We recently published a study (1) in which we used colony size distribution (CSD) to estimate the in vitro proliferative potential of a large number of male and female donors aged 19–90+ years of age. In a commentary published in this issue of the Journal, Cristofalo and colleagues (2) have commented on some differences between the data and interpretation presented in our report and those of other authors. In this article, we respond to those comments in the order in which they were presented.

First, Cristofalo and colleagues “suggest that CSD is not useful as a method for estimating total replicative life span” (2). This observation is based primarily on the failure of the authors to find a significant relationship between the estimated in vitro life span, which we calculated using CSD, and the proliferative potential obtained by technicians at the Coriell Institute for Medical Research, using standard subcultivation techniques (3). They suggested that the different results might have been due to a cohort effect in the cultures we examined.

We consider this unlikely for the following reasons. The correlation between CSD and in vitro life span was observed in studies for numerous cell types over a period of several years. Furthermore, the linear relation [PD (population doublings) remaining = a × (% colonies with at least 16 cells) + b] has very similar coefficients in all cell types tested (4–6). We first showed that the CSD could be used to predict the proliferative potential of normal human cells in culture (4). In these experiments, we used WI-38 (human fetal lung fibroblasts) cells grown and cloned in MCDB 102 with 10% FBS (fetal bovine serum); 10–20 WI-38 cells were seeded in each of several 60 mm tissue culture dishes and incubated at 37°C, 5% CO₂, and 98%–99% relative humidity (20% O₂) undisturbed for 2 weeks. Three independent subcultivation series of WI-38 cells were cultured to the end of their in vitro life span and the CSD determined at 4 different PD for each subcultivation series. Colony size distribution was determined by counting the number of cells in each colony up to a maximum of 256 cells. All colonies with more than 256 cells were considered as a single group. The number of colonies of 1, 2–3, 4–7, 8–15, and so forth, cells were plotted. We found empirically that only the number of colonies with 16 or more cells was linearly related to the number of population doublings remaining (PDR) in the proliferative potential of the culture at the time the CSD was determined. Linear regression analysis revealed the relationship PDR = 0.56 (% > 16) + 0.6, r² = 0.98.

While we have no specific explanation for the inability of Cristofalo and colleagues to reproduce our results, several possibilities exist. In 1980 (6), we noted that since the in vitro life span of mass cultures can vary considerably depending on culture conditions, this exact relationship cannot be expected to apply to mass cultures maintained under culture conditions significantly different than those used in our studies. Since 30% to 50% of the cells fail to reattach at each subcultivation, the larger the number of subcultivations the lower the number of cumulative PD one would expect to obtain (7). Culture conditions that permit wide pH fluctuations and/or significant medium depletion during a subcultivation period might result in substantial cell death and also result in an erroneously low value for measured PD. Thus, the CSD loses its predictive value, as does prior life span determination by serial subculture, when cell culture conditions vary significantly from those originally used. If the culture conditions used at the Coriell Institute were uniformly better or worse than those used by us, the CSD would consistently underestimate or overestimate the actual in vitro life span, but the data actually appear to be random with respect to the estimated life span. Random variation could be caused by any of a number of cell culture conditions. For example, differences between various lots of serum or cell culture media used during the serial subculturing experiments could cause significant variation. Another variable is split ratio. Cells were cultured at a 1:2 or 1:3 split ratio in 15% FBS (3). The 1:2 or 1:3 split ratio could result in fewer apparent PD than the 1:4 ratio we used because of the failure of a substantial fraction of cells to attach at subcultivation. Also, the use of 15% FBS
versus 10% that we used could result in a longer in vitro life span (8,9) or a shorter one if the FBS contained any toxic elements. Thus, while it is not possible to ascertain the precise reason that the results of Cristofalo and colleagues differ from ours, the uncertainties associated with in vitro life span determinations could easily account for major differences.

Cristofalo and colleagues (2) also propose that the CSD measures proliferation rate and cloning efficiency and that, while these parameters may correlate with donor age, they may give an artificially high estimate of proliferative potential of cells derived from young donors or fetal tissue. While it is true that the CSD could be used to measure those two parameters, we took special care to devise experiments that ensured that this was not the case (6). With regard to cloning efficiency, we counted all the clones present, without reference to the number seeded. Concerning growth rate, we based our estimates on the percentage of clones containing 16 or more cells. This value did not change after 2 weeks (6), which was the time allowed for clonal growth. The percentage of colonies with 16 or more cells increased from week 1 to week 2 but not week 2 to week 3. Any cells that had not doubled at least 4 times during a 2-week period would have a doubling time of >88 hours—something seen in a very small percentage of cells in both high- and low-passage cultures (10). A cell culture consists of millions of individual cells, each with a unique life history. While its ultimate proliferative potential is determined by the small fraction of cells with the greatest capacity for division (7), that value is unknowable until the culture has ceased dividing. At any point in time, each cell has the capacity to give rise to a certain number of progeny (11). It is well known (12,13) that the percentage of nonreplicating cells increases with increasing PDL of a culture. Cristofalo and others (12,14) have shown that there is a close relationship between the percentage of nondoning cells and percentage of life span completed. This represents an extreme case of the CSD in that the percentage of cells capable of zero doublings and those capable of 1 or more doublings is determined.

The proliferative potential of the individual cells in a mass culture is dynamic and changes with each cell division (11,15,16). The CSD is a basic property of a cell culture and is a snapshot of the distribution of clonal life span at any particular time in the life history of a culture. As such, it can be used to predict the number of PD remaining in the culture’s life span (6). The percentage of clones of any particular size has a predictive value. For example, a colony consisting of a single cell represents the percentage of nondividing cells. The major difference between CSD and percent nondividing cells is that the relationship of CSD to PDR is linear, whereas that of nondividing cells is logarithmic. Also, in labeling experiments the percentage of nondividing cells will be diluted out by the dividing cells so that long labeling periods are not possible, which may give rise to an artificially low percentage of nondividing cells observed due to failure to label long enough or by dilution.

Cristofalo and colleagues were surprised that we did not see a decreased proliferative potential in cell cultures derived from donors with type I or type II diabetes. However, the relationship between proliferative potential of fibroblasts from diabetic versus normal individuals is, as is the case for the decline of proliferative potential as a function of age, quite controversial. For example, in the 4 references cited by Cristofalo and colleagues in support of decreased proliferative potential in diabetic cells, strong support for this relationship is not presented. In one case (17), it was concluded that there was no significant difference in proliferative potential of fibroblasts from diabetic and normal individuals. In another report (18), by the same authors, a p value of <.04 was obtained, when the data were adjusted for mean age of the groups studied earlier (17). Morocutti and colleagues (19) showed a strong decline in proliferative potential for cultures derived from donors who had diabetic nephropathy but no significant difference between normal donors and diabetic donors without nephropathy. In the fourth citation (20), proliferative potential was not determined. Based on a few positive finding and reinforced by studies examining various cell growth parameters (growth rate, confluent cell density, etc.) in which cells from diabetics were found to resemble those of late-passage normal cells, it has become common for investigators to believe that cells from diabetic donors have diminished proliferative potential compared with comparable cells from normal donors. However, this belief is not well supported by experimental findings.

Cristofalo and colleagues also assert that there are no reports of senescent cells in normal tissue in vivo. Until recently it was impossible to identify senescent cells in vivo due to the lack of reliable markers for senescence. Senescence-associated β-galactosidase (SA β-gal) has emerged as a reliable marker for replicative senescence and so-called stress-induced senescence. Using this marker, several investigators have demonstrated that SA β-gal-positive cells increase with increasing age. It is of particular interest that this occurs in healthy human dermis and epidermis (21), human articular chondrocytes (22,23), human vascular endothelial cells (24), and the epidermis of rhesus monkeys (25). SA β-gal-positive cells have also been found in normal liver, livers from chronic hepatitis cases, and nontumor tissue of hepatocellular carcinoma cases (26). It is of interest that SA β-gal-positive cells are also prominent in prostate epithelial cells of tissue from patients with benign prostatic hyperplasia (27). The presence of SA β-gal-positive cells was found to correlate strongly with high prostate weight, suggesting that this marker occurs more rapidly in diseases that may be due to increases in cell proliferation, thus exhausting the cells’ proliferative capacity earlier in life. Since SA β-gal has proven so reliable in identifying senescent cells in vitro, it seems reasonable to assume that SA β-gal-positive cells in vivo have some characteristics in common with senescent cells in vitro. What would be the definition of senescent cells in vivo? In the context of this discussion, it would seem reasonable to define them as cells that have lost the capacity for further division under physiological circumstances. For this definition to have any meaning, we must also stipulate that the SA β-gal-negative cells of the same tissue should be capable of further division. One approach for identifying such cells in vivo
might be to use a wound-healing model. The animals could be infused with $^3$H-TdR or bromodeoxyuridine to label any cells that went through S phase and, after a suitable labeling period, labeled nuclei and SA β-gal-positive cells would be scored. One would expect that if the SA β-gal cells were indeed senescent, one would rarely see a β-gal-positive cell with a labeled nucleus, whereas a large fraction of the β-gal-negative cells should have labeled nuclei.

**The Use of Fibroblasts as a Model to Study Aging**

What can the study of fibroblast cells in culture tell us about the aging processes in humans? It is clear from numerous studies that there is not a close correlation between the in vitro life span of fibroblasts derived from an individual and the age of the individual. However, the number of PD that cells from a donor can achieve is a blunt instrument that cannot detect significant changes in the life history of cells from the original sample. As we pointed out before (1), (as an extreme example) complete loss of the ability to divide in 999 out of 1000 cells in a donor sample would result in a decrease of only 10 PD (which is generally considered to be within the range of experimental variation) in the measured proliferative potential of the resulting culture. Therefore it is easy to see that major changes in the proliferative state of skin fibroblasts would remain undetected in experiments that seek to determine whether there is a correlation between donor age and proliferative potential of their fibroblast using traditional methods. Are there other experimental paradigms that can give more-definitive results? In vitro labeling to detect DNA synthesis during wound healing is hardly practical in humans but perhaps possible in nonhuman primates. CSD of cells dissociated from the tissue may give a snapshot of the proliferative state of the cells in a sample, but there is a possibility that cell damage during dissociation may create artifacts. Although it may be difficult to resolve this question, as noted by Cristofalo and colleagues, it is clear that a number of important results have been obtained from the study of in vitro cellular aging and senescence. Furthermore, as we pointed out (1), many cell types (28) exhibit a stronger decline in proliferative potential as a function of donor age than do fibroblasts.

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