrided by Goldsby and Zipser (1969) and an example of the application of this technique to detect and isolate the nutritionally deficient variant cells from cultured Chinese hamster *hai* cells. In this paper we shall describe the results of similar experiments in the same Chinese hamster *hai* cells after about a year and the characteristics of the isolated variant cells.

MATERIALS AND METHODS

Cells and medium. A Chinese hamster *hai* cell line derived from the lung of a newborn male Chinese hamster was kindly supplied by Dr. I. Yamane of Tohoku University, Sendai, Japan. This cell line was maintained originally in his laboratory in a medium composed of 90% Eagle's MEM (supplemented with 0.05% lactalbumin hydrolysate and 10^{-3}M sodium pyruvate) and 10% calf serum (the original medium). After arrival in our laboratory, the cells were grown in a complete medium composed of 90% Eagle's MEM+N18 medium (Suzuki and Horikawa 1973) and 10% dialyzed calf serum (Suzuki and Horikawa 1973). This medium was recently developed in the authors' laboratory for the study of the nutritional requirements of cells. When cultured in a

humid atmosphere of 5% CO_2 and 95% air at 37°C, the average doubling time of the cells in this medium was about 13 hours as determined by cell growth.

For detecting and isolating alanine-, asparagine-, proline-, aspartic acid-, serine-, glycine-, hypoxanthine-, or thymidine-deficient variants (auxotrophic variants) from the original Chinese hamster *hai* cells, various media were prepared, each of which lacked one of the ingredients——L-alanine, L-asparagine, L-proline, L-aspartic acid, L-serine, glycine, hypoxanthine, or thymidine——from the Eagle's MEM+N18 medium (Suzuki and Horikawa 1973), respectively. These media supplemented with 10% dialyzed calf serum (Suzuki and Horikawa 1973) were used as selection media.

Seeding of single Chinese hamster hai cells in a master plate and preparation of replica plating cultures. MicroTest II tissue culture plates were employed as a master plate for single Chinese hamster *hai* cell cultures as well as for replica plates for replica plating cultures, as previously described (Suzuki *et al.* 1971; Suzuki and Horikawa 1973). Seeding of single Chinese hamster *hai* cells in a master plate and preparation of replica plating cultures were carried out as described in the previous paper (Suzuki and Horikawa 1973).

Aliquots of 0.2 ml cell suspension containing 8 cells/ml in the complete medium were added to each of 96 wells of a MicroTest II tissue culture plate. The culture medium in wells which received no cells was sucked up by a syringe and 0.2 ml of cell suspension was added again. In this way it is possible to seed a single cell into 38 to 42 of the 96 wells of a plate. The master plate was incubated at 37°C for 13 days in a humidified CO_2 incubator, and 28 to 38 clones originating from single cells were obtained.

The culture medium in the wells was discarded and 0.015 ml of warmed 0.25% trypsin and 0.25% EDTA solution was added to each well to detach the monolayer cells from the flat bottom. Two to three minutes after the addition of trypsin and EDTA solution, 0.2 ml of Eagle's MEM alone was added to each well, and the cells in the wells were dispersed and transferred, by a hand replicator, to 17 replica plates devoid of culture medium. Then 0.2 ml of the complete medium was added to 28–38 wells of the master plate containing cell parts of cell suspension which had not been transferred and to a replica plate containing transferred cells, and the plates were incubated at 37°C for 10 days in a humidified CO_2 incubator. In addition, 0.2 ml of various selection media lacking L-alanine, L-asparagine, L-proline, L-aspartic acid, L-serine, glycine, hypoxanthine, or thymidine from the complete medium, was added to 28–38 wells of each of two plates of the remaining 16 replica plates, respectively, and the plates were incubated at 37°C for 10 days in a humidified CO_2 incubator. After incubation, the growth of clones transferred to identical positions (wells) on the replica plates from a master plate was determined under an inverted microscope.

Examination of the stability of nutritional requirements of variant cells isolated. In order to examine the stability of a nutritionally deficient clone isolated from the original Chinese hamster *hai* cells by the procedure mentioned above, 500 or 5×10^4 cells were inoculated into 60- or 90-mm glass petri dishes containing 5 or 10 ml of each selection medium supplemented with 10% dialyzed calf serum (Suzuki and Horikawa

1973). After incubation at 37°C in a humidified 5% CO_2 incubator for 10 days, the number of colonies per dish was determined.

Analysis of chromosome number and karyotype in the original and variant Chinese hamster hai cells. Cells in the logarithmic phase of growth were used for chromosome analysis. The monolayer cells in 200 ml square culture bottles were pretreated for 5 hours with colcemid at a final concentration of 0.025 μg/ml and then collected by centrifugation at 1500 rpm for 10 min. After this, cell preparations for chromosome study were obtained by an air-dry method and Giemsa staining and by a slight modification of the Giemsa banding technique of Seabright (1971) as well.

RESULTS

Detection and isolation of the nutritionally deficient variant cells from the original Chinese hamster hai cells by a replica plating method. The results of the first survey (Suzuki and Horikawa 1973), performed about one month after the original cells were transferred to the complete medium composed of 90% Eagle's MEM+N18 medium and 10% dialyzed calf serum, are shown in Table 1: 38 clones transferred to identical positions (wells) on the replica plates from a master plate from a master plate can be divided into seven classes, according to their nutritional requirements. Clones 17, 18, 20, 25, 26, 28, 37, and 38, which belong to Class 1, were alanine-, asparagine-, proline-,

Table 1. Properties of 38 clones classified into 7 classes according to nutritional requirements in the first survey

Class	Clone no.	Required substances						
1	17, 18, 20, 25, 26, 28, 37, 38							
2	36		Pro					
	6, 29, 33					Gly		
	10, 11, 27							TdR*
3	2, 3, 4, 16, 22, 31					Gly		TdR
	5, 12	Asn				Gly		
	1			Asp		Gly		
	8		Pro		Ser			
4	23, 30, 32				Ser	Gly		TdR
	19	Asn				Gly		TdR
	24		Pro			Gly		TdR
5	21	Asn		Asp	Ser			TdR
	13	Asn			Ser	Gly		TdR
	7, 35		Pro	Asp		Gly		TdR
	9		Pro		Ser	Gly		TdR
	34			Asp	Ser	Gly		TdR
6	15		Pro	Asp	Ser	Gly	Hyp	TdR
7	14	Asn	Pro	Asp	Ser	Gly	Hyp	TdR

* Thymidine

aspartic acid-, serine-, glycine-, hypoxanthine-, and thymidine-sufficient clones, which did not require any of these eight nutritional substances for their growth (prototrophic clones). On the other hand, clones 36, 6, 29, 33, 10, 11, and 27 (Class 2) were proline-, glycine-, or thymidine-deficient clones which required proline, glycine, or thymidine for their growth (one-substance-auxotropic clones). Clones 2, 3, 4, 16, 22, 31, 5, 12, 1, and 8 (Class 3) were two-substance-deficient clones which required any two among asparagine, proline, aspartic acid, serine, glycine, and thymidine (two-substance-auxotrophic clones). Clones 23, 30, 32, 19, and 24 (Class 4) were three-substance-deficient clones which required any three among asparagine, proline, serine, glycine, and thymidine (three-substance-auxotropic clones). Clones 21, 13, 7, 35, 9, and 34 (Class 5) were four-substance-deficient clones which required any four among asparagine, proline, aspartic acid, serine, glycine, and thymidine (four-substance-auxotrophic clones). Finally, clone 15 (Class 6) was proline-, aspartic acid-, serine-, glycine-, hypoxanthine-, and thymidine-deficient (six-substance-auxotrophic clone), and clone 14 (Class 7) was asparagine-, proline-, asparatic acid-, serine-, glycine-, hypoxanthine-, and thymidine-deficient (seven-substance-auxotrophic clone). On the basis of these results it can be concluded that the majority (about 58-63%) of the 38 clones obtained from the Chinese hamster *hai* cells grown in the complete medium for about a month after having been supplied by Dr. I. Yamane was glycine- and thymidine-auxotrophic clones, and that only two clones were hypoxanthine-auxotrophic clones which required hypoxanthine for their growth.

The second survey was performed about one year after the cells had been transferred to the complete medium from the original medium. However, at this time, three independent experiments were repeated simultaneously to detect and lsolate the nutritionally deficient clones from 28, 33 and 32 clones obtained from three master plates. Similar results were obtained from these three experiments. Two results among the three are shown in Tables 2 and 3. The properties of 28 and 33 clones in each replica plate of the two experiments, cultured with the complete medium or the various selection media for 10 days, are shown in Tables 2 and 3, respectively, according to the

Table 2. Properties of 28 clones classified into 6 classes according to nutritional requirements in the second survey

Class	Clone no.	Required substances
1	6, 7, 28	
2	1, 2, 3, 4, 5, 9, 10 11, 12, 13, 14, 16, 20, 21 27	TdR*
3	8, 15, 22, 23, 24	Gly TdR
4	17	Asn Ser TdR
5	18	Asn Ser Gly TdR
6	25, 26	Asn Pro Asp Ser TdR
	19	Asn Pro Ser Gly TdR

* Thymidine

Table 3. Properties of 33 clones classified into 7 classes according to nutritional requirements in the second survey

Class	Clone no.	Required substances
1	19, 23	
2	1, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 18, 20, 21, 22, 24, 27, 28, 31, 32	TdR*
3	2, 4, 13, 25, 26, 29	Gly TdR
4	30	Pro Asp TdR
5	33	Asn Pro Asp Ser TdR
6	3	Ala Asn Pro Ser Gly TdR
7	17	Ala Asn Pro Asp Ser Gly TdR

* Thymidine

same classification as in Table 1.

As seen in these tables, the results of the two experiments show almost the same tendency. The 28 and 33 clones obtained from the two experiments can be divided into six and seven classes, respectively, according to their nutritional requirements. However, these results are different from those of the first survey shown in Table 1. The numbers of prototrophic clones (Class 1) which did not require any of eight nutritional substances (alanine, asparagine, proline, aspartic acid, serine, glycine, hypoxanthine and thymidine) for their growth, and of auxotropic clones which required several or all substances among asparagine, proline, aspartic acid, serine, and glycine for their growth, decreased in the second survey. On the contrary, all auxotrophic clones obtained in the second survey were thymidine-deficient. Furthermore, hypoxanthine-deficient clones obtained in the first survey could not be obtained in the second survey, while two alanine-deficient clones were obtained in the second survey.

The stability of the nutritionally deficient cells isolated. Clone 23 (in Table 1) obtaind in the first survey was isolated as a prototrophic clone (Ala^+, Asn^+, Pro^+, Asp^+, Hyp^+) for studying forward mutation in cultured mammalian cells in the future. This cell line was stable and grew in a medium which lacked alanine, asparagine, proline, aspartic acid, and hypoxanthine for about two years after isolation. On the other hand, clone 3 (in Table 3), obtained in the second survey was isolated as an auxotropic clone (Ala^-, Asn^-, Pro^-, Ser^-, Gly^-, TdR^-) for studying reverse mutation. Before studying the reverse mutation, we examined the stability of this nutritionally deficient clone. At various intervals after isolation, the nutritional requirements of the cells were studied for L-alanine, L-asparagine, L-proline, L-serine, glycine, and thymidine. Table 4 shows the results of these experiments. As seen in this table, the colony-forming ability (the number of colonies per dish) of clone 3 cells a short time after isolation, in a selection medium which lacked any one of L-alanine, L-asparagine, L-proline, L-serine, and glycine, was very low. However, as the cells were maintained longer in

Table 4. The stability of a nutritionally deficient variant (clone 3[1]) isolated from the original Chinese hamster *hai* cells by a replica plating method

Date of examination	Number of colonies per dish[2]						
	Complete medium	Substance omitted from complete medium					
		Ala	Asn	Pro	Ser	Gly	TdR[3]
June 12, '73	164.5[4] (100)[5]	4.0 (2.43)	0.0 (0.00)	2.0 (1.22)	0.0 (0.00)	3.5 (2.13)	0.0 (0.00)
July 19, '73	241.0 (100)	44.0 (18.3)	0.0 (0.00)	0.0 (0.00)	22.0 (9.13)	1.0 (0.41)	0.0 (0.00)
August 4, '73	259.0 (100)	49.0 (18.9)	8.5 (3.28)	64.0 (24.7)	18.3 (7.07)	0.0 (0.00)	0.0 (0.00)

1) Isolated on April 12, 1973 and then cultured in the complete medium.
2) 500 cells were inoculated into each dish.
3) Thymidine
4) Each number shows the mean of the three dishes.
5) Each number in parentheses shows the percentages of the plating efficiency in each selection medium when the plating efficiency in the complete medium is defined as 100.

Table 5. Colony-forming ability of clone 3 cell[1] in a medium which lacked thymidine from the complete medium and their reverse mutation frequency

Date of examination	Number of colonies per dish[2]								Mutation frequency ($\times 10^{-6}$)
August 16, '73	0	0	0	0	1	0	0	1	5.0
August 17, '73	1	0	0	1	0	0	0	0	5.0
August 20, '73	0	0	0	1	0	0	0	0	2.5
August 23, '73	2	1	0	3	0	0	0	1	17.5
October 5, '73	0	0	0	0	0	0	0	0	0.0
November 11, '73	0	0	0	0					0.0
November 20, '73	0	0	0	1	0	0	0	0	2.5
November 25, '73	0	0	0	0	1	0	0	0	2.5
November 28, '73	0	0	0	0	0	0	0		0.0
December 2, '73	0	0	1	0	0	1			6.7
December 10, '73	0	3	0	0	0	1	0	0	10.0
								Mean	4.7

1) Isolated on April 12, 1973 and then cultured in the complete medium.
2) 5×10^4 cells were inoculated into each dish.

the complete medium, the colony-forming ability in these selection media gradually increased. On the other hand, the requirement of the cells for thymidine was very stable. Even about four months after isolation, we could not observe any colony in three dishes when 500 cells were inoculated into a dish containing a thymidine-less medium. In order to confirm this further, 5×10^4 cells were inoculated into 90-mm glass petri dishes containing 10 ml of a selection medium which lacked thymidine, at various intervals thereafter. After incubation at 37°C in a humidified 5% CO_2 incubator for 10 days, the number of colonies per dish was determined. Table 5 shows the results

of these experiments. As can be seen in this table, the colony-forming ability (the number of colonies per dish) of clone 3 cells in a medium which lacked thymidine was very low, and the requirement of the cells for thymidine was relatively stable even about eight months after isolation.

Karyotype of the original and variant Chinese hamster hai cells. Karyotypes of a prototrophic clone (clone 23) and an auxotrophic clone (clone 3) were investigated 9 or 10 months after their isolation. The modal chromosome number of the original Chinese hamster *hai* cells, clone 23 cells and clone 3 cells, analyzed by an air-dry method and Giemsa staining, was the same, 26. Similar patterns in the distribution of chromosome numbers were observed in these three cell lines. Furthermore, we could not find any significant differences in karyotypes among the three cell lines, as analyzed by a slight modification of the Giemsa banding technique of Seabright (1971).

On the other hand, there were also no significant differences in the average doubling times among these three cell lines The average doubling times of the original Chinese hamster *hai* cells and an auxotrophic clone (clone 3) in the complete medium at 37°C were 13 and 13.2 hours as determined by cell growth, whereas that of a prototrophic clone (clone 23) in a medium which lacked alanine, asparagine, proline, aspartic acid, and hypoxanthine from the complete medium was 14 hours.

DISCUSSION

In this paper we have reported the results of experiments designed to survey the number of nutritionally deficient cells from cultured Chinese hamster *hai* cells by our replica plating method (Suzuki *et al.* 1971; Suzuki and Horikawa 1973) and have described the characteristics of the isolated variant cells. This cell line was maintained originally in a medium composed of 90% Eagle's MEM (supplemented with 0.05% lactalbumin hydrolysate and 10^{-3}M sodium pyruvate) and 10% calf serum, at Tohoku University. After reaching our laboratory, the cells were grown in the complete medium composed of 90% Eagle's MEM+N18 medium (Suzuki and Horikawa 1973) and 10% dialyzed calf serum (Suzuki and Horikawa 1973). The surveys were performed about one month and again one year after the cells were transferred to the complete medium from the original culture medium. It is interesting that a very high incidence of various types of nutritionally deficient cells was observed in a cell line, so far detected by a replica plating method, although the factors inducing these phenotypic characteristics remain unclear.

Among the cell clones obtained, clone 23 (Table 1) and clone 3 (Table 3) obtained in the first and second survey, which was performed about one month and one year after the cells were transferred to the complete medium from the original medium, were isolated as a prototrophic and an auxotrophic clone. The prototrophic characteristics (Ala^+, Asn^+, Pro^+, Asp^+, Hyp^+) of clone 23 cells were stable. After isolation, this cell line was maintained in a medium which lacked alanine, asparagine, proline, aspartic acid, and hypoxanthine for about two years. We examined them by a modification of the 5-bromodeoxyuridine-visible light method described by Puck and Kao (1967)

about two years after isolation, and could not find any spontaneous auxotrophic cells requiring any one of alanine, asparagine, proline, aspartic acid, or hypoxanthine for their growth, in at least 5.1×10^6 cells of clone 23 (Details of this study will be published elsewhere).

On the other hand, the majority of the requirements (Ala^-, Asn^-, Pro^-, Ser^-, Gly^-) among the auxotrophic characteristics (Ala^-, Asn^-, Pro^-, Ser^-, Gly^-, TdR^-) of clone 3 cells were unstable, as shown in Table 4. Only the thymidine-deficient characteristic of this clone was stable. Thus the surviving cells in the deficient medium may be assumed to be revertants. The mean reverse mutation frequency for this characteristic is calculated to be 4.7×10^{-6} (Table 5). It is not yet clear why the alanine-, asparagine-, proline-, serline, and glycine-deficient nature of clone 3 cells tends to disappear during the short period of cultivation after isolation. These results resemble those of experiments which were recently performed by Kuroki (personal communication). He isolated seven ultraviolet-sensitive clones from cultured mouse cell lines, FM3A and L5178Y, by the Lederberg replica culture method (Kuroki 1973). However, he found that these UV-sentitive clones also tend to lose their UV-sensitivity as culture progress, in terms of increasing Do values or increasing in the shoulder of the survival curve. The instability of apparently genetic characteristics in mammalian cells indicate that epigenetic changes may result in the acquisition or lack of some enzyme system for a definite period of time as expected from a genetic change. By the replica plating method the cells having apparently genetic characteristics altered by epigenetic as well as genetic changes can be well detected equally. Of course, it is still unknown what is the nature of epigenetic changes in mammalian cells. However, it can be supposed that such an epigenetic change may play an important role in the process of differentiation of mammalian cells.

Furthermore, no significant differences were observed in the distribution of chromosome numbers and in karyotypes among the original Chinese hamster *hai* cells, prototrophic cells (clone 23) and auxotrophic cells (clone 3). These results indicate that the thymidine-deficient nature of clone 3 cells may be due to gene mutation.

On the other hand, there were some differences in the nutritional requirement of various clones between the two surveys, which were performed about eleven months apart. These may be due to adaptation or selection of cells with various clonal characteristics or further epigenetic and genetic changes induced during their maintenance in the complete medium. Since all the auxotrophic clones obtained one year after the cells were transferred to the complete medium were thymidine-deficient (Tables 2 and 3), and the thymidine requirement was shown to be rather stable (Table 5), thymidine deficient mutants induced during maintenance may accumulate. Auxotrophic characteristics, except the thymidine requirement, may be epigenetically controlled. The mechanism of the induction of epigenetic changes remains unclear. But it is speculated from the present findings that the changes of the culture medium may result in induction of opigenetic changes, and after prolonged maintenance of cells in the same medium, the cells may adapt to the medium and the rate of induction may be decreased. In any case such a survey of cell population by a replica plating method may be useful to establish a model for cell population genetics which may be important for the study

of carcinogenesis and aging in mammals. In addition, studies of forward mutation and reverse mutation, using a prototrophic clone (Ala^+, Asn^+, Pro^+, Asp^+, Hyp^+) and an auxotrophic mutant clone (TdR^-) obtained from the original Chinese hamster *hai* cells in the present studies, are now in progress.

SUMMARY

Nutritionally deficient cells were detected and isolated from cultures of Chinese hamster *hai* cells by our replica plating method. The nutritionally sufficient characteristics (Ala^+, Asn^+, Pro^+, Asp^+, Hyp^+) of a prototrophic clone isolated in the first survey, which was performed about one month after the cells were transferred to the complete medium from the original medium, were stable. On the other hand, the majority among the nutritionally deficient characteristics (Ala^-, Asn^-, Pro^-, Ser^-, Gly^-, TdR^-) of an auxotrophic clone isolated in the second survey, which was performed about one year after the cells were transferred to the complete medium, were unstable. Only the thymidine-deficient characteristics of this clone were stable, and the mean reverse mutation frequency of this characteristic was 4.7×10^{-6}. These results indicate that epigenetic as well as genetic changes may be related to the changes in nutritional requirement of mammalian cells and that our replica plating method might equally detect the cells having apparently genetic characteristics altered by epigenetic as well as genetic changes.

ACKNOWLEDGMENTS

The authors wish to thank Dr. I. Yamane, Tohoku University, for his kind gift of Chinese hamster *hai* cells, and Dr. T. Sugahara, Kyoto University, for his many helpful discussions and reading of the manuscript. This study was supported financially by grant for Cancer Research from the Japanese Ministry of Education (No. 801019).

LITERATURE CITED

Barski, G., S. Sorieul, and F. Cornefert, 1960 Production dans des cultures *in vitro* de deux souches cellulaires en association, de cellules de caractère "hybride". Compt. Rend. Acad. Sci. **251**: 1825-1827.

Barski, G., S. Sorieul, and F. Cornefert, 1961 "Hybrid" type cells in combined cultures of two different mammalian cell strains. J. Natl. Cancer Inst. **26**: 1269-1291.

Chu, E. H. Y., and H. V. Malling, 1968 Mammalian cell genetics, II. Chemical induction of specific locus mutations in Chinese hamster cells *in vitro*. Proc. Natl. Acad. Sci. U. S. **61**: 1306-1312.

Ephrussi, B., and M. C. Weiss, 1965 Interspecific hybridization of somatic cells. Proc. Natl. Acad. Sci. U. S. **53**: 1040-1042.

Gartler, S. M., and D. A. Pious, 1966 Genetics of mammalian cell cultures. Humangenetik **2**: 83-114.

Goldsby, R. A., and E. Zipser, 1969 The isolation and replica plating of mammalian cell clones. Exptl. Cell Res. **54**: 271-275.

Kao, F. T., and T. T. Puck, 1968 Genetics of somatic mammalian cells, VII. Induction and isolation of nutritional mutants in Chinese hamster cells. Proc. Natl. Acad. Sci. U. S. **60**: 1275-1281.

Kao, F. T., and T. T. Puck, 1969 Genetics of somatic mammalian cells, IX. Quantitation of mutagenesis by physical and chemical agents. J. Cell Physiol. **74**: 245-258.

Kuroki, T., 1973 Colony formation of mammalian cells on agar plates and its application to Lederberg's replica plating. Exptl. Cell Res. **80**: 55-62.

Littlefield, J. W., 1964 Selection of hybrids from mating of fibroblansts *in vitro* and their presumed recombinants. Science **145**: 709-710.

Naha, P. M., 1969 Temperature sensitive conditional mutants of monkey kidney cells. Nature **223**: 1380-1381.

Puck, T. T., and P. I. Marcus, 1955 A rapid method for viable cell titration and clone production with Hela cells in tissue culture: The use of X-irradiated cells to supply conditioning factors. Proc. Natl. Acad. Sci. U. S. **41**: 432-437.

Puck, T. T., P. I. Marcus, and S. J. Cieciura, 1956a Clonal growth of mammalian cells *in vitro*: Growth characteristics of clones from single Hela cells with and without a "feeder" layer. J. Exptl. Med. **103**: 273-284.

Puck, T. T., and H. W. Fisher, 1956b Genetics of somatic mammalian cells, I. Demonstration of the existence of mutants with different growth requirements in a human cancer cell strain (HeLa). J. Exptl. Med. **104**: 427-434.

Puck, T. T., and F. T. Kao, 1967 Genetics of somatic mammlian cells, V. Treatment with 5-bromodeoxyuridine and visible light for isolation of nutritionally deficient mutants. Proc. Natl. Acad. Sci. U. S. **58**: 1227-1234.

Seabright, M., 1971 A rapid banding technique for human chromosomes. Lancet **II**: 971-972.

Suzuki, F., M. Kashimoto, and M. Horikawa, 1971 A replica plating method of cultured mammalian cells for somatic cell genetics. Exptl. Cell Res. **68**: 476-479.

Suzuki, F., and M. Horikawa, 1973 A replica plating method of cultured mammalian cells. In Method in Cell Biology (D. M. Prescott, ed.) Vol. VI, pp. 127-142, Academic Press, New York and London.

Thompson, L. H., R. Mankovitz, R. M. Baker, J. E. Till, L. Siminovitch, and G. F. Whitemore, 1970 Isolation of temperature-sensitive mutants of L-cells. Proc. Natl. Acad. Sci. U. S. **66**: 377-384.

Weiss, M. C., and B. Ephrussi, 1966 Studies of interspecific (rat×mouse) somatic hybrids. I. Isolation, growth, and evolution of the karyotype. Genetics **54**: 1095-1108.