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EFFECT OF TEMPERATURE ON NORMAL AND SV40-TRANSFORMED HUMAN FIBROBLASTS

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Summary

The effect of a temperature shift from low (36°C) to high (40°C) temperature on human fibroblasts (IMR90) at various population doubling levels and IMR90 cells transformed by SV40 virus infection at a population doubling level of 30 (SV40/IMR90) was examined. Both IMR90 and SV40/IMR90 cells showed a decrease in cell saturation density at confluency, whereas an increase in population doubling time and protein content was noted when the cells were shifted up to 40°C from 36°C.

The modification of IMR90 chromosomal proteins by arginyl-tRNA transferase was increased by the temperature shift, whereas NH_2-terminal arginylation of SV40/IMR90 chromatin was not altered.

Similarly, no appreciable change in 2-deoxyglucose uptake was noted with SV40/IMR90 cells at either temperature, although 2-deoxyglucose uptake by IMR90 cells was increased by the temperature shift. Additionally, the rate of 2-deoxyglucose uptake showed no difference between IMR90 and SV40/IMR90 cells.

The above results support previous findings that environmental alterations, such as temperature shift can cause acceleration of cellular senescence. These findings also imply that cellular senescence remains fixed when viral transformation occurs and is rendered refractory to further age-associated alterations.

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Abbreviation: SDS, sodium dodecyl sulfate.

Introduction

Human fibroblasts in culture have been used for the study of cellular senescence since they were shown to have a finite lifespan by Hayflick in 1965 [1]. The process of cellular senescence consists of three phases, phase I for an initial explantation period, phase II for a period of rapid proliferation with the cells dividing logarithmically and finally phase III for a period of declining proliferative capacity with granular cell morphology and no cell division. In contrast to normal diploid fibroblasts, cells transformed by SV40 virus exhibit various morphological and biochemical alterations as well as exhibiting the characteristics of an immortal cell line in vitro [2,3]. These two concepts have been incorporated into a model for the study of aging-dependent and aging-independent characteristics. A recent finding from this laboratory indicates that exposure of normal human fibroblasts such as IMR90 and WI38 cells to SV40 viruses arrests the age-dependent alteration of various biochemical parameters, such as growth rate, population doubling time, cellular protein content, the activity of arginyl-tRNA transferase and the post-translational modification of chromosomal proteins directed by arginyl-tRNA transferase [4].

In contrast to the viral transformation of human cells, an increased environmental temperature has been implicated as an accelerator of aging in cultured human fibroblasts [5,6], as well as in various other invertebrates and vertebrates such as *Drosophila subobscura*, *Daphnia magna* and *Cynoledis adoffi maynard* [7—9]. The current study was designed to determine whether increased temperature does accelerate the aging processes in normal fibroblasts, what effect raising the incubation temperature has on SV40-transformed fibroblasts and whether SV40-initiated transformation can arrest the age-associated alterations in the above-mentioned parameters at a higher temperature. Furthermore, since increased uptake of 2-deoxyglucose in transformed cells as compared to normal cells has been considered as an accepted marker of transformation [10], we examined 2-deoxyglucose uptake with regard to SV40-initiated transformation of human cells and shifts in the environmental incubation temperature.

Materials and Methods

Cell cultures

Human fibroblasts strains, IMR90 and an SV40-transformed cell line of IMR90 cells, AG2804 (referred to as SV40/IMR90 below) were maintained in minimum essential medium with 10% fetal calf serum containing 100 μg/ml streptomycin and 2000 U/ml penicillin G. The cells were grown at 36°C in a humidified, 5% CO_2 atmosphere on 10-cm culture dishes and subcultivation was carried out at an 8 : 1 split ratio using Puck's EDTA-trypsin solution. As the cells approached confluency, the medium was aspirated, the cells washed twice with phosphate-buffered saline (137 mM NaCl/8.1 mM Na_2HPO_4/2.6 mM KCl/1.4 mM KH_2PO_4) and harvested with a rubber policeman.

Determination of 2-deoxyglucose uptake

After cells had grown to 1—5 · 10^4 cells/cm^2 in 3.5-cm tissue culture dishes,

the dishes were gently washed three times with glucose-free Hanks' solution and incubated with 0.5 μCi 2-[^{3}H]deoxyglucose (8.26 Ci/mmol) supplemented glucose-free Hanks' solution for 10 min at 37°C and washed three times with ice-cold glucose-free Hanks' solution. The cells were dissolved in 0.3% SDS and aliquots were counted in toluene/Triton X-100 scintillation mixture.

Preparation of arginyl-tRNA transferase and chromatin

Purified arginyl-tRNA transferase was prepared essentially by the method of Soffer [18] except that a 70% $(NH_4)_2SO_4$ precipitate of postmicrosomal supernatant of calf kidney was used.

Chromatin was obtained from nuclei of cells homogenized and purified as described [11]. Transfer RNA and ^{3}H-labelled arginyl-tRNA were prepared as described earlier [12] except that tRNA was extracted from normal rat kidney cells transformed by temperature-sensitive mutant LA24 of Rous sarcoma virus and grown at 36°C.

Transfer of arginine from arginyl-tRNA to chromatin

The transfer of arginine from ^{3}H-labelled arginyl-tRNA to chromatin acceptor proteins (12 μg) was carried out in a 50-μl reaction mixture at 37°C containing 100 mM Tris-HCl (pH 7.8)/45 mM KCl/5 mM $MgCl_2$/50 mM 2-mercaptoethanol/3 μg purified calf kidney arginyl-tRNA transferase/various amounts of chromosomal proteins and 10 000 cpm ^{3}H-labelled arginyl-tRNA. 15 μl aliquots were treated with hot trichloroacetic acid as described earlier [13].

Determination of population doubling time, cell saturation density and protein content

$1 \cdot 10^4$ cells from various population doubling levels (the number of doublings attained by the cell population since the plantation of the primary culture) were plated in a Linbro 24-well dish and the number of cells were counted after trypsinization at various times (Fig. 1). The concentration of protein in the well was determined by dissolving the total contents of the well in 0.3% SDS before processing for Folin-Ciocalteau reaction [14]. Population doubling levels were calculated according to the formula

$$2^X = \frac{\text{The number of cells harvested}}{\text{The number of cells plated}}$$

X representing the population doubling increase at each subcultivation. Population doubling times were calculated from the slopes of the growth curves. Cell saturation density was calculated from the number of cells grown in a 2-cm^2 Linbro well after the cells reached the plateau stage.

Results

Effect of transformation and high temperature on growth characteristics

Comparison of the cellular growth and population doubling level of normal IMR90 fibroblasts and its SV40-transformed cells (SV40/IMR90) at 36°C as well as 40°C are shown in Fig. 1. After a 2-day initial lag-period, non-transformed young cells exhibited exponential growth for no more than 5 days,

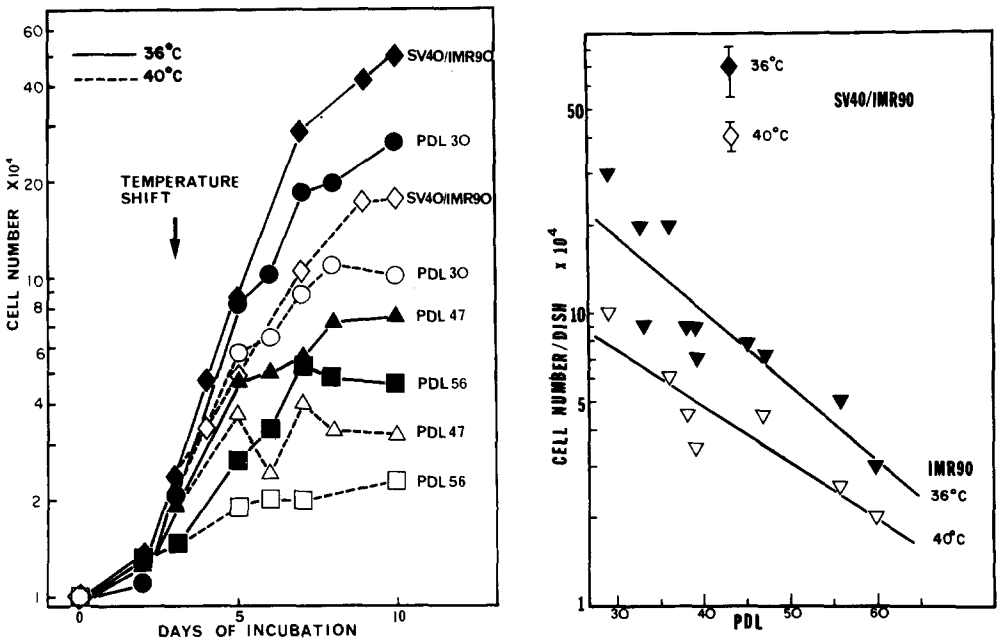

Fig. 1. Temperature effect on growth rate of IMR90 cells at various population doubling levels (PDL) and of SV40/IMR90 cells. Linbro plates having 24 wells were plated initially with $1 \cdot 10^4$ cells/well and the cell number per well was determined at various time intervals as indicated in the figure after trypsinization. Temperature shift from 36°C (closed symbols) to 40°C (open symbols) was carried out two days after innoculation. These data are the average of two determinations.

Fig. 2. Temperature effect on cell saturation density of IMR90 cells at various population doubling levels (PDL) and SV40/IMR90 cells. Saturation density was calculated from number of harvested cells grown in a Linbro well (2-cm^2) after the cells reached plateau level on the growth curves, such as drawn in Fig. 1. IMR90 (▼) and SV40/IMR90 (◆) grown at 36°C and 40°C are indicated by closed and open symbols, respectively.

whereas the SV40-transformed cells (SV40/IMR90) grew exponentially even after 8 days. In contrast to these young and transformed cells, the growth rate of older cells decreased progressively. The growth rates of cells at population doubling levels of 30, 47 and 56, as quantitated by the slope of the lines during expotential growth were 2.37, 1.00 and 0.33 respectively and the cell saturation densities of these cells were $1.4 \cdot 10^5$ cells/cm^2, 0.74 and 0.42 respectively. These values are in good agreement with the values on the slope drawn in Fig. 2. Transformed cells were infected with SV40 virus at a population doubling level of 30 (Personal Communication with Dr. E. Toji of the Institute for Medical Research, Camden, NJ) and the growth rate of these SV40/IMR90 cells was slightly higher than normal IMR90 cells at a population doubling level of 30. Although the growth rate of SV40/IMR90 was similar to non-transformed cells at a popualtion doubling level of 30, its maximum saturation density was nearly 5-times that of IMR90 cells at a population doubling level of 30.

Increasing incubation temperature caused a lowering of the growth rate for cells at population doubling levels of 30, 47 and 56 and SV40/IMR90 cells to

1.60, 0.44, 0.13 and 1.72, respectively. These values were substantially lower than those obtained from the cells incubated at 36°C. When the growth rate of cells transformed by SV40 virus (SV40/IMR90) was compared to normal cells grown at 40°C, a population doubling level of 27 was obtained. Saturation density of cells grown at 36°C and 40°C were 0.63 and 0.20, indicating that the cells can reach saturation densities 3-times those obtained at 40°C.

Population doubling time

The population doubling time of normal fibroblasts increased from a value of 25 h for cells at a population doubling level of 30 to 62 h for cells at a population doubling level of 60 (Fig. 3). The population doubling time of cells transformed by SV40 virus (SV40/IMR90) is slightly higher than that of cells at a population doubling level of 30. Extrapolation of the line beyond the population doubling time of cells at a population doubling level of 30 indicated that the population doubling time of the transformed cells fell approximately at a population doubling level of 33. The SV40/IMR90 cells were transformed at a population doubling level of 30, again showing that SV40-initiated transformation of human cells can arrest a biological parameter at the time of cell exposure to virus.

Raising the culture temperature to 40°C from 36°C caused an increase in

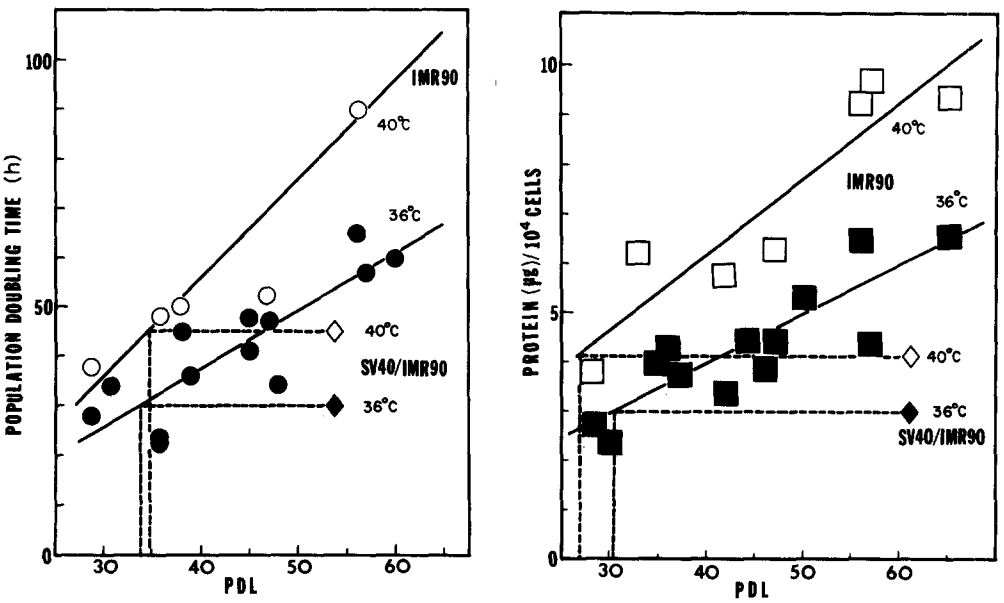

Fig. 3. Temperature effect on population doubling time of SV40/IMR90 cells and aging IMR90 cells. Population doubling time at 36°C (closed symbols) and 40°C (open symbols) was calculated from the slope of growth curves of IMR90 (●) and SV40/IMR90 (◆) cells at logarithmic growing phase.

Fig. 4. Temperature effect on cellular protein content of SV40/IMR90 and IMR90 cells at various population doubling levels (PDL). The total contents of the well were dissolved in 0.3% SDS after washing the cell layer twice with phosphate-buffered saline and the concentration of protein in aliquots of the solution was measured by the method of Lowry et al. [11]. IMR90 (■) and SV40/IMR90 (◆) grown at 36°C and 40°C are indicated by closed and open symbols respectively.

population doubling time of both normal and SV40-transformed cells. The population doubling time of cells at a population doubling level of 30 was 35 h and that of cells at a population doubling level of 56 was 90 h. The increase in the population doubling time due to temperature increase was more evident in the older cells than in the younger cells, as can be seen from the slope of the line in Fig. 3. The population doubling time of SV40/IMR90 cells at the higher temperature was 45 h, which extrapolates back to a population doubling level of 34 in the non-transformed cells. This suggests that viral transformation causes a fixation of population doubling time even at higher temperature.

Cellular protein content

As has been shown previously [4,15], aging senescent cells enlarge and spread out. Quantitative measurement of cellular protein content (Fig. 4) indicates that cells at a population doubling level of 60 contain approximately twice the amount of protein when compared with that of cells at a population doubling level of 30. Cells transformed by SV40 virus (SV40/IMR90) contained total protein content similar to the cells at a population doubling level of 30, the time at which cells were exposed to SV40 virus. Shifting the temperature up to 40°C from 36°C induced even the young cells to expand and spread out. Consequently, cellular protein was increased and the extent of increase was even greater with senescent cells. SV40-transformed cells (SV40/IMR90) were also affected with increasing culture temperature but the protein content remained the same as that of non-transformed cells of a population doubling level of 27.

2-Deoxyglucose uptake

The size of human cells increases with the increasing culture age. This morphological change is evident not only in their appearance but also in cellular protein content and the degree of saturation density as shown in the preceding sections of this report. Coinciding with these observations is a general tendency of an increase in the rate of 2-deoxyglucose uptake which was seen during senescence with IMR90 cells but not with IMR90 cells transformed by SV40 virus (SV40/IMR90). The rate of uptake is accelerated slightly by shifting up the incubation temperature to 40°C from 36°C. The increase of the rate of uptake due to the temperature shift was generally evident in the senescent cells. However, SV40-transformed cells have a rate of 2-deoxyglucose uptake similar to the rate of non-transformed IMR90 cells of a population doubling level of 30, the time at which the cells were exposed to SV40 virus.

Chromatin acceptor activity

Arginyl-tRNA transferase, which functions by transfering arginine from arginyl-tRNA to appropriate acceptor proteins [12], has been implicated in cell regulatory and/or proliferative processes [11,16—19] by acting as a post-translational modulator of protein function. Transferase activity, as well as the transferase-directed modification of chromosomal proteins serves as a marker of senescent phenomena in cultured human fibroblasts, with both parameters decreasing as a function of increasing culture age [4]. Furthermore, transformation of human fibroblasts by SV40 viruses appears to arrest the age asso-

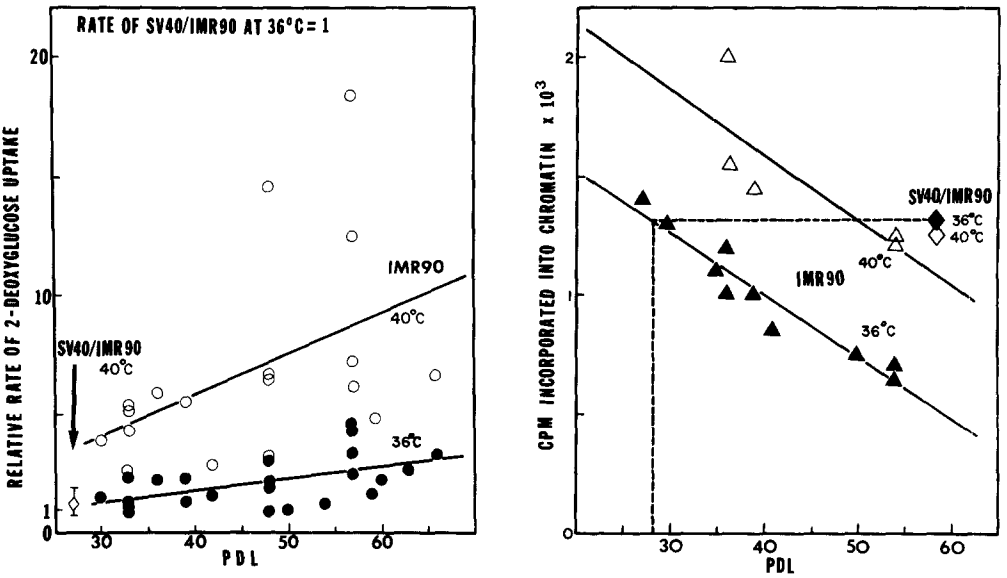

Fig. 5. Relative rate of 2-deoxyglucose uptake by IMR90 cells at various population doubling levels (PDL). Relative rate of IMR90/SV40 cells at 40°C (◇) is also shown. After cells had grown to $1-2 \cdot 10^5$ cells/cm^2 in 3.5-cm tissue culture dishes (lux), the cell layers were gently washed three times with glucose-free Hanks' solution and incubated with 1.0 ml of 0.5 μCi/ml 2-[^{3}H]deoxyglucose (8.26 Ci/mmol)-supplemented glucose-free Hanks' solution for 10 min ans washed three times with ice cold glucose-free Hanks' solution. All procedures were carried out in water bath at 37°C. The cell layers were dried, dissolved in 0.3% SDS and aliquots were counted in toluene/Triton X-100 scintillation mixture. Relative rate was calculated by normalizing the rate of IMR90/SV40 cells at 36°C to the value 1. IMR90 cells (●) grown at 36°C and 40°C are indicated by closed and open symbols, respectively.

Fig. 6. Effect of temperature on the acceptor capacity of chromatin directed by arginyl-tRNA transferase. The radioactivity incorporated at 12 min is shown. IMR90 cells at various population doubling levels grown at 36°C and 40°C are indicated by closed and open triangles. Acceptor capacity for SV40/IMR90 cells grown at 36°C and 40°C are indicated by ♦ and ◇ respectively.

ciated alterations in transferase-related activities, as well as in various other biochemical parameters and renders them refractory to subsequent aging processes [4].

High temperature produced a characteristic shift in the age-associated chromatin acceptor capacity, resulting in an increase in the observed protein modification at the higher temperature (Fig. 6). However, this stimulation of chromatin acceptor capacity induced by high temperature was not evident in the cells transformed by SV40 virus (SV40/IMR90), such that no apparent differences were observed between transformed cells grown at 36°C and those grown at 40°C. Furthermore, a decrease in protein modification was observed with the aging cells even though the protein content of cells was increased.

Discussion

The growth of human fibroblasts at high temperature has been proposed as a means to accelerate the aging processes in vitro [6]. The cellular parameters described in this report, which are subject to age-associated alterations, were

also affected by a temperature shift from 36°C to 40°C.

(1) This temperature shift induced an increase in population doubling time, cellular protein content and 2-deoxyglucose uptake and a decrease in cell saturation density at confluence. Similar alterations in these characteristics were observed during senescence and our findings on specific age-dependent parameters are consistent with the report of Thompson and Holliday [6], who have shown that the longevity of human fibroblasts cultured at 40°C is shorter than cells cultured at a lower temperature. The lifespan of the human diploid fibroblasts MRC-5 was found to be 57.2 and 29.2 doublings at 37°C and 40°C respectively. Cultures grown at 32°C had a reduced lifespan compared to the control, but this was not attributed to aging since the transfer of a dying culture back to 37°C allowed them to reach the normal lifespan.

Litwin [20] found that cells grew well at temperatures between 34°C and 37°C and some cells could be adapted to grow at 40°C. We could not find such cells adapted to the high temperature in dealing with IMR90 cells. Very limited growth occured at 30 to 31°C, although confluent monolayers of cells could be maintained for months at 30°C and still produce actively growing cultures. Although the exact mechanism of cellular senescence is still unknown, the above observations suggest that environmental temperature may contribute to accelerated aging processes in vitro. Comparison of the arginyl-tRNA transferase-directed chromatin modification between cells grown at 36°C and 40°C indicated an increase, rather than a decrease in the availability of chromosomal proteins for modification by arginyl-tRNA transferase. This increase was proportionately the same for both young and old cells, therefore, the age-dependent decrease in acceptor capacity was still evident even at the high temperature. The temperature shift had no effect on those cells transformed by SV40 virus (SV40/IMR90), such that the chromatin acceptor capacity remained constant. The increase in acceptor capacity in non-transformed cells at 40°C could be due to a conformational change of the chromatin or of individual proteins associated with it, activation of proteases dependent on high temperature, stimulation of an acceptor protein that is temperature-sensitive rather than aging-dependent, or a decrease in nuclear arginyl-tRNA transferase activity which would result in the accumulation of available acceptor proteins. Whatever the mechanism producing increased acceptor capacity in non-transformed cells, it appears that SV40-initiated transformation eliminates or masks this effect due to increased temperature.

(2) Transformation of IMR90 cells by SV40 viruses increased population doubling time and cellular protein content at high temperatures in a similar manner to non-transformed IMR90 cells at a population doubling level of approx. 30. This observation further supports the notion that the transformation of IMR90 cells by SV40 viruses arrests the process of cellular senescence at the age when the cells are exposed to the viruses and render it refractory to further age-associated alterations [4].

The study of 2-deoxyglucose is of particular interest, since in most cell types, except leukocytes and macrophages, this analog interacts only with the plasma membrane and with hexokinase and is not further metabolized. Furthermore, the earliest manifestation of the transformation in avian or mammalian cells infected by tumor viruses are known to be sugar uptake enhance-

ment [10]. Human Fanconi and mouse cells both exhibited a 3-fold or greater increase in uptake of 2-deoxyglucose after transformation by SV40 wild type and mutant A28 viruses as reported by Brugge and Butel [21]. In contrast to this and other reports, the rate of 2-deoxyglucose uptake by SV40/IMR90 cells was similar to that by non-transformed IMR90 cells at a population doubling level of 30. Therefore, the rate of 2-deoxyglucose uptake may also be used as a measure for 'fixation of cellular senescence'. This observation implies that the rate of 2-deoxyglucose uptake is not a transformation marker in transformed human fibroblasts in IMR90 cells as well as in WI38 cells [22] as has previously been reported for other transformed cell lines. The above finding on the comparable rates of 2-deoxyglucose uptake in control and transformed cells, together with the observation that non-transformed human fibroblasts have high plasminogen activator activity [23] which has been used as a transformation marker [24], suggests that some human fibroblasts promote an altered expression of SV40-initiated transformation characteristics.

We are now in a process of studying the effects of viral transformation and temperature shifts on the cells transformed at different population doubling levels.

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