Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 2302–2306, April 1987
Cell Biology

Three clonal types of keratinocyte with different capacities for multiplication

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Contributed by Howard Green, December 12, 1986

ABSTRACT **Colony-forming human epidermal cells are heterogeneous in their capacity for sustained growth. Once a clone has been derived from a single cell, its growth potential can be estimated from the colony types resulting from a single plating, and the clone can be assigned to one of three classes. The holoclone has the greatest reproductive capacity: under standard conditions, fewer than 5% of the colonies formed by the cells of a holoclone abort and terminally differentiate. The paraclone contains exclusively cells with a short replicative lifespan (not more than 15 cell generations), after which they uniformly abort and terminally differentiate. The third type of clone, the meroclone, contains a mixture of cells of different growth potential and is a transitional stage between the holoclone and the paraclone. The incidence of the different clonal types is affected by aging, since cells originating from the epidermis of older donors give rise to a lower proportion of holoclones and a higher proportion of paraclones.**

The epidermis is a stratified squamous epithelium whose differentiated cells are the progeny of proliferative cells located mainly in the basal cell layer. Although many of the basal cells are capable of multiplication (1), few of them are thought to be self-renewing stem cells (2–4).

We have recently shown that the clone-forming ability of a human keratinocyte in culture can be estimated from its size: small keratinocytes give rise to clones with high frequency, larger ones do so with lower frequency, and still larger ones, not at all. But once a colony has formed, its growth potential is not specified by the size of the founding cell (5).

We describe here a method of analysis that reveals the growth potential of individual clones. We inoculate a single founding cell, and 7 days later we transfer the progeny, while they are still growing exponentially, to indicator dishes, where they are allowed to grow for a further period of 12 days. According to the growth in the indicator dishes, we can classify the original clone. Holoclones (*holo* = entire) form large rapidly growing colonies; fewer than 5% of the colonies abort and terminally differentiate. Paraclones (*para* = beyond) are programmed for limited growth and consequently form uniformly small, terminal colonies on the indicator dishes. Meroclones (*mero* = partial) form two kinds of colonies on the indicator dishes—growing and terminal. The meroclone therefore contains a proportion of cells that have degraded to paraclone-formers.

MATERIALS AND METHODS

Cell Culture. Human epidermal keratinocytes were cultivated as previously described (6), using lethally irradiated supporting 3T3 cells (7). The epidermal growth factor used to promote multiplication (8) was the cloned human polypeptide kindly provided by Chiron (Emeryville, CA) (9). All experiments were carried out with a single batch of serum tested for its ability to support colony formation. The medium was changed every 4 days. Strains AY and YF 19 were derived from foreskin, strain GMA from thigh, and strain BW from breast.

Single Cell Inoculation and the Scoring of Clonal Types. Primary cultures were derived from epidermis and stored frozen in liquid N_2. Single cells were isolated from subconfluent secondary cultures prepared from the frozen stock, and each cell was inoculated into a 35-mm Petri dish (5). Seven days later, each resulting colony was photographed under a Zeiss 1M35 inverted photo microscope, using either a ×6.3 or a ×10 objective. The area of the colonies was measured on prints by using an image analyzing system (Microplan II, Nikon) and corrected for microscopic and photographic enlargement. Each clone was transferred by trypsinization to two indicator dishes. Twelve days later, these cultures were fixed and stained for classification of clonal type.

Measurement of Colony Area and Determination of Cell Number. Microscopic colonies were stained with 1 μM Hoechst nuclear dye 33342 for 30 min at 37°C (10). They were then fixed with 3.7% (vol/vol) buffered formaldehyde. The nuclei of small colonies were counted directly under a Zeiss microscope equipped for epifluorescence, using a ×25 water-immersion objective. Keratinocyte nuclei were identified by their homogeneous fluorescent pattern characteristic of human cells, in contrast to the spotted pattern of (mouse) 3T3 cells. When a colony contained too many nuclei for direct counting, it was photographed with 3000 ASA high-speed Polaroid film and the nuclei were counted directly on the print. Where the colony was very large, composite photographs were made. In order to measure the area of a microscopic colony, it was stained with 1% rhodamine B and magnified with a ×2.5 or ×6.3 objective, and the image was projected through a Zeiss monocular tube onto the digitizing tablet.

The area of a macroscopic colony was outlined on the bottom of the culture dish. After careful removal of the supporting 3T3 cells by forcefully pipetting the medium over the cells, the colony was trypsinized, and the cells were counted in a hemacytometer. The outlined area was then measured directly on the digitizing tablet.

Immunoperoxidase Staining for the Detection of Involucrin. Cultures were stained by the peroxidase–anti-peroxidase technique (11, 12), using the immunoglobulin fraction (13) of a rabbit antiserum to involucrin (14).

RESULTS

Morphology of Keratinocyte Colonies. When supported by lethally irradiated 3T3 cells, human keratinocytes can give rise to macroscopic colonies within 6 days after inoculation. By 12 days, three colony types can be recognized:

(i) *Large, with smooth perimeter.* Such a colony is 10–30 mm^2 in area and contains 2–5 × 10^4 cells. Its perimeter is nearly circular (Fig. 1 *A–D*); this is particularly evident as the colony grows larger. It contains mainly small cells, and these

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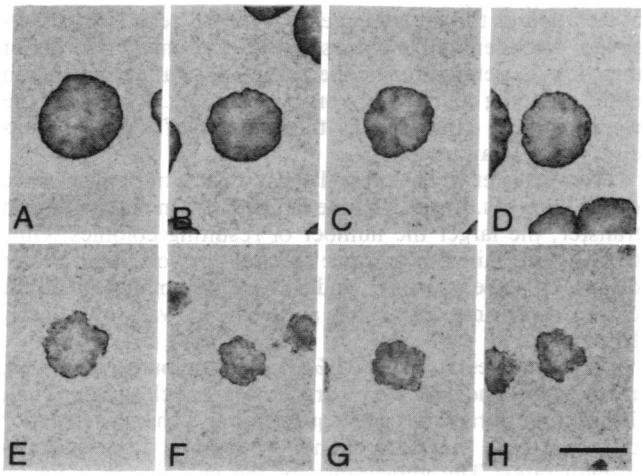

FIG. 1. Macroscopic colony types formed by keratinocytes. Twelve-day colonies formed by strain AY were fixed and stained with rhodamine. Note the smooth perimeter of the colonies in *A–D*, and the wrinkled colonies in *E–H*. The former are typically formed by holoclones and the latter by meroclones. (Bar = 5 mm.)

may be concentrated near the perimeter. The interior of the colony is stratified, the upper terminally differentiating layers consisting of large flattened cells covering the basal layer of small cells. Colonies of this type are typically formed by the cells of a holoclone.

(ii) *Small, highly irregular, and terminal.* The colony area is less than 5 mm^2 and the perimeter of the colony is drawn out into marked irregularities. All proliferation has ceased. The cells are large and flattened (Fig. 2 *C–E*). This is clearest at the perimeter, which consists predominantly of a single layer of such cells. All the cells, including those at the perimeter, contain involucrin, a protein marker characteristic of terminally differentiated cells (14, 15). In contrast to a terminal colony, a growing colony has a perimeter whose small cells do not contain involucrin (Fig. 2 *A* and *B*); as in the epidermis, where it is present only in the outer half of the stratified epithelium, involucrin is confined, in a growing colony, to the suprabasal layers of the central region.

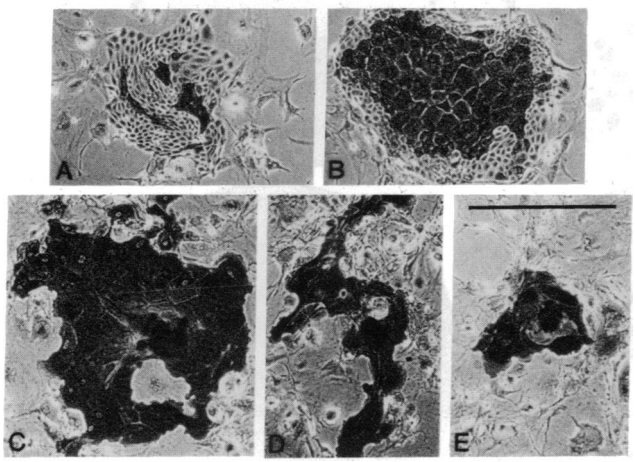

FIG. 2. Involucrin distribution in colonies of different types. Colonies of strain GMA were stained for involucrin. All cells staining black are large and squamelike. In 6-day growing colonies (*A* and *B*), involucrin-containing cells are in the most superficial layer and are either distributed in patches or confined to the central region of the colony. Terminal colonies (12 days old) are highly irregular in outline (*C–E*). Even at the periphery of the colony, the cells are large and flattened and contain involucrin. The large unstained cells lying outside the colonies are the supporting lethally irradiated 3T3 cells. (Bar = 0.5 mm.)

A pool of colonies appearing to be terminal was transferred to fresh dishes and incubated further to test for proliferation. Out of an estimated 10^5 cells transferred in this way, none gave rise to colonies. Terminal colonies are typically formed by the cells of a paraclone.

(iii) *Wrinkled.* This colony type grows progressively to macroscopic size but does not reach the same size as the typical colonies produced by holoclones. It has a wrinkled perimeter (Fig. 1 *E–H*), suggesting some kind of heterogeneity within the colony; as explained below, such a colony will soon become terminal. It is typically formed by meroclones.

Classification of Clonal Types. The distinction among clonal types was based on the frequency of terminal colonies produced when the clone was transferred to two indicator dishes. After fixation and staining with rhodamine at 12 days, the colonies were scored under the low-power microscope.

Table 1. Classification of clones (strain AY)

Original clones at time of transfer (7 days)			Indicator colonies at 12 days			
Clone ident. no.	Area, mm^2	No. of cell divisions	Total no. obtained	Term. diff. colonies, fraction of total	Nature of clone	Mean size of five largest, no. of cell divisions
47	0.388	9.3	682	0.05	M	15.3
12	0.340	9.1	624	0.01	H	15.6
3	0.321	9.0	598	0.02	H	15.4
33	0.293	8.9	426	0.35	M	14.6
36	0.269	8.8	294	0.11	M	15.3
24	0.265	8.7	380	0.01	H	15.6
56	0.256	8.7	552	0.03	H	15.2
54	0.248	8.6	342	0.35	M	14.9
23	0.245	8.6	186	1.0	P	8.8
44	0.235	8.6	480	0.02	H	15.5
57	0.229	8.5	388	0.10	M	15.9
21	0.219	8.5	212	0.39	M	14.5
35	0.191	8.3	262	0.14	M	16.0
8	0.191	8.3	312	0.85	M	14.4
50	0.191	8.3	214	0.51	M	14.5
1	0.183	8.2	226	0.04	H	15.5
11	0.179	8.2	286	0.18	M	15.5
39	0.173	8.1	206	0.07	M	15.7
66	0.166	8.0	162	1.0	P	9.4
45	0.164	8.0	224	0.26	M	15.0
25	0.163	8.0	358	0.03	H	15.5
52	0.159	8.0	284	0.01	H	15.6
27	0.158	8.0	228	0.42	M	14.7
59	0.158	8.0	300	0.12	M	15.2
26	0.152	7.9	196	0.22	M	15.0
49	0.151	7.9	170	0.38	M	14.7
38	0.144	7.8	234	0.06	M	15.2
34	0.143	7.8	222	0.13	M	15.4
46	0.130	7.7	106	0.46	M	14.8
40	0.129	7.7	46	0	H	15.5
62	0.129	7.7	232	0.04	H	15.6
42	0.113	7.5	102	0.33	M	15.4
31	0.102	7.4	120	1.0	P	9.4
61	0.078	7.0	18	1.0	P	9.3
9	0.073	6.9	28	1.0	P	8.5
58	0.048	6.3	0	–	P	–
43	0.043	6.1	28	1.0	P	7.5
63	0.029	5.5	0	–	P	–
60	0.025	5.3	0	–	P	–

Term. diff., terminally differentiated; H, holoclone; M, meroclone; P, paraclone.

When 0–5% of colonies were terminal, the clone was scored as a holoclone. When no colonies formed or when all colonies formed were terminal, the clone was classified as a paraclone. When more than 5% but less than 100% of the colonies were terminal, the clone was classified as a meroclone.

Determination of Number of Cell Generations from Measurements of Colony Area. In order to determine the number of cells in a colony and, from this value, the number of cell generations since inoculation of the founding cell, standard curves were established to relate cell number to colony area. Single cells were isolated from secondary cultures and reinoculated. At different times, colony areas and cell number were determined. Over a large range of colony size, the number of cells was proportional to colony area and equal to 1800 cells per mm^2. For small growing colonies (less than 1 mm^2) the best visual fit for the relation was at 1600 cells per mm^2; this may be accounted for by the limited stratification in small colonies. Terminally differentiated colonies contain predominantly large cells, and the cell number per unit area was therefore lower. Cell number for colonies of each type was read from the appropriate standard curve.

Frequency of Clonal Types in Different Strains of Keratinocytes. An 8-day secondary culture of strain AY (donor age = 0 years) was trypsinized and the cells were suitably diluted. A series of 66 cells was isolated under the microscope (5), and each cell was transferred to a different 35-mm Petri dish containing 2.2×10^5 irradiated 3T3 cells. On the seventh day, the dishes were scanned and each colony was photographed for later measurement of its area. Each colony was then trypsinized and cells were inoculated into two 100-mm indicator Petri dishes, already containing irradiated 3T3 cells. These dishes were fixed and stained 12 days later.

Table 1 lists each clone, numbered according to the order of isolation of the founding cell. Of the 66 cells isolated, 39 gave rise to identifiable clones. These are ranked in order of their size, as determined by their area 7 days after inoculation of the founding cell, and just prior to transfer. The size of the clones is also expressed as the number of cell doublings during the 7-day period.

After transfer to indicator dishes, most clones again gave rise to colonies. In general, the larger the clone at the time of transfer, the larger the number of resulting colonies. Only three clones failed to produce any colonies on the indicator dishes. Of these, clones 63 and 60 were the smallest of all the clones at the time of transfer, and clone 58 was only slightly larger.

Of the clones studied, 28% were holoclones, 49% were meroclones, and 23% were paraclones. It is clear from Table 1 that some paraclones grow quite rapidly, and classification of clonal type therefore cannot be derived from study of growth rates. The appearance of the colonies in indicator dishes for each of the three clonal types is shown in Fig. 3.

Similar experiments were carried out on cells derived from another newborn and from two elderly donors. The founding cells gave rise to clones that grew about as rapidly in all cases. When these clones were classified it was found that the frequency of the three clonal types when the cells were derived from elderly donors was different from that observed when the cells were derived from newborns: few or none of the clones were holoclones and most were paraclones (Table 2).

Growth Potential of Paraclones and Meroclones. Paraclones: The data on growth potential of the paraclones of two strains are summarized in Table 3. From the number of generations that occurred in the original colony and the mean number of generations that occurred in the indicator plates

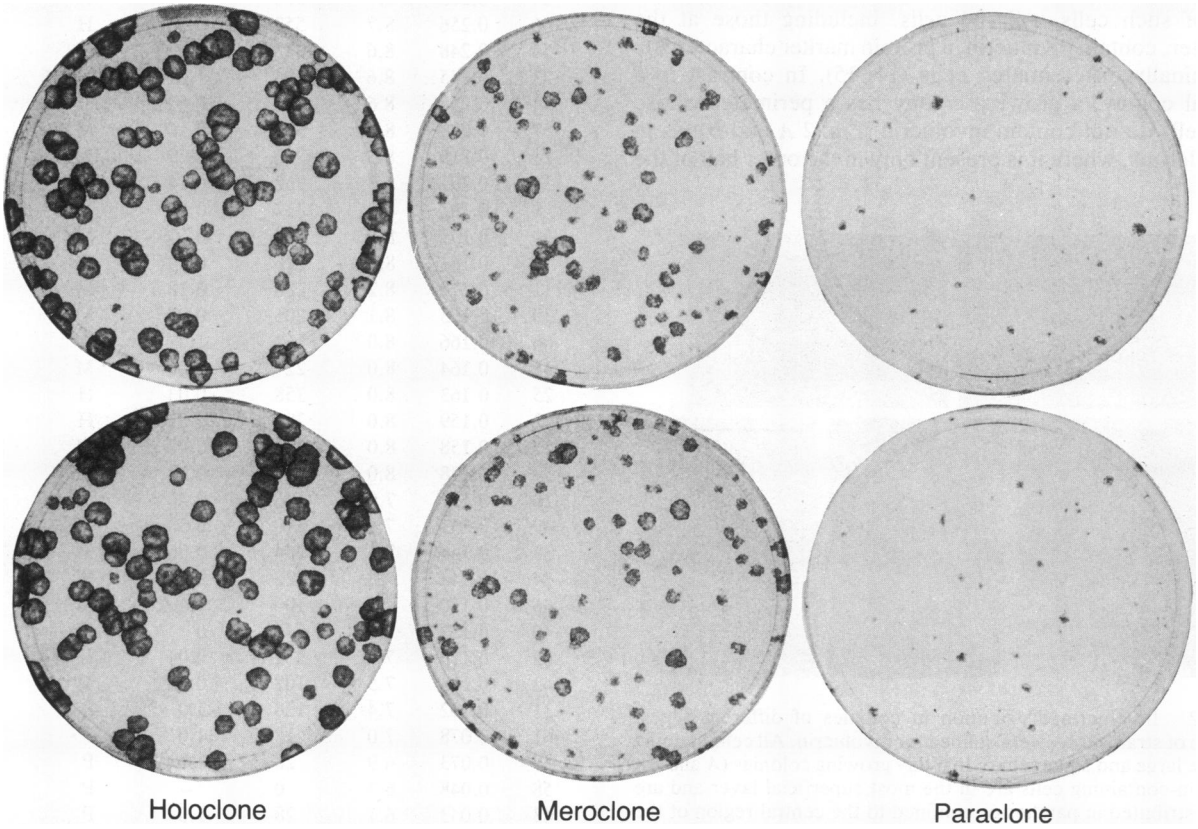

FIG. 3. Colonies produced in indicator dishes by different clonal types of strain AY. Each clone was disaggregated, and one-quarter of the cells was inoculated into each of two indicator dishes containing irradiated 3T3 cells. The cells were allowed to grow for 12 days, when the dishes were fixed and stained with rhodamine. The clones shown are those identified in Table 1 as nos. 44 (holoclone), 33 (meroclone), and 66 (paraclone).

Table 2. Abundance of clonal types in epidermal cultures derived from young and aged donors

Strain	Donor age, yr	Clonal type, % of total clones		
		Holoclones	Meroclones	Paraclones
AY	0	28.2	48.7	23.1
YF19	0	31.0	65.6	3.1
BW	64	3.1	37.5	59.4
GMA	78	0	9.4	90.6

the total lifetime of each paraclone was calculated. It can be seen that the maximal lifetime for paraclones of two strains derived from donors of widely differing ages was about 15 generations.

It might be expected that, depending on how much growth they had undergone prior to their isolation, our analysis would reveal paraclones with measured lifetimes extending over a continuous distribution from the maximum of 15 generations down to 1 generation. Actually, there were some gaps in the distribution of paraclone lifetimes (Table 3). Such gaps might be expected if paraclones that have nearly exhausted their growth potential do not complete the last few generations when trypsinized and transferred.

Table 3. Growth potential of paraclones

Strain	Clone ident. no.	No. of cell generations		
		In original colony	In indicator dishes	Total
AY	9	6.9	7.6	14.5
(0 yr)	43	6.1	6.8	12.9
	66	8.0	7.2	15.2
	23	8.6	6.7	15.3
	61	7.0	7.8	14.8
	31	7.4	7.5	14.9
	58	6.3	0	6.3
	63	5.5	0	5.5
	60	5.3	0	5.3
GMA	72	10.2	5.3	15.5
(78 yr)	92	9.5	5.6	15.1
	74	9.2	6.5	15.7
	25	9.0	4.7	13.7
	21	8.4	5.2	13.6
	23	8.1	5.0	13.1
	45	7.7	7.1	14.8
	68	7.2	5.5	12.7
	14	6.5	4.7	11.2
	48	9.3	0	9.3
	40	8.8	0	8.8
	90	8.7	0	8.7
	1	8.4	0	8.4
	3	8.3	0	8.3
	37	7.9	0	7.9
	84	7.8	0	7.8
	94	7.5	0	7.5
	46	7.5	0	7.5
	60	7.2	0	7.2
	5	6.9	0	6.9
	85	6.8	0	6.8
	76	6.8	0	6.8
	33	6.8	0	6.8
	80	6.6	0	6.6
	6	6.4	0	6.4
	44	6.1	0	6.1
	95	5.9	0	5.9
	18	5.0	0	5.0
	13	4.2	0	4.2

Meroclones: Cultures of three different meroclones of strain GMA were subcultivated every 7 days. At each transfer, one subculture was allowed to grow for 12 days and was then fixed and stained for scoring of clonal types. All three meroclones gradually converted to paraclones. Two meroclones that began with more than half the colonies terminal converted completely to paraclones in two transfers (21 and 23 generations, respectively). The third, which contained fewer than 20% terminal colonies, converted completely to paraclones in four transfers (38 cell generations).

Colonies with wrinkled perimeters (Fig. 1 *E–H*) are very common in the indicator dishes inoculated with meroclones and are absent from those inoculated with holoclones. Wrinkled and smooth colonies were isolated from indicator dishes produced by a meroclone and subcultured. The cells of wrinkled colonies gave rise to few colonies, and these all became terminal while still very small. The wrinkled colonies are therefore subterminal paraclones. In contrast, the cells of smooth colonies isolated from the same dish gave rise to colonies with high efficiency, and these colonies grew progressively. It is concluded that meroclones give rise to paraclones and are consumed by this conversion.

DISCUSSION

The experiments described here reveal three clonal types of multiplying epidermal keratinocytes distinguishable by the marked difference in the frequency with which they give rise to terminal progeny. Holoclones have the greatest growth potential. They are likely to be stem cells, according to the definition of Lajtha (16). In contrast, paraclones may grow rapidly at first, but their total lifetime is no more than 15 cell generations, after which their growth is arrested and the cells are found to contain involucrin, a marker of terminal differentiation.

The meroclone is a clone of mixed composition, as it gives rise to paraclones with appreciable frequency. A paraclone generated early in the growth of an isolated meroclone will exhaust its growth potential in the indicator dishes and give rise only to terminal colonies. A paraclone generated late in the growth of the isolated meroclone will not exhaust its growth potential before the indicator dishes are fixed, and it will give rise to larger colonies with a wrinkled perimeter. The proportion of terminal colonies in the indicator dishes (from 5% to approaching 100%) should depend on the rate of generation of paraclones in the isolated meroclone. As meroclones are eventually consumed by the generation of paraclones, they must be continuously generated from holoclones.

The transitions from holoclone to meroclone to paraclone are unidirectional and result in progressively restricted growth potential. These clonal keratinocyte types may have analogs in other differentiated cell types. For example, the paraclone may correspond to subterminal multiplying cell types in the hematopoietic system (17, 18), the nervous system (19), and muscle (20). Although it does not give rise to multiple differentiated subtypes, the holoclone is the only clonal type comparable in its self-renewing ability to the spleen colony-forming units of the hematopoietic system. When they are grafted to humans, keratinocyte cultures containing holoclones can regenerate epidermis that persists for years (21). Paraclones may be useful over the short term in regenerating epidermis, but they can obviously not contribute to its long-term survival. The relation, if any, between the clonal types we have described and the multiplying cell types defined by thymidine labeling kinetics (22–26) remains to be clarified.

It has been known for some time that fibroblast clones differ in their culture lifetimes (27–29) and that the average

culture lifetime declines with age of donor (27, 30, 31). Intraclonal diversity in growth potential is known to develop within cultured fibroblast clones (32). It has been proposed that the limited culture lifetime of the fibroblast is associated with terminal differentiation (28), but numerous arguments against this interpretation have also been put forward (33).

As in the case of fibroblasts, the culture lifetime of keratinocyte populations declines with age of donor (7, 34). The experiments described indicate that this is due to changes in the abundance of the three clonal types. Determination of the distribution of clonal types in a keratinocyte population therefore gives information on its growth potential much more easily than estimates of culture lifetime by serial subcultivation.

All clone-forming cells studied were isolated from secondary subcultures. This was necessary in order to repeat experiments on comparable materials: primary cultures were preserved by freezing, and clone-forming cells were isolated from the secondary cultures prepared from the frozen stock. Since the clonal composition changes during serial cultivation, as in natural aging, the frequency of holoclones isolated directly from the epidermis should be higher than in cells isolated from secondary cultures, and the frequency of paraclones should be lower. The rate of transition of holoclone to meroclone to paraclone during serial cultivation will likely be affected by the culture conditions, and the scoring of clonal types is likely to provide a sensitive assay for monitoring the comparative efficacy of different culture conditions in maintaining growth potential.

We thank Dr. Nicholas O'Connor for providing us with skin samples for study. These investigations were aided by grants from the National Institute of General Medical Sciences and the National Cancer Institute and by a gift from Johnson and Johnson.

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