Chapter 3

Aging of Cultured Human Skin Fibroblasts

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1. Introduction

There is substantial evidence that aging is related to the finite ability of somatic cells to divide and repair damaged tissue. Since the seminal observation of Hayflick and Moorhead (*1*) that cultured human fibroblasts have a finite replicative lifespan, a great deal of basic biological research on aging has been based on the model of cellular senescence in vitro (2).

There are three ways in which cultured cells can be used in gerontological research. First, cells can be isolated from donors of various ages or, in longitudinal studies, from one donor at various times. Molecular and cellular features of the cells can then be compared at corresponding early times in culture. Differences are related to the age of the donor. Similarly, a second method of investigation involves comparison of cell cultures from individuals with accelerated aging syndromes such as progeria or Werner's syndrome to cultures at corresponding passages in vitro from aged-matched normal donors. These types of comparisons do not make use of the in vitro senescence of cells. Thus, in the third model, cultures initiated from normal donors are followed as a function of cell generations in vitro, and comparisons are made between early and late passages of the culture lifespan. Differences seen as a function of in vitro aging may be related to cellular aging within the organism. Each of these methods has advantages and limitations; it is probably best to use more than one (3).

Studies on cellular senescence in vitro demand careful attention to the establishment of the primary culture and ongoing estimation of population doublings. These are the points that will be emphasized in this chapter. For example, periodically throughout the lifespan of a culture, the number of viable cells used as the inoculum (N_o), the fraction that attaches (f_a), the fraction of attached cells that divide (f_d), and the cell number at confluence (N_c) should be measured (*4*). These numbers determine the incremental cell doublings within one passage, provided there are not dramatic changes in other parameters such as cell size and interdivision time:

$$\text{Doublings} = \log_2[(N_c - (1-f_d)f_aN_o)/f_af_dN_o] \quad (1)$$

In practice, however, the fraction of normal skin fibroblasts that attach and divide is close to 1 for most of the lifespan of the culture, and these effects are ignored or assumed in the term MPD (mean population doublings):

$$\text{MPD} \approx \log_2(N_c/N_o) \approx \log_2(1/\text{split ratio}) \quad (2)$$

Thus, if cells are split at confluence at a 1:4 or 1:8 ratio, for example, the number of mean population doublings to refill the dish is simply 2 or 3, respectively. It should be realized that this estimate of cell generations can be substantially in error, especially toward the end of the culture when the fraction of cells that attaches and divides becomes significantly less than one, and the number of cells at confluence declines.

Other chapters in this volume provide greater detail on maintenance of cell strains and special techniques relevant to specific cell types. Cells other than fibroblasts have in fact been used in studies of aging in culture (*2*). The methods outlined below for careful determination of mean population doublings can be applied to any cell growing on a solid matrix.

2. Materials

1. Regular growth medium (RGM): α-minimal essential medium (GIBCO) supplemented with 15% fetal calf serum. Store the components as indicated by suppliers. Prepare bottles of RGM as needed, and keep at 4°C (up to 2 mo) except during use, when it should be prewarmed to 37°C. It is important not to change lots of serum. Therefore, order a small amount of two or three lots, asking for an appropriate number of bottles of each to be kept on hold. Test for optimal growth of cells at high and low cell density, and then place a large order for the best batch (Notes 1,2).

2. Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS): 0.14*M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, 8.1 m*M* Na_2HPO_4.
3. Trypsin: Crude Difco or Sigma trypsin at 0.125% (w/v) in PBS. Prepare 5–10 mL aliquots, and store at –20°C. Thaw as needed. Unused portions of the aliquot may be refrozen and used once or twice more (Note 3).

3. Methods

3.1. Primary Culture

1. A standard 2–4 mm punch biopsy of epidermis plus dermis from the abdominal or inner forearm surface is taken sterily by a qualified person (Note 4).
2. Place the tissue piece into a 100-mm Petri dish containing a small amount of RGM. Cut it in two, and place one-half in a vial containing 10 mL of RGM. Place this vial at 4°C as a backup sample. It will remain viable for many days.
3. Dice the remaining half into pieces about 1 mm^3 or less in size, and place three pieces dermis-side down (if possible) in each of several 35-mm Petri dishes. Use a siliconized Pasteur pipet to transfer the tissue. Place a sterile 25-mm coverslip over the skin pieces as shown in Fig. 1, add 2 mL RGM, and incubate at 37°C.
4. Monitor the explants for cell growth from around the edges of the tissue every day or two. Refeed weekly. The first cells to appear are the irregular, closely packed keratinocytes, which terminally differentiate and die in RGM. Spindle-shaped fibroblasts appear within several days.
5. When a dish has a total of about 1 cm^2 of fibroblast outgrowth (2–4 wk), loosen and invert the coverslip, aspirate the RGM, rinse well twice with PBS, and add 0.3 mL trypsin. Tilt the dish several times until trypsin covers all cells. Set at 37°C for 10 min or until cells start to detach from the dish and/or coverslip. It may be necessary to give the dish one or two gentle shakes during the digestion period.
6. Add 1.5 mL RGM, pipet up and down to suspend cells, remove the coverslip and tissue debris, and count an aliquot of the cell suspension. Pool cells from several dishes.
7. Estimate the mean population doublings (MPD) in the primary culture as shown in Table 1.

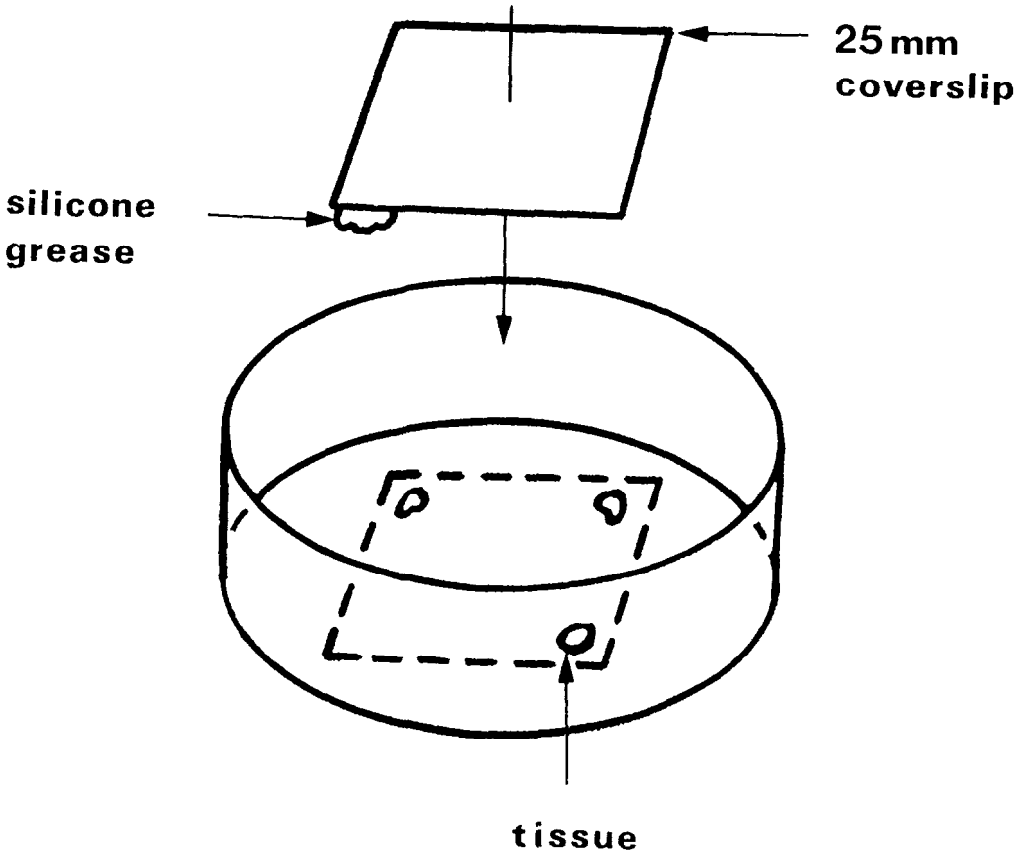

Fig. 1. Establishing a primary culture. Tissue fragments are transferred with a siliconized pipet containing a drop of medium into a dry 35-mm dish. A dab of sterile silicone grease (e.g., high vacuum lubricant) holds a 25-mm coverslip over the tissue fragments. Medium is then carefully added to the dish. The tissue fragments anchor themselves to the dish and/or coverslip, and cells begin to grow out onto these surfaces usually within 1–2 wk.

8. Place the remaining cells into fresh 35- or 60-mm dishes. Record on the lid the date, strain designation, and expected MPD at confluence. The latter is obtained by adding to the MPD of the primary culture 3.3•log (N_c/N_o), where N_c is the expected number of cells at confluence (Table 2) and N_o is the initial number of cells (Note 5).

3.2. Secondary Culture and Subsequent Passages

1. When the culture just reaches confluence, aspirate the medium, rinse once with PBS, add trypsin (Table 2), and incubate at 37°C for 10 min

Table 1
Estimated MPD During Primary Culture*

Cell number/dish	MPD
30,000	6
60,000	7
120,000	8
250,000	9
500,000	10

*This estimation assumes that about 500 cells initiate outgrowths from the tissue fragments in one 35-mm dish. Some heterogeneity in population doublings is introduced into the culture because of density-dependent inhibiton of growth (4).

Table 2
Tissue Culture Dishes

	35 mm	60 mm	100 mm
Surface area (cm^2)	8	21	55
Cells at confluence (N_c)	5×10^5	1.3×10^6	3×10^6
Trypsin (mL)	0.3	0.6	1.0
RGM (mL)	1.7	3.4	9.0

or until cells loosen from the plate. It is often necessary to shake or rap the dish gently.

2. Add RGM (Table 2) and pipet up and down until all cells are freed from the dish and a single-cell suspension is achieved. Count an aliquot of this suspension, and inoculate a fraction of it into a fresh dish (or dishes) containing an appropriate volume of RGM. The inoculum should represent 1/8 to 1/2 the number of cells required to form a monolayer of cells in the new dish. Shake the dishes gently in an irregular fashion to ensure a homogeneous dispersion of cells. Record date, strain designation, and expected MPD at confluence.
3. Refeed the culture at least once per week until confluence is again reached.

3.3. Senescence (Phase III)

1. Visually monitor cells at each passage and estimate growth rate by days to confluence from a standard inoculum size (e.g. 1/8 of final confluent cell number). The culture is approaching its terminal passage when growth rate drops and cells become larger and irregular in shape (Fig. 2).

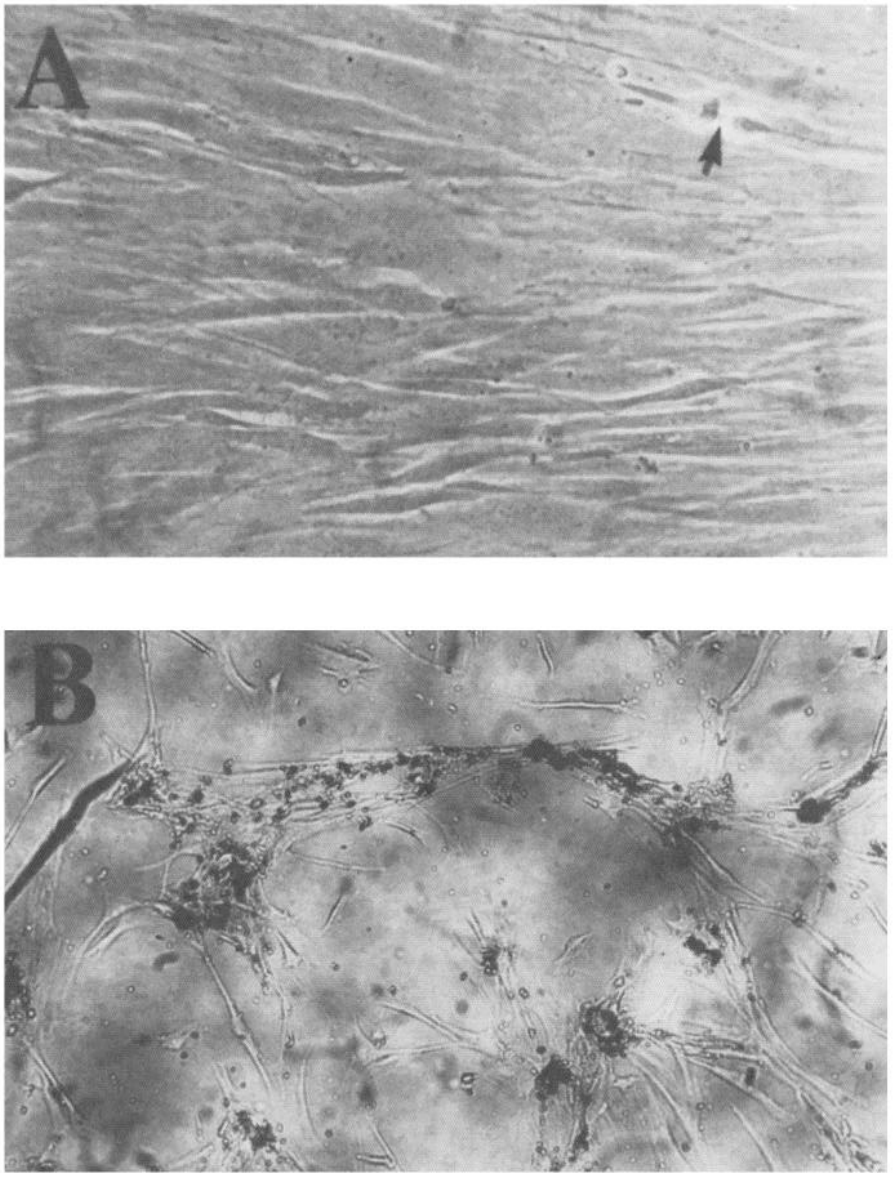

Fig. 2. Cultured human fibroblasts from an adult forearm skin biopsy. The clean, spindle appearance of cells aligned in parallel or spiral arrays at confluence during early passage (A) is gradually replaced in late passage by nonaligned, irregular-shaped cells containing opaque degradation products (B). Early-passage cultures contain numerous mitotic cells (A, arrow) during log phase and early confluence, whereas terminal passage cultures have essentially no dividing cells and, thus, fail to reach confluence even after numerous weekly refeedings.

2. Senescence, also known as MPD_{max}, Phase III, or terminal passage, is the point at which one MPD takes longer than one week. For example, if a culture split at a 1:8 ratio is not confluent in 3 wk, it may be considered terminal.

4. Notes

1. Do not use dialyzed or heat-treated serum for routine culturing of normal human fibroblasts. However, fibroblasts are fairly tolerant of media changes for short-term culture, and it is often necessary to use defined or special media lacking certain components for biochemical studies. To ensure that cells are not adversely affected, conduct a growth curve in regular vs experimental medium, or, if less than one population doubling is involved, assay the rate of protein or DNA synthesis. For incorporation assays, it is important that the specific activity of the precursor be the same in each medium tested.
2. Avoid the use of antibiotics and antimycotics, since they may have unexpected effects. They are not needed if sterile technique is diligently practiced.
3. Crude preparations of trypsin may contain other proteases useful in dissociating cells. However, they also vary in activity. Therefore, adjust the concentration up (or down) by a factor of 2 if cells take more than 15 min (or less than 5 min) to detach from the dish.
4. Fibroblast cultures can be initiated from biopsies taken almost any where on the body. Considerations include sun exposure, environmental variation, nature of the study, and compliance from donor to donor. Abdominal and the non-sun-exposed forearm surfaces are commonly used, as are foreskins and other surgical pieces of skin.
5. If cultures or ampules of cells are supplied, one must estimate the previous mean population doubling of the culture based on data from the supplier. Assume 9 MPD for the primary culture and 1–3 MPD for each passage depending on whether cells were previously split at 1:2, 1:4, or 1:8 ratios. Upon arrival, subculture the cells at a split ratio of 1:2 into fresh dishes and add an appropriate number of MPD to this estimate based on how many cells appear to have attached after 6–12 h. For example, if about 1/2 of the cells attached, count this initial 1:2 split as 2 MPD.
6. Biopsy fragments can be disaggregated with proteases, and cell suspensions rather than outgrowths are then used to establish the primary culture. However, this often requires extensive proteolytic

treatment to liberate cells, and it is difficult to estimate the number of viable founder cells of the fibroblast culture. The advantage of this is that it eliminates the heterogeneity in doublings within the primary cell population, which arises from density-dependent arrest of cells in the outgrowth technique (4). However, this effect is reduced in the technique described here by harvesting the primary culture before outgrowths become large and heterogeneous.

7. Values of N_c (Table 2) reflect an average value for human forearm skin fibroblasts. Each laboratory should determine N_c and N_o periodically throughout the lifespan of each strain studied and use these values for MPD calculations, which are not based on valid "split ratio" methods.
8. In reporting comparisons of early and late passage cells, it is useful to report culture age as "percent lifespan completed," i.e., MPD/MPD_{max}, together with the value of MPD_{max} for the culture.
9. Various criteria have been used to define senescence, or Phase III. It is wise to monitor the fate of the terminal passage cells to determine if additional MPD occur. However, we have not been able to further passage cells that fail to reach confluence at a 1:8 split ratio within 3 wk.
10. Other methodology relevant to the culture of human skin fibroblasts has recently been described (5).

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