Loss of Division Potential in vitro: Aging or Differentiation?

Departure of cells from cycle may not be a sign of aging, but a sign of differentiation.

E. Bell, L. F. Marek, D. S. Levinstone, C. Merrill
S. Sher, I. T. Young, M. Eden

Summary. We have examined the hypothesis that diploid cells grown in vitro age, and propose that only proliferative potential and not life-span is telescoped. We suggest that explanted or transplanted diploid cells are driven to divide by the process of subculturing in vitro or in vivo and, in response to this pressure, also complete their differentiation and become refractory to further mitotic stimulation. We conclude that differentiation rather than "mortality" distinguishes diploid from transformed cells and that the former may not age in vitro, but are lost because culture methods are selective for cycling cells.

The notion that diploid cells age in vitro is based on the observation that they undergo only a limited number of population doublings (*1*). It has been assumed (i) that the life-span of young or even embryonic cells can be telescoped if the cells are pushed to divide by subculturing them at regular intervals, suggesting that cells age as some alloted number of divisions is used up; and (ii) that as cells approach limiting population doubling levels (PDL's) and pass out of cycle they undergo degenerative changes and die. In this article we examine these assumptions and provide evidence for an alternative interpretation—namely, that cessation of proliferation of diploid cells, particularly fibroblasts, in culture represents a step of differentiation and not one of senescence.

The population dynamics of cultures that have a limited life-span have been described as consisting of three phases (*2*): a first phase during which primary cells establish themselves as a confluent sheet; a second in which division of the culture into two parts yields a constant number of cells; and a third during which cell numbers diminish with each succeeding subdivision of the culture.

These three phases, however, which describe population dynamics, do not reflect the states of the cells which populate the culture during each phase.

An Alternative View

1) Our first point is that nondividing "phase 3" cells must be the descendants of many different lineages among which the average number of generations can vary greatly; this, in part, is because of the heterogeneity of interdivision times observed in mass cultures and in clones. Macieira-Coelho *et al.* (*3*) reported variability of interdivision times (IDT's) after labeling mass cultures with [^{3}H]thymidine in short-term experiments and attributed the variability to prolonged G_1 and G_2 periods at high PDL's, but data for individual cells giving the range of variation were not provided. The IDT's for cells of various PDL's are available from the work of Absher and co-workers (*4, 5*). We determined IDT's from cinematographic histories of cells in lineages analyzed by a new interactive computer system and found them to be heterogeneous for cells of all PDL's (Fig. 1) (*6*). We observed similar intraclonal variability of IDT's in human foreskin fibroblasts, whether they were of low or medium PDL's. Cells of a 36th PDL clone, for example, had an average IDT of 42 ± 25 hours. The IDT's of cells of a 15th PDL clone, strain 1519, were also highly variable (Fig. 1).

From the genealogies provided (*4, 5*) and from our own data, we also calculated distributions of the ages of all cells of colonies at the end of 200 hours and found them to be highly variable for both high and low PDL's (Fig. 2). We show that the large variance values for IDT's and age are significant because sister cells have very different numbers of descendants. Other cell lineage data (*4, 5*) as well as our own provide the evidence. Table 1 indicates the number of descendants of each member of daughter, granddaughter, and great granddaughter pairs for several lineages started with founder cells taken at the indicated PDL. It is clear that the population present when the clones were terminated, as well as at other times, consisted of individuals with markedly different cell division histories, some cells being the products of many fewer divisions than others.

2) Our second point is that departure of cells from cycle as well as variability of IDT's is responsible for clone size heterogeneity. Cloning experiments show that cells of any PDL are heterogeneous with respect to proliferative potential. Founder cells taken at any PDL fall into several classes with respect to proliferative potential. The distribution of clone size (cells per clone) of human skin fibroblasts of PDL 8.5 was found by Martin *et al.* (*7*) to be bimodal. Secondary clones generated from the most rapidly proliferating clones after the 22nd PDL and quarternary after the 37th PDL also gave rise to two general classes of clones: small and large. Merz and Ross (*8*) also demonstrate a wide range of sizes for clones of WI-38 cells of the 20th PDL. Similar results were reported by Smith and Hayflick (*9*) who followed clones to the completion of their "life-span" in vitro. Even at relatively low or intermediate PDL's only 50 percent of the founder cells were capable of making clones with more than 2^8 cells.

We analyzed a colony of cells at the 36th PDL in detail and found that after 10.5 days no members of the colony underwent further divisions. The cessation of divisions appears to be unrelated to cell density; that is, contact inhibition seems to play no part in limiting population growth. In fact most cells of small colonies in which divisions are no longer occurring appear to be well separated from each other. The oldest cell in the field had not divided for 16.4 days and the youngest for 8.0 days. The average

E. Bell is professor of biology; L. F. Marek and C. Merrill are in the Department of Biology; T. Young and M. Eden are professors of electrical engineering and computer science; D. S. Levinstone is in the Department of Electrical Engineering and Computer Science; and S. Sher holds appointments in the departments of Biology and Electrical Engineering and Computer Science, Massachusetts Institute of Technology, Cambridge 02139.

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age (out of cycle time) was 12.8 ± 1.7 days. We observed similar departures of cells from cycle in clones arising from low PDL founder cells. The range of interdivision times was 17.5 hours to 5.5 days. This suggests that the variability of clone size is due to two factors: (i) departure of cells from cycle and (ii) heterogeneity of interdivision times. Moreover, it suggests that no rigid causal nexus exists between cell divisions and aging—that is, cells are not required to pass through a fixed number of divisions before leaving cycle permanently.

3) Our third point is that heterogeneity of features such as size and motility is also characteristic of diploid cells in culture. We examined the fates of individual cells in lineages for periods up to 3 weeks and determined that cells derived from founders of low as well as high PDL's can leave cycle at some time after division and remain out of cycle. In a 36th PDL lineage, all of whose cells in the field eventually fell out of cycle, we compared features of cells that left cycle with those of cells that were cycling. Cells in cycle remained much smaller than most of those that left cycle, many of which became giant cells (Fig. 3). However, a few very large cells were seen to divide (Fig. 3). Similar results have been reported by others (*5*). Only a small difference (15 percent) in cell velocity (corrected for the time during which no translocation occurs) was observed between cells which were in cycle and those which were not (Fig. 4). On the other hand, a large difference was observed in stationary time (Fig. 5). Cells that left cycle were stationary on the average nearly 50 percent of the time, whereas cycling cells spent only 12 percent of their time in a stationary mode. So in addition to cell cycle heterogeneity, differences of size and motility exist within the clonal population.

4) Our fourth point is that "phase 3"

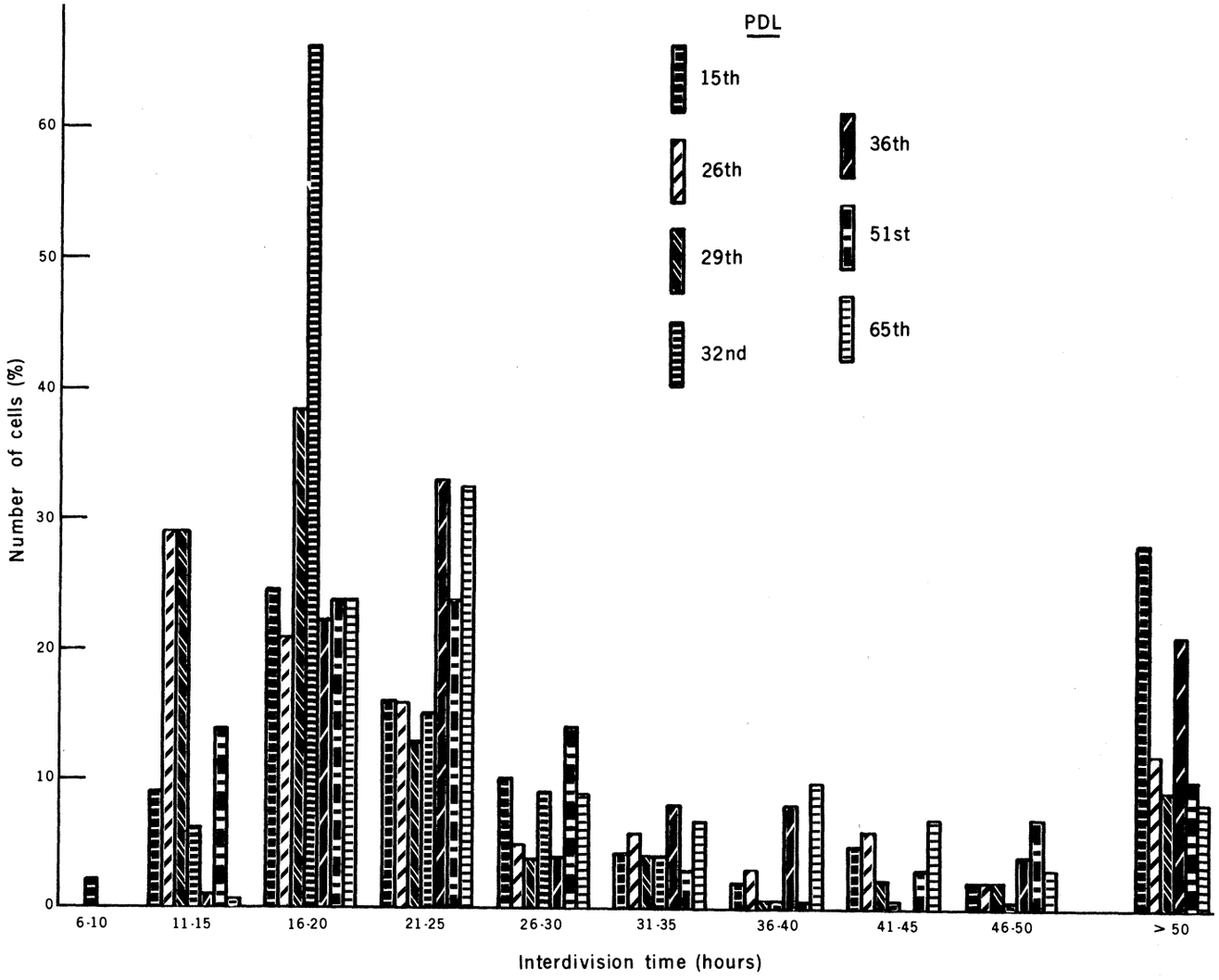

Fig. 1. The heterogeneity of interdivision times (IDT's) of human diploid fibroblasts in clones derived from founder cells of varying population doubling levels (PDL's) is presented as a bar graph. The distributions of IDT's from clones of cells of the 26th, 29th, 32nd, 51st, and 65th PDL's were derived from published clonal geneologies of WI-38 cells (*4*, *5*). The 15th and 36th PDL distributions were derived from clones of neonatal foreskin fibroblasts (strain 1519) (*36*). Nonconfluent cultures at varying PDL's are shaken to remove dividing cells that have detached from the substrate. Approximately 50 cells are transferred to each Lux tissue culture dish (60 mm). After the cells have settled and attached (2 to 24 hours), the old medium is replaced (McCoy's 5A with 20 percent fetal calf serum). The Lux top is replaced with a Cooper tissue culture dish top (Falcon 3009) and sealed with sterile silicone grease. The dish is placed on the stage of a microscope equipped with Hoffman modulation contrast optics in a 5 percent CO_2 atmosphere at 37°C. A cell selected for study out of the one or two in the camera field of view is photographed every 5 minutes (16 mm H&W VTE Panchromatic film; ×6.1) for 2 to 3 weeks. The colony derived from the founder cell is analyzed interactively with computer assistance (*37*). Basically, the trajectories, mitotic histories, contacts, velocities and stationary times, areas, and other features of all cells are recorded and analyzed by the TRACK program (*6*). The distributions of IDT's presented here show similar heterogeneities despite the wide range of PDL's and the source of the diploid founder cells (*38*). Consequently, mass cultures of cells at any PDL are likely to contain dividing cells whose generation numbers vary greatly.

cells cannot be assumed to be moribund because they have ceased to divide. Although it is well established that populations of diploid cells in vitro at some point cease to increase, one of the least documented features of the aging-in-vitro hypothesis is the accepted conclusion that phase 3 cells are moribund and eventually die. No hard data support this conclusion. It is based on the observation that cell proliferation ultimately stops and further division of the culture into two or more parts results in a decline in the number of cells per culture vessel. It is also true that with most standard media cells become covered with refractile cell debris and often detach from the substrate. We observed the survival of foreskin fibroblasts (strain 1519) in culture for long periods after cell divisions no longer occurred. Our first cultures of 1519 cells were kept alive in Ham's medium (MCDB 104) (*10*) for 22 months, more than a year to 14 months after cell divisions terminated. Parallel cultures grown in McCoy's medium (*11*) were abandoned after 12 to 14 months, when too few cells remained after replating to carry the cultures forward. In our experience, replating itself inevitably results in loss of cells.

Nor can we assume that embryonic cells that leave cycle at any PDL prior to "phase 3" in culture have become senescent. We observed instances of sister cells, one of which leaves cycle while the other generates many descendants, as well as sister cells with widely different interdivision times. Further studies of cells that leave cycle early in the history of the population will have to be conducted to discover whether they remain healthy and are retained in subculturing. Biochemical studies have shown that while phase 3 cells differ in some respects from phase 2 cells, they are essentially normal. All phase 3 cells are active in RNA synthesis (*12*); their DNA content cannot be distinguished from that of phase 2 cells (*13, 14*); they can ligate x-ray damaged DNA strands at the same rate as that of cells in young cultures (*15*); their carbohydrate metabolism is normal (*16, 17*); so is their permeability (*13*); respiratory and other enzymes (*17, 17a*) are present in the same concentration as that of low PDL cells. Other biochemical similarities have been tabulated by Cristofalo (*18*).

Reported biochemical differences between phase 2 and phase 3 cells reflect differences of differentiation rather than of age. We refer to differences such as the concentration of lysosomes and lysomal enzymes (*19*), the character of the propyl hydroxylation system associated with collagen synthesis (*20*) and the capacity to catabolize proteins (*15, 21*), as well as differences of size, adhesiveness, motility, and ease of culturing (*22*). Biochemical and other reported differences between cells of various PDL's in phase 2 and early phase 3, based on analyses of mass cultures, may represent only averages because at each PDL there are mixtures of cells which have left cycle and those which have not.

Table 1. Heterogeneity of the numbers of descendants in intraclonal lineages.

Starting PDL	Source	Descendants			
		Founder	Daughters of founder	Granddaughters	Great granddaughters
26th	WI-38 (*4, 5*)	112	51	24	12
					12
				27	13
					14
			61	27	9
					18
				34	15
					19
			22	11	4
					7
				11	5
					6
29th	WI-38 (*4, 5*)	57	35	8	3
					5
				27	11
					16
			33	12	5
					7
				21	9
					12
32nd	WI-38 (*4, 5*)	67	34	17	6
					11
				17	8
					9
			8	1	—
					—
				7	2
					5
36th	1519 (*6*)	26	18	7	1
					6
				11	5
					6
			11	3	1
					2
				8	2
					6
51st	WI-38 (*4, 5*)	30	19	4	1
					3
				15	4
					11

Further Differentiated Fibroblasts

The four points we have made lead us to propose that diploid fibroblasts in culture consist of two classes of cells: cycling fibroblasts (CF cells) and further differentiated fibroblasts (FDF cells). At low PDL's, cell strains such as WI-38, WI-26 or MFC-5 derived from embryos or 1519 cells derived from a neonate would consist mainly of CF cells, whereas cultures of higher PDL's would have an increased proportion of FDF cells. Since fibroblasts grown in vitro can be stimulated to divide, they may also be stimulated to leave cycle and differentiate further as fibroblasts do in the granulation tissue of a wound (*23*). There may, in fact, be a strong tendency to do so, one which we represent by the following:

$$CF \rightleftarrows FDF$$

Cells in an organism must have this tendency, but to a lesser extent than cells in vitro, where ultimately all cells of the population may be forced to complete differentiating.

When the number of differentiating cells in vitro exceeds the number of cells arising from division, a critical inflection occurs in the population growth curve, after which division of the culture into two or more parts results in a decline of cell numbers.

The factors that drive CF cells into the FDF state in vitro are still unknown. Small perturbations, both intra- and extracellular, may be responsible. For example, the conditions of cloning may constitute one such set of perturbations, since many founder cells even at low PDL's exhibit severely limited doubling potential. This seems very clear in the experiments of Smith and Hayflick (*9*) where large numbers of cells drop out of cycle early. The "bottleneck" conditions of Holliday *et al.* (*24*) which reduce population doubling expectations by up to ten generations may in fact be like cloning conditions. Another experimental trigger of differentiation may be temporary agar overlays that prevent cells from dividing and reduce their capacity to proliferate in proportion to the period during which the cells are maintained under agar (*25*). Still another may be a transient decrease in serum concentration from 10 to 0.1 percent (*26*)—which also reduces doubling potential. A transient decrease from 10 to 0.5 percent had no such effect (*27*). In addition to a built-in tendency to complete their differentiation, inadequate serum or some other culture condition could drive CF cells into the FDF state and be responsible for the considerable variation in the "life-span" of mass cultures of fibroblasts in vitro reported by Holliday *et al.* (*24*).

Studies of the serial transplantation of mouse mammary-gland tissue in vivo have brought out the distinction between "growth-span" or proliferative potential and "life-span" (*28*). When mammary tissue is subcultured at short intervals (3 months) the growth-span is contracted and subculturing is no longer possible after a period of 22 months; but if the interval between transplants is 1 year, subculturing is still possible after 8 years and, as Daniel suggested, possibly indefinitely (*29*). We interpret the data to mean that cells repeatedly pressed to divide attain a differentiated state in which they are refractory to further mitotic stimulation and to subculturing under the conditions employed. Being refractory to mitotic stimulation need not mean they are moribund. Similarly, if phase 3 cells are not moribund, why then are they so difficult to culture? Our answer is that present techniques and media favor proliferating and not differentiated cells. By the end of phase 3 most cells, by our designation, are FDF cells which may require special conditions for their maintenance. Culture and handling conditions which favor differentiated as well as cycling cells need to be developed. It is possible that the continued survival of cultures in Ham's medium (MCBD 104), after all cells have differentiated, is due to the presence of the polyamine putrescine. It has been reported that aminoguanidine can enhance survival of terminal nondividing WI-38 cultures by at least 4 months (*30*). Aminoguanidine inhibits diamine oxidase which deaminates putrescine. Synthesis of putrescine may be reduced, or synthesis of diamine oxidase may increase in cells that leave cycle and differentiate further. Surely it is diluted as the number of cells per culture vessel decreases and the cultures consisting of nondividing cells are split into two or more parts.

It is of more than passing interest that Dell 'Orco *et al.* (*27*) extended the chronological "life-span" of human foreskin fibroblasts for 6 months by keeping them in low concentrations of serum in a nondividing condition. Given an adequate environment these further differentiated fibroblasts might have an expected life-span equal to that of muscle, nerve, or other cells which leave cycle in the process of completing their differentiation and live as long as the individual of which they are normally a part. Gelfant and Smith (*31*) suggested that some tis-

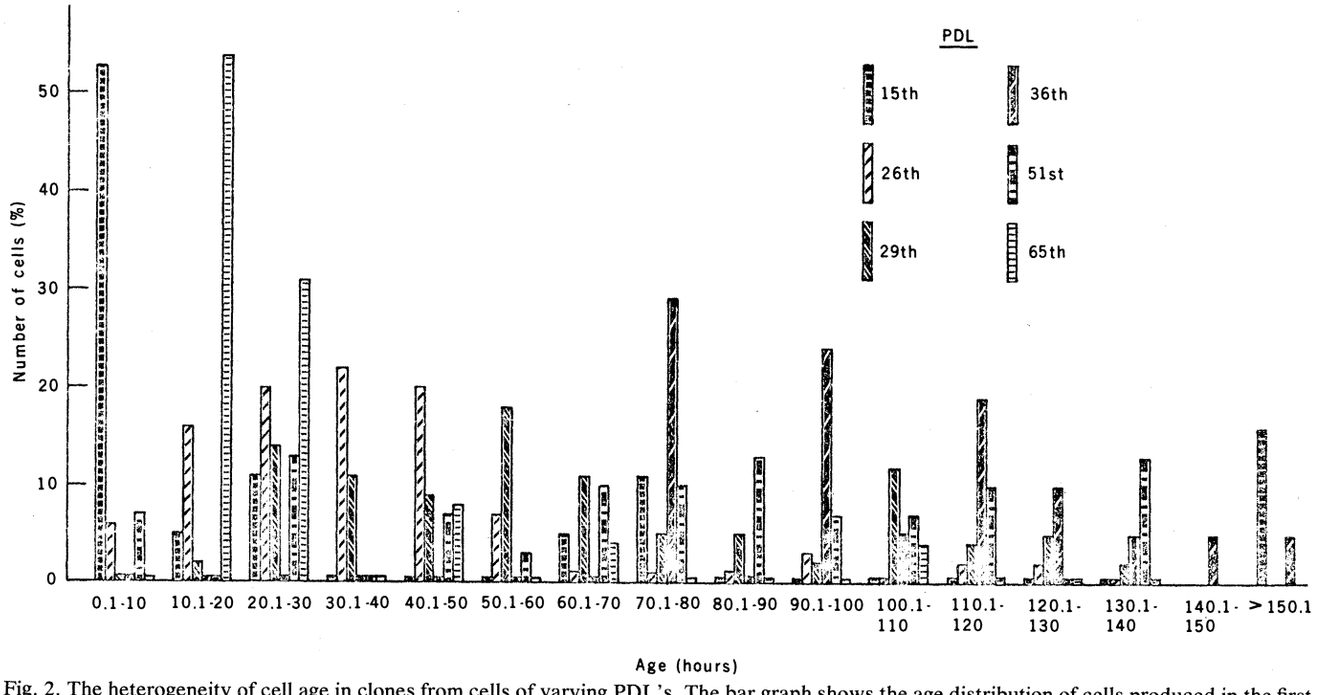

Fig. 2. The heterogeneity of cell age in clones from cells of varying PDL's. The bar graph shows the age distribution of cells produced in the first 200 hours of filming. Age is here defined as the elapsed time since the last division. Founder cells of all PDL's give rise to colonies of different sizes with about 50 percent of them generating less than 2^8 cells (*9*). The age distribution of cells in clones depends in part on the proliferative potential of the founder cells. Large clones (due to a founder of high proliferative potential) will have, on the average, young cells (that is, cells having short IDT's). Small clones (due to a founder of low proliferative potential) will have old cells (*37*).

sues complete their "cellular aging" during embryogenesis, whereas others do so later in life; in both, however, the process consists of a conversion of cells from a cycling to a noncycling state, the latter, although not irreversible, being "synonymous with cellular aging."

Similarly, Martin and co-workers (*7*) proposed that the bimodality of clonal size can be explained by the presence of a subpopulation of differentiating cells that undergo metaplasia; their view is that the cultures "differentiate themselves to death." However, if the cessation of cell division is not taken as a sign of aging but one of differentiation, the event is akin to what occurs during development when virtually all cell types undergo divisions and then differentiate further, and during later life when cells such as fibroblasts leave cycle after some divisions, in the course of a specialized activity such as wound healing, and then complete their differentiation. Since the fibroblast population in the adult organism will have had more time to complete its development than the population in the embryo, but is not forced to divide as cells in vitro may be, it too should have a high concentration of FDF cells, but only moderately so. This idea is supported by results of Marciera-Coelho (*32*), who found in sister cultures labeled continuously that 97 percent of embryonic cells at a low PDL synthesized nuclear DNA while only 66 percent of adult cells at a low PDL did so. There is no reason to believe that 34 percent of the adult cells that did not divide were moribund or had undergone more divisions than the dividers. In the organism, maintaining a balance of CF and FDF cells would ensure the availability of a pool of cells capable of dividing. It seems unlikely that high concentrations of FDF cells like those in "phase 3" cultures would occur anywhere except possibly in scar tissue. In the organism, therefore, CF cells might exist in yet another state—one of preparedness for division; we designate them as potential CF cells or PCF cells. We picture the interrelationship among the three classes of fibroblasts as follows:

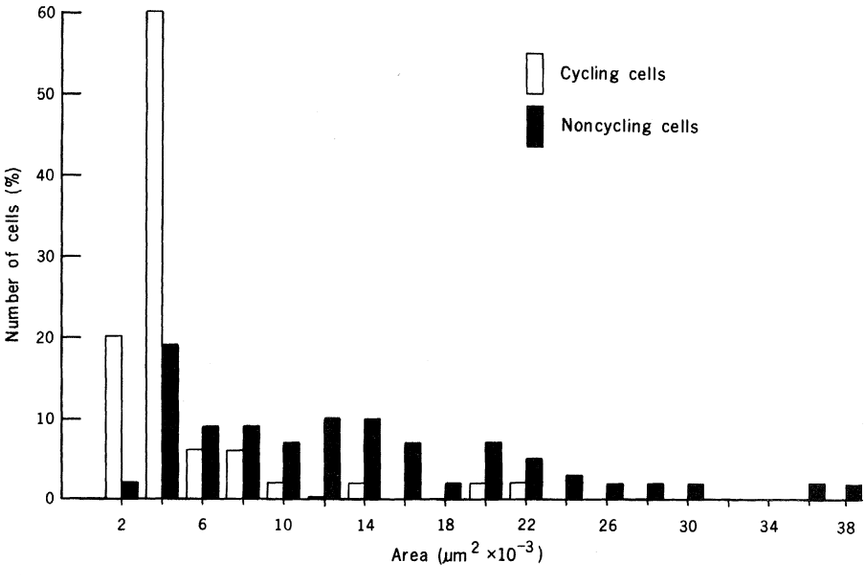

Fig. 3. Comparison of areas of cycling and noncycling cells of a 36th PDL (strain 1519 clone). The area of dividing and noncycling cells (cells out of cycle for more than 10 days) was calculated with computer assistance (*39*). Measurements were made when cells were flattened and separated from other cells. For dividing cells, two measurements were made under similar conditions: one immediately after division and one prior to the subsequent division. For noncycling cells, three measurements were made over the "life" of the cell. All measurements are plotted in the histogram. The noncycling cells have a much larger area than the cycling cells. Although the large size of noncycling cells can be attributed in part to continued growth with increased life-span of the individual cell, it might also reflect changes in cell adhesiveness and motility. Volume measurements might yield somewhat different results.

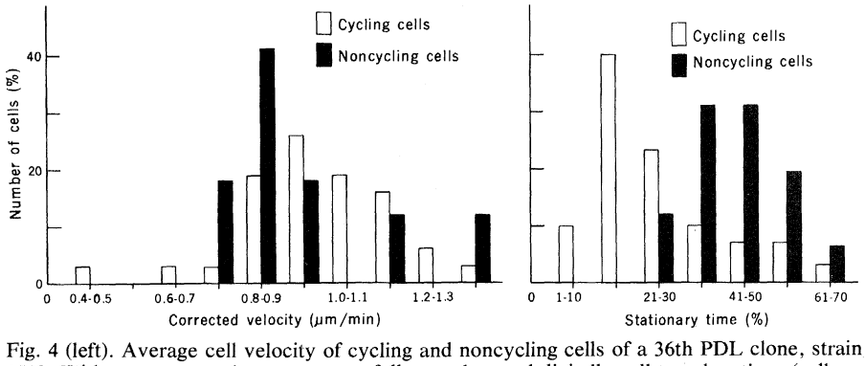

Fig. 4 (left). Average cell velocity of cycling and noncycling cells of a 36th PDL clone, strain 1519. With computer assistance we can follow and record digitally cell translocations (cell nucleus) from frame to frame over the life of each cell in a clone. The average velocity of a cell is defined as the total time during which the cell nucleus shows no net translocation (stationary time, Fig. 5) subtracted from the total life-span of the cell divided into the total distance traveled by the cell (sum of translocations between each frame). As seen here, cycling and noncycling cells show very little difference in their average velocity when moving. This result is different from those reported by others (*4, 5*), in which stationary time is not taken into account. Fig. 5 (right). Stationary times of cycling and noncycling cells of a 36th PDL clone, strain 1519. While cycling and noncycling cells show surprisingly little difference in their average velocities, they differ considerably in the amount of time spent in a stationary mode. Noncycling cells spend nearly half their time without translocating, whereas cycling cells spend only a little more than a tenth of their time without moving.

$$\text{PCF} \rightleftharpoons \text{CF} \rightleftharpoons \text{FDF}$$

Conclusion

Normal diploid fibroblasts grown in vitro may constitute a system in which cells regularly leave cycle and enter a further differentiated noncycling state. Just as in the embryo (and for fibroblasts during adulthood) cells may pass through a finite but not fixed number of divisions before completing differentiation; so in vitro fibroblasts may perform similarly, the main difference being that in vitro eventually all of them differentiate further. If our hypothesis is correct, cultured fibroblasts from embryonic or neonatal sources alone are not an appropriate subject for the study of aging. Instead, as Schneider and Mitsui (*33*) have suggested, comparisons of fibroblast cultures derived from human donors of different ages may be more useful.

Recently it has been proposed that while most diploid cells in culture are committed to aging, a subpopulation that is not so committed and that is essentially immortal as judged by its capacity to proliferate is lost because of handling methods (*24*). This view would endow at least some diploid fibroblasts with a

property enjoyed by transformed lines, namely immortality in the sense of unlimited proliferative potential. Our reasoning suggests that the difference between transformed cells and normal diploid cells lies in the inability of the former to differentiate rather than in some intrinsic capacity to remain immortal which, given the right conditions, might be a property of noncycling diploid cells as well.

With certain exceptions (*34*), notably cells of embryos whose death contributes to the execution of tissue morphogenesis and cells of the adult—such as keratinocytes and erythrocytes which exchange immortality for specialization—cells of organisms need not be programmed intrinsically to die (*35*). Cellular death, then, may be a consequence of organismic death, and cellular aging, where it occurs, a product of organismic or systemic as well as environmental attrition.

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37. E. Bell, P. Yanover, D. Levinstone, C. Merrill, S. Sher, L. Marek, I. T. Young, *J. Cell Biol.* **75** (No. 2, pt. 2), 6a (1977).
38. The finding of cells with large IDT's (> 50 hours) is probably due to the long period over which we have followed colonies (up to 3 weeks). The longest IDT we recorded was 10.5 days for a cell in a 15th PDL clone. Absher and Absher (*5*) have reported cells with IDT's of 120 hours in their studies of WI-38 clones.
39. A reference mark on a Numonics tracing arm (Numonics Corp., Lansdale, Pa.) is superimposed on some point of a cell's perimeter and is moved around the projected image of the cell. As it is moved, the coordinates of the mark are converted to digital form and stored in a PDP9 computer. Fifteen coordinate pairs define a cell. A computer program calculates the area directly from the coordinate pairs by the rectangular planimetry formula:

$$\text{Area} = \frac{1}{2} \sum_{i=1}^{n} (x_{i+1}y_i - x_i y_{i+1})$$

where the $(n + 1)$th point is equivalent to the first point of the cell contour.

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