SV40-Transformed Human Diploid Cells that Remain Transformed throughout Their Limited Lifespan

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SUMMARY

Of ninety-one subcultured loci of SV40-transformed WI-38 human diploid fibroblasts, none yielded cells that grew indefinitely, but all cells in each subculture continued to produce T antigen and to look morphologically transformed throughout their lifespan. These results are consistent with the commitment theory of fibroblast senescence, but predict that a special transformation event is necessary to account for the rare survivors.

Cells transformed by simian virus 40 (SV40) show the profound changes in growth and physiology characteristic of the transformed state (Shein & Enders, 1962; Risser & Pollack, 1974). It has not been determined, however, whether these changes are related to the transition of normal cells to indefinite growth. This ambiguity has persisted because transformation studies have most often started with established, indefinitely growing cell lines (like BALB c/3T3) or with cells easily established in culture (Syrian hamster, for example) which have tumorigenic capacities of their own (Earle, 1943; Stoker & Macpherson, 1964).

Ordinarily, diploid human fibroblasts cultured in vitro have a limited lifespan before they deteriorate, cease dividing and die (Hayflick & Moorhead, 1961). Koprowski et al. (1966a, b) have shown that SV40 infection of fibroblast cultures will induce the survival of rare cells which can subsequently grow into permanent cell lines (see also Potter et al. 1970; Shein & Enders, 1962; Todaro et al. 1966). However, it is not known what connection exists between the 'rescue' of the dying fibroblasts and transformation per se. One possible explanation for the rarity of surviving cells, despite the relative frequency of apparent transformants, is that the overwhelming majority of these foci are abortive transformants. The abortive transformants would lose the transformed phenotype before they reached crisis and thus cease cellular division much as uninfected cells. As a first step in testing this possibility, we infected human diploid fibroblasts, identified and subcultured apparently transformed foci and observed their properties as they approached crisis. The results reported here have a bearing on some current models for cellular senescence and argue against abortive transformation as a reason for the rarity of transformed cells that grow indefinitely.

Normal human diploid fibroblasts (WI-38) were obtained at passage 18 from the American Type Culture Collection and inoculated into 60 mm Petri dishes (6 x 10^5 cells per dish). The cells were grown in alpha-modified Eagle's minimal essential medium (MEM-alpha) supplemented with 20% foetal calf serum (FCS: K.C. Biological, Inc., Lenexa, Kan.) in a 5% CO₂, 95% air environment at 37 °C. The medium was changed every other day. At passage 26, based on 1:2 split ratios, the medium was removed and 5 x 10^5 p.f.u. of SV40 (obtained as a plaque-purified small plaque variant from Dr D. Nathans, Johns Hopkins University, Baltimore) were added in 0.5 ml MEM-alpha at a multiplicity of 50 p.f.u./cell. After an adsorption period of 3 h, the virus suspension was removed, fresh
medium added and incubation resumed. BALB c/3T3 cells, grown in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum (Colorado Serum Co., Denver, Colo.), were infected and handled in a similar manner to serve as transformation controls.

Three to 6 weeks after treatment of subconfluent WI-38 cells with virus, 91 well-separated foci of heaped-up cells were identified morphologically, 5 to 10 on each dish. Each focus was encircled with a 4 mm glass cylinder and the cells were trypsinized and transferred to a single 6 mm well in a Falcon microtitre plate. When cells had reached confluence in the 6 mm well, they were split 1:2, a ratio that was maintained as long as the subcultures continued to grow and expand to larger cultures. The ‘cell age’ of each subcultured focus was conveniently estimated as the total number of passages at 1:2 dilution, augmented by the approximate number of divisions required for the initial encircled cells to reach confluence in a 6 mm microtitre well. The latter number was calculated from: (1) The number of confluent established SV40-transformed WI-38 cells [WI-38(K) cells], kindly supplied by Dr H. Koprowski (Wistar Institute, Philadelphia, Pa.), contained in the area of the cloning cylinder; and (2) the assumption that each focus originated from a single transformed cell. Because each focus certainly also contained non-transformed cells of lower viability, and because the plating efficiency was only 30 to 60%, the total number of cell divisions is likely underestimated. However, this underestimation does not affect the inferences in this study.

During subculture, cells were grown in conditioned medium which contained anti-SV40 serum to prevent re-infection by any virus particles shed during growth. Conditioned medium was prepared by plating $2 \times 10^7$ WI-38(K) cells (or WI-38 cells younger than culture generation 22) in a flask and removing the medium 24 h later (Holley & Kiernan, 1968). This medium was filtered through a Millipore filter (0.45 μm) and supplemented with 1 ml goat anti-SV40 serum (titre 1:1000; Biological Assoc., Bethesda, Md.) per litre before use.

Of the 91 foci isolated, only 46 grew sufficiently to be expanded to a confluent culture in

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**Fig. 1. Lifespan of subcultures of SV40-transformed WI-38 Cells.** Ninety-one transformed WI-38 foci were subcultured and followed until division ceased. The histogram represents the number of foci whose subcultures died at each culture generation number. Three foci inadvertently lost at generation 45 were excluded from the figure.
Fig. 2. Subculture doubling times as a function of culture age. Culture doubling times versus cell age for four representative subcultured foci (A to D) are plotted. Subculture A was followed both continuously (○—○) and after four months storage in liquid nitrogen at culture generation 54 (×—×).

In general, the fastest-growing (earliest-appearing) foci were the most successfully subcultured. These foci could be subcultured at dilutions of up to 1:50, while the slower growing foci died out even after they were plated without dilution.

The initial criterion for transformation was morphological; every focus showed characteristics of transformed cells comparable to WI-38(K) cells. These cells also contained SV40 T-antigen, which was not detected in control WI-38 cells.

Saturation density, a third criterion for transformation, was also increased in these subcultures as compared to control cultures. For example, at confluency, untransformed WI-38 cells numbered $9.3 \times 10^4$ cells/cm$^2$; WI-38(K) cells, $3.6 \times 10^5$ cells/cm$^2$; and a subculture of a typical transformed focus (52) averaged $3.8 \pm 0.1 \times 10^5$ cells/cm$^2$ at culture generations 49, 53, 55 and 57.

None of the apparently transformed SV40-infected human fibroblast foci were able to grow in soft agar, even in conditioned medium fortified with 20% foetal calf serum (Macpherson & Montagnier, 1964). However, cloning in soft agar could easily be accomplished with both the SV40-infected BALB/c3T3 foci and the WI-38(K) cells. It was this inability to grow in soft agar that necessitated the use of glass cylinders for the isolation of transformed WI-38 foci.

The longevity of the subcultured foci varied widely. Twenty-four grew only through passage 41 (Fig. 1). Confluent uninfected WI-38 cells were also isolated in cylinders in 10 experiments; none of those subcultures survived any longer than the mass culture.

The remaining 22 subcultures survived up to a maximum of 64 to 65 passages at 1:2 dilution (Fig. 1, 2). The doubling time of cultures increased sharply in the last few generations (Fig. 2); this was especially precipitous and occurred within 1 generation in the longest-lived subculture (A). When cells of this subculture frozen at passage 54 were thawed and passed once again, crisis occurred in the same abrupt fashion. The cell density of confluent cultures also fell in the last few passages. For example, the subculture from focus 52 at confluence dropped from $3.6$ to $3.7 \times 10^5$ cells/cm$^2$ to $2.1 \times 10^5$ cells/cm$^2$ at generation 58 and $1.8 \times 10^5$ at generation 61.

Two independent mass cultures of SV40-infected WI-38 cells passaged without intermediate subculture survived no longer than the individual foci, with division ceasing at...
culture generations 50 and 51. These findings contrast markedly with the results of three independent trials with WI-38(K) cells, which were easily subcultured and passaged for more than 140 generations.

During the entire growth period, including the time in crisis, the cells of each subculture uniformly showed the morphology and SV40 T-antigen positivity of transformed cells. We therefore infer that SV40 transformation per se is insufficient to confer indefinite growth on human diploid fibroblasts. The limited life span of these SV40-transformed cells is thus distinct from the abortive transformation of mouse cells by SV40. In the latter case (Smith et al. 1972), T-antigen is lost and cell morphology reverts to normal with time; and SV40 genetic information is also lost.

Since we were unable to 'rescue' any of the transformed cells, we cannot exclude the possibility that the failure of our transformed cells to survive was related to some technical problem. However, the ease with which WI-38(K) cells were subcultured, even after great dilution, and their subsequent long-term survival argues that the technique was probably adequate. It is likely, then, that the finite lifespan of these transformed cells is an intrinsic property.

Why is the survival of SV40-transformed cells so rare? To answer this question, it is first necessary to consider how these results relate to current theories of senescence in untransformed fibroblasts.

The commitment theory of cellular aging (Holliday et al. 1977) suggests that fibroblasts tend to lose the capacity for indefinite growth by an irreversible change ('commitment') which occurs many generations before their cell division actually ceases. The finite lifespan of human fibroblasts in culture is due, according to this view, to dilution and loss of remaining uncommitted cells during subculture. Consistent with this theory are the observations of considerable variation in the growth potential of untransformed human fibroblasts separated at an early passage (Smith & Hayflick, 1974). Likewise, our findings that subcultured foci did not survive crisis can be explained if each focus was derived from cells already committed at the time of transformation, and the variation in lifespans (Fig. 1) could again reflect the different division potential remaining in the cells at the time of transformation.

The commitment theory has emphasized that the gradual increase in doubling time of a mass culture reflects its content of cells of markedly different reproductive capacities (Holliday et al. 1977). In our experiments, however, each subcultured focus is essentially a clone consisting of cells of the same 'age'. Cell division would then be expected to cease abruptly. This is in agreement with our observation (see particularly subculture A, Fig. 2). It strongly supports commitment as opposed to theories which propose the cessation of cellular division as a result of cell damage accumulating from heterogeneous, random events even in cohorts of cells of the same age (see Hayflick, 1973).

The commitment theory does not, however, explain how SV40 transformation promotes the survival of any cells from mass culture. From the studies of Jensen et al. (1963), one can infer that SV40 transformation provides some function required for the indefinite growth of human fibroblasts. Our data suggest that the cell which survives must be special in some other way. Perhaps a human fibroblast destined to survive indefinitely must be 'uncommitted' at the time of transformation. Transformation could then specifically increase the growth rate of the uncommitted cell, or lessen the probability for later commitment, or both, thereby tending to conserve these cells during subculture. And our study, beginning at passage 18 and following only 91 subcultured foci, would have been unlikely to produce a transformed uncommitted cell. However, Koprowski et al. (1966a, b) were
able to rescue some fibroblasts with SV40 transformation even in the crisis phase, at a
time when the commitment theory states that no uncommitted cells remain. Thus, either
uncommitted cells can exist in cultures in crisis but cannot overgrow the mass of undividing
cells; or SV40 transformation can reverse the terminal cessation of cell division in rare
cells.

That SV40 transformation can selectively increase growth potential is directly indicated
by the findings that mass cultures of transformed fibroblasts survive, on the average,
many generations longer than cultures of untransformed cells (Koprowski et al. 1966a)
and by our additional finding that the fastest-growing transformed subcultures were
precisely those that continued to divide the longest.

If reversal of commitment is required to promote survival, a special transformation
event must also be postulated or survivors would be more common. Among the possibilities
that might produce indefinite growth are integration at a rare site (Botchan et al. 1976;
Gelb & Martin, 1973; Ketner & Kelly, 1976; Prasad et al. 1975) or infection of a cell in
a rare physiological state. Transformation, then, would be a relatively frequent event;
but the indefinite growth associated with tumorigenesis would be much more rare and
only occasionally induced by the transforming virus. The only hint of a possible physio-
logical correlate of this 'special' transforming event noted was the inability of our 91 sub-
cultures, unlike SV40(K) cells, to grow in soft agar. Perhaps selection of transformants in
soft agar would reveal rare cells destined to survive indefinitely.

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Short communications


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