hibitory effect of progesterone on nuclear estrogen receptor in vitro (*11*). Among other possibilities, we have hypothesized that progesterone modulation of nuclear estrogen receptor retention involves the induction or stimulation of a factor (estrogen receptor regulatory factor) that directly alters the nuclear estrogen receptor. The presence of a progesterone-induced modulator capable of altering the occupied form of estrogen receptor within the target cell nucleus may provide a fundamental mechanism for regulation of cellular response to hormone action. Furthermore, the regulation of such modulators by exogenous agents would provide a specific and selective means of controlling hormone-induced responses. Progestin therapy is useful in the management of certain types of endometrial cancer (*12*). Our results suggest that the responses of occupied nuclear estrogen receptor or of estrogen receptor regulatory factor to progestin may provide a novel approach for selecting patients with hormone-dependent endometrial carcinoma.

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Cellular Aging: Further Evidence for the Commitment Theory

Abstract. *A large, transient reduction in the population size of human fibroblasts in early passages significantly increases the variability of the life-spans of cultures in comparison to control cultures, as predicted by the commitment theory of cellular aging. The theory also predicts that a constant population of noncycling cells will appear in the later part of the culture life-span. This was confirmed by labeling the cells in culture with tritiated thymidine.*

Diploid human fibroblasts have a finite growth potential when they are cultured in vitro (*1*). Hayflick and Moorhead (*2*) defined three growth phases of cells in culture: phase 1 being the establishment of the primary culture, phase 2 being a long period of proliferation during which the cells are outwardly normal, and phase 3 being a period in which the growth rate slows down, cell morphology is abnormal, the yield of cells at confluence declines irreversibly, and the culture can no longer be propagated. The life-span of human fetal lung fibroblasts varies from about 40 to 70 population doublings (*3*).

A number of features of the growth of diploid cells and the existence of permanent lines led us to formulate the commitment theory of fibroblast aging (*3, 4*). We suggested that at some early stage, prior to the establishment of fibroblasts in culture, there exists a subpopulation of cells that can undergo an unlimited number of divisions (*5*). We call these "uncommitted" cells and assume that during their division they can become committed to finite growth with a probability of value P. Thus a committed cell is one that gives rise to a clone with strictly limited replicative potential. We also assume that each committed cell and its descendants multiply at the same rate as uncommitted ones through an incubation period, M, consisting of a fixed number of cell divisions, after which no further growth occurs. If P is sufficiently high (approximately .25) and M is sufficiently long (approximately 55 cell divisions), then for normal laboratory populations the cultures will eventually die out, since all the uncommitted cells will necessarily be lost by dilution from every culture. However, the exact time at which the last uncommitted cells are lost will fluctuate considerably from population to population, and this will greatly affect the final life-span of the culture in question.

The theory can thus explain the variability in life-span of parallel cultures set up at an early passage and grown under identical conditions. For example, among 24 such cultures of the fetal lung strain MRC-5, the average life-span was 57 passages, with a standard deviation of seven passages, which corresponds to more than a 100-fold difference in growth potential (*6*). In other experiments, populations of early-passage fibroblasts consisting of a 1:1 mixture of two heritable phenotypes were grown and then scored at intervals for the proportion of each type of cell. In many cases the ratio remained constant for many population doublings, showing that there was no direct selection for one type, but frequently there was a sudden transition to a predominance of one or other phenotype at the end of the life-span (*7*). This result is explained by the random and independent dilution of the two subpopulations of uncommitted cells. From studies of individual clones, it has also been concluded that there is an important underlying stochastic process in the aging of fibroblasts (*8*). The commitment theory predicts that population size can have an important effect on the longevity of fibroblast cultures, and evidence for this has been reported (*3*). We describe here further results that conform with other predictions of the commitment theory and we comment on the discordant report by Harley and Goldstein (*9*).

Since we assume that committed cells divide normally for many cell generations, initially all the cells in the culture will be viable. We refer to this as stage 1, and if the culture is finally to die out, all the uncommitted cells must be lost at some time during this stage. When the cells that first become committed cease growth, the viable cells in the culture must divide more than once to double the population number. Thus, the population growth rate will decrease. We call this stage 2 of culture growth. Our early experiments were with several populations of MRC-5 cells and one of these growth curves was published previously (*4*). In 29 cultures of another fetal lung strain, designated MG-4, the period of rapid growth was always followed by a period of constant slower growth (*10*) (Fig. 1). The same transition has also been observed in cultures of adult human skin (*11*). The reduction in growth rate in stage 2 is determined by the value of P (*4*). For MRC-5, MG-4, and adult skin fibroblasts we estimate from the change in growth rate that P is in the range of .25

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to .35. From other longevity experiments with MRC-5, during which population size was drastically reduced, we estimated that $P = .275$ (*3*). As the last cells to become committed reach the end of their incubation period, the constant growth rate during stage 2 will be superseded by further slowing down and final cessation of growth of the whole culture, which we refer to as stage 3. Stage 3 corresponds to Hayflick and Moorhead's phase 3, but our stages 1 and 2 should not be confused with their phases 1 and 2 of fibroblast growth.

During stage 2 there should be a constant number of nondividing cells, which provides another easily testable prediction of the commitment theory. In experiments in which the number of dividing cells is scored by measuring the uptake of [^{3}H]thymidine by autoradiography, we would expect that all nuclei would be labeled in stage 1 of culture growth (apart from deaths due to age-independent factors); there would be a constant proportion of nonlabeled nuclei in stage 2, and labeled nuclei would rapidly disappear in stage 3.

We have measured the [^{3}H]thymidine labeling index throughout the life-span of cultured MG-4 cells. We found that during stage 1 the proportion of nondividing cells was 5 percent, but when the population entered stage 2 there was a sudden transition to about 20 percent nonlabeled cells (see Fig. 1). Most of the cells (approximately 80 percent) were still cycling until one or two passages before the end of the life-span. Many other investigators who have used this technique have obtained this result (*12*), although Cristofalo and Sharf (*13*) reported that the labeling index declines gradually throughout the life-span.

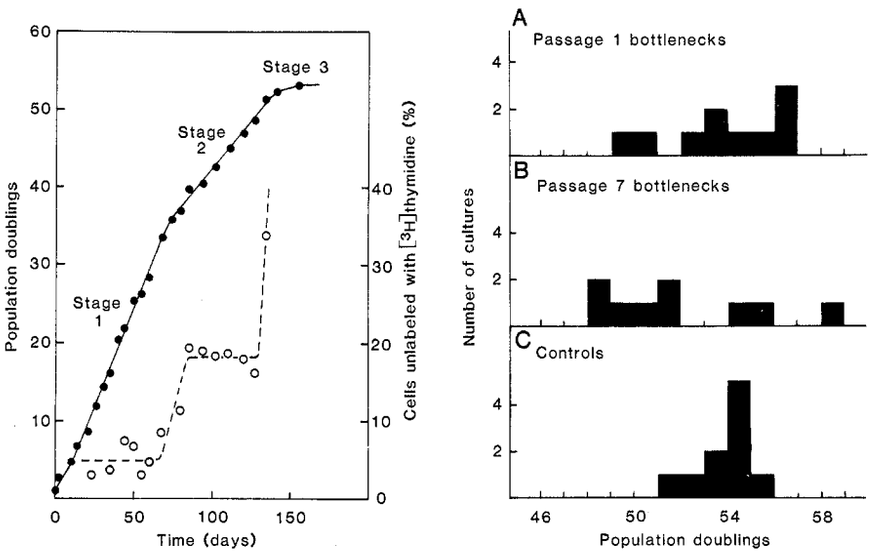

Fig. 1 (left). The growth and [^{3}H]thymidine labeling index of human fetal lung strain MG-4. Lung tissue from a spontaneously aborted 11-week male fetus was cut into fragments and treated with successive portions of 2.5 percent trypsin until it had disintegrated. Cells in suspension were decanted, spun, and washed, and the pooled population resuspended in Eagle's basal medium supplemented with 10 percent fetal bovine serum, 1 percent nonessential amino acids, penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 m*M* glutamine. Approximately 10^6 cells were seeded into a 25-cm^2 flask and the subsequent confluent layer was designated passage 1. Thereafter the cells were cultured in the same medium with a 1:8, 1:4, or 1:2 split ratio (depending on the age of the culture). Confluent cells were harvested with trypsin-Versene (*2*) and counted with a Coulter counter prior to subculture to calculate cumulative population doublings. For autoradiography, 1×10^5 to 2×10^5 cells were seeded onto cover slips in 35-mm petri dishes. The cells were allowed to become attached overnight, and after the addition of 0.5 μCi of [H^3]thymidine (specific activity, 50 Ci/mmole) per milliliter they were left for 48 hours. The cells were washed once in phosphate-buffered saline and then fixed twice, for 20 minutes each time, in a mixture of methanol and glacial acetic acid (3:1 by volume). Each cover slip was then rinsed in absolute alcohol, sequential lower concentrations of alcohol, and then distilled water, and was finally air-dried. The slides were then dipped in photographic emulsion (K5 Ilford U.K.) and kept dark at 4°C for 1 week, after which they were developed with D19 Kodak and fixed with 30 percent sodium thiosulfate. After they were stained with Harris's hematoxylin solution, 600 to 700 cells of each population were scored for labeled and unlabeled nuclei independently by two of us (L.I.H. and R.H.). There were no statistically significant differences between the two sets of observations, and the open circles are the average of the two measurements. Fig. 2 (right). The life-spans of bottleneck and control mass cultures of MG-4. Bottlenecks were set up at passage 1 and passage 7 by seeding 10^4 cells into Linbro wells (1.75 cm^2; Flow Laboratories, Scotland). The primary culture transferred to wells is defined as passage 1; passage 7 corresponds to approximately six further population doublings, or two 1:8 splits. Confluent cells in the wells were subcultured without dilution until standard populations (~ 2.5×10^6 cells) in 25-cm^2 flasks were obtained. Thereafter they were subcultured in these flasks to the end of their life-span (see Fig. 1). Passage 1 cells were subcultured until sufficient cells were available to set up 12 control mass cultures in 25-cm^2 flasks. One of these was lost from contamination and one which grew significantly more slowly than the rest was excluded from the data. All cultures were screened routinely for mycoplasma contamination (*21*) and all tests were negative.

In formulating our original model, we assumed that cells that have ceased division are passively transmitted, that is, they always reattach to the new container surface after trypsinization. However, we noted that this assumption is not critical to our model, since the growth kinetics of the populations and the final life-span are not significantly affected if nondividing cells do not reattach to the surface, or, for example, they are only transmitted once before they are lost from the culture (*4*). The number of nondividing cells in stage 2 depends on the extent of their transmission and also on whether they cease growth in G_1 or G_2 of the cell cycle. For $P = .275$, Table 1 shows the expected proportions of nondividing cells in stage 2 under various assumptions. For reasons of simplicity we also assumed that growth rate is constant throughout the incubation period. This is unrealistic, because as the cells approach the end of their replicative life-span they grow more slowly (*14*); the consequence would be to reduce all the values in Table 1. When Harley and Goldstein (*9*) tested the commitment theory they did not distinguish between assumptions that are crucial to our model and those that were made solely to simplify the mathematical treatment. Adopting our assumptions literally and further assuming that all cells cease growth in G_1, these workers measured the proportion of nonlabeled cells in the later part of the culture life-span of MRC-5 and found that it was much less than 55 percent. As we have explained, this result is not inconsistent with the commitment theory.

The time when uncommitted cells are lost by dilution depends on the population size (N). This provides the means to test our theory more directly, since N can be manipulated experimentally. If N were sufficiently large during stage 1, uncommitted cells would never be lost prior to stage 2 and we could predict that the population would grow indefinitely. Such a result would, in effect, establish the validity of our theory. Unfortunately, if $P = .275$ and $M = 55$ [see (*3*)], for immortal growth (if uncommitted cells do have an unlimited replicative potential) N would have to be at least 10^{10}. Such an experiment would not be feasible in a conventional laboratory, since it would require approximately 1000 large

flasks, or 100 Winchester bottles, at every subculture (*15*).

The theory could also be tested by drastically reducing population size at different times during the life-span, allowing the cells to grow up to the normal population size, and determining their longevity in population doublings. The commitment theory makes specific predictions about the effect of such bottlenecks in reducing life-span. [A bottleneck experiment is one in which the population size is reduced to 10^3 to 10^4 cells which are then allowed to grow back to the normal population size; for a discussion of predictions from such experiments, see (*3*).] We previously used this technique with MRC-5 cells, reducing the population size by about 1000-fold to 2×10^3 cells at passages 8, 13, 21, and 31. In all, the longevity of 51 cultures was measured, including eight controls. We found that bottlenecked populations at the three earlier passages had an average reduction in life-span of eight population doublings, whereas those bottlenecked at passage 31 were not significantly different from controls. Computer simulations showed that this was directly consistent with the result expected, if $P = .275$ and $M = 55$ (*3*). If our interpretation is correct, even cells at passage 8 would have < 0.01 percent uncommitted cells left in the population and these would almost always be lost in the bottleneck. A more critical test of the theory which we have now carried out is to use the bottleneck method at even earlier passages. In this case, we expected that uncommitted cells would sometimes be lost by a 1000-fold reduction in population size, so there would be a subsequent reduction in life-span, but sometimes they would be retained. These later cultures would have a normal or even an increased life-span if, by chance, several uncommitted cells were included in one bottleneck. The upshot would be a wide distribution in culture life-spans, with a few "rejuvenated" cultures. At an even earlier stage of population growth, the number of uncommitted cells would be sufficiently high for bottlenecks to have little or no effect. Cultures would then behave in the same way as control mass cultures (*16*).

It is not possible to obtain MRC-5 cells earlier than at passage 8, since all were frozen at this passage (*17*). We therefore obtained a new primary culture of fetal lung fibroblast strain MG-4, as closely matched to MRC-5 as possible (male, lung tissue at 11 weeks of gestation). At passage 1, the cells were seeded into Linbro wells (1.75 cm^2) at various dilutions. About 70 percent attached to the wells and the smallest populations that grew rapidly to confluence were from inocula of 10^4 cells. Ten such populations were grown to the normal size of 2×10^6 cells and the longevity of these was determined. The same procedure was carried out with passage 7 cells and the longevity of nine bottlenecked cultures was determined. In addition, we monitored ten control mass cultures. Figure 2 shows that passage 1 bottleneck cultures had a wider range of longevities than the controls, but the difference was not statistically significant. The passage 7 bottleneck cultures showed an even broader distribution: there were four cultures with a shorter life-span than any of the controls and one with a longer life-span. Statistical comparison of these distributions (Kolmogorov-Smirnov two-sample test) showed that, despite the small numbers of cultures, this difference was significant ($P < .05$). The results of this second bottleneck experiment are therefore consistent with another prediction of the commitment theory, since bottlenecks with passage 1 cells had no obvious effect on life-span, but a few passages later there was a significant increase in life-span variability (*18*). Our results suggest that MG-4 cells at passage 7 are at a slightly earlier stage in their life-span than MRC-5 cells at passage 8. However, there is no reason to believe that primary or passage 1 fetal lung cells obtained in different laboratories are exactly equivalent, since the number of cells growing from the fragments of tissue cannot be controlled quantitatively.

Table 1. The expected percentage of cells unlabeled with [^{3}H]thymidine during stage 2 of growth (see text), if one assumes that the probability of commitment is .275.

Fate of noncycling or dead cells	Cell death in	
	G_1	G_2
Immediate detachment	27.5	0
Transferred once	39.1	20.7
Transferred indefinitely	55.0	45.7

Although the commitment theory can, in principle, explain the variation in the growth potential of individual cells within a culture (*19*), we recognize that our model, as currently formulated, is not compatible in detail with all the results obtained. For example, we would not predict that the daughters of committed cells would produce clones of different size (*8*). Obviously, the assumption of a rigid incubation period for all committed cells is an oversimplification that experimental data may help to modify into a more realistic form. In any case, the commitment theory rests for its validity on events relatively early in cell culture life-span, rather than on the terminal growth of clones.

If the commitment theory is correct, the finite growth of laboratory populations of diploid fibroblasts is due only to the dilution out of uncommitted cells and not to an intrinsic limitation in the replicative potential of all individual fibroblasts. Contrary to the assertion of Harley and Goldstein (*9*), we never stated, or even implied, that fibroblasts do not age in culture, or that such cells do not provide excellent material for studying cellular or molecular changes during aging. One of our aims in formulating the theory was to try to explain the difference in growth potential between diploid and transformed cells. It is easy to show that a reduction in either of the parameters P (the probability of commitment) or M (the incubation period) can change a population of a finite life-span into one that can be propagated indefinitely (*3*, *4*). We predict that permanent lines may contain many cells with limited replicative potential, as indeed seems to be the case for HeLa (*20*).

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Calcium-Induced Cell Death: Susceptibility of Cardiac Myocytes Is Age-Dependent

Abstract. *Rat hearts perfused with calcium-free media exhibit an age-dependent response when a normal calcium concentration is restored to the perfusate. The response of 15- and 26-day hearts is an explosive cell death which includes immediate cessation of contractile activity, as well as release of large amounts of creatine phosphokinase activity and myoglobin. Between birth and 15 days of age, the sensitivity of the heart to this calcium-induced cellular disruption changes from a relative insensitivity before 6 days of age to a slight but constant sensitivity from 7 to 11 days of age, and to full sensitivity at 15 days of age. This pattern of sensitivity may be due to the rapid increase in complexity and quantity of the membrane systems of cardiac myocytes during the first 15 days of growth.*

Calcium ions are important in the maintenance of normal cell structure and function. They are thought to be integral constituents of the cell membrane, both within the lipid bilayer and in the adjacent basement membrane (*1*). The cardiac myocyte is dependent on the presence of calcium ions in the extracellular fluid for excitation-contraction coupling (*2–4*) and for the structural integrity of its surface membrane (*5–7*). A sufficient concentration of calcium ions (approximately 50 μM) must be present in the extracellular fluid to preserve the structural integrity of the adult cardiac sarcolemma and its adjacent basement membrane (*7–10*). Perfusion of adult hearts with calcium-deficient solutions results in alterations in the capacity of individual myocytes to control the flux of calcium ions across their surface membrane (*7, 9, 11*). In a typical experiment, perfusion of the heart with calcium-free solution, followed by reperfusion with solutions containing normal plasma levels of calcium, leads to rapid cessation of contractile activity, to contracture of the heart, and to massive release of intracellular components such as creatine phosphokinase (CPK) and myoglobin (*9, 10, 12–15*).

This explosive cell death, termed the "calcium paradox" by Zimmerman and colleagues (*12, 13*), has been studied extensively in perfused preparations of adult hearts. The sensitivity of young hearts to calcium-deficient solutions has not been studied by perfusion techniques. However, myocytes prepared from neonatal hearts according to standard procedures (with calcium-free media and selective proteolytic enzymes) do survive when subsequently cultured in calcium-containing medium (*16*). Since myocytes prepared from adult hearts do not survive long when cultured in calcium-containing media (*17*) we speculated that the neonatal heart has a different sensitivity to a transient exposure to calcium-free medium than does the adult heart. Therefore, we applied the nonrecirculating Langendorff perfusion technique to hearts from 3- to 26-day-old rats and examined the development of sensitivity to calcium-induced cell death. We report here that the sensitivity of the rat heart to calcium-induced cell death is absent before 7 days of age and is fully developed by 15 days. From 7 to 11 days the heart exhibits a constant but minor sensitivity to changes in extracellular calcium concentrations.

As shown in Fig. 1, the 26-day hearts showed rapid tissue disruption and contracture when calcium was reintroduced into the perfusing medium after 20 minutes of calcium-free perfusion. The amounts of CPK activity and myoglobin released into the perfusing medium during the calcium repletion period represented 36 percent of the total CPK activity and 35 percent of the total myoglobin released by the 26-day hearts during the 70-minute collection period used in our study (Table 1).

In contrast to the adult heart (*13, 15*) and the 26-day heart, the 3- to 6-day hearts resumed and maintained weak rhythmic beating during the entire 20-minute calcium repletion period. The 3- to 6-day hearts did not undergo contracture or release CPK activity and myoglobin during the calcium repletion period (Fig. 1 and Table 1). The total amounts (per milligram of heart, dry weight) of CPK activity and myoglobin released by the 3- to 6-day hearts were not significantly different from the total amounts released by hearts of rats in the other age groups studied (Table 1). If hearts from all of the age groups were affected similarly by the calcium depletion-repletion procedure, the amount of CPK activity released (per milligram of heart, dry weight) during calcium repletion would be the same for each heart studied. Thus the data in Table 1 indicate that, unlike the 26-day hearts, the 3- to 6-day hearts are not sensitive to a transient depletion of extracellular calcium.

Of the 7- to 11-day hearts, 65 percent resumed very weak beating during the calcium repletion period. The beating differed for each heart studied; that is, the beating was unstable with respect to rate, rhythm, and strength and often did not continue for the entire calcium repletion period. Although these hearts did not undergo contracture, they did release small amounts of CPK activity during calcium repletion (Table 1 and Fig. 1). The release of CPK activity (Table 1) by the 7-, 8- to 10-, and 10- to 11-day hearts suggests that a slight sensitivity to the depletion of extracellular calcium first appears in the rat heart at 7 days of age, and that this sensitivity remains unchanged from 7 to 11 days of age. The sensitivity was characterized as slight between 7 and 11 days, since less than 5 percent of the total CPK activity and no detectable myoglobin were released by these hearts during the calcium repletion period (Table 1).

The 15-day hearts developed severe contracture and released large amounts of CPK activity and myoglobin at the start of the calcium repletion period (Fig.

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