A Question of History

In the May, 1993, issue of BioEssays, *a review of several recent books on senescence by Dr C. S. Downes was published in our features column, 'What the Books Say'* (BioEssays, *vol.* ***15****, pp. 357-360). This article touched on the contributions of Dr Leonard Hayflick, the discoverer of the 'Hayflick Limit', as discussed and interpreted by the joint authors of one of the books reviewed. Dr Hayflick has taken exception to this interpretation and we print his response below. The authors of the book in question, Drs L. A. Gavrilov and N. S. Gavrilova, were invited to reply to his statement, and their remarks are printed here, followed by Dr Hayflick's response to their comments. Finally, this correspondence is completed with a statement from an outside observer, Dr Robin Holliday, an acknowledged expert in the field of cellular senescence.*

Ed.

From Leonard Hayflick

Sir,

In his review of '*The Biology of Life Span: a Quantitative Approach*' by L. A. Gavrilov and N. S. Gavrilova (*BioEssays*, **15**, No. 5, May 1993, page 359), Dr C. S. Downes, the Book Review Editor, devotes more than half of his review to a discussion of my work and the Gavrilovs' attack on me personally. For reasons best known to Downes, he has chosen to emphasize the 13 pages in which the Gavrilovs have attacked me, out of the book's 385-page length.

Furthermore, Downes fails to distinguish clearly his own views from those of the Gavrilovs so that readers are left with the impression that he agrees with many of the sentiments of the Gavrilovs. I leave to readers to decide whether the quotations below make clear that the opinions expressed are solely those of the Gavrilovs. The repetition of unsubstantiated allegations in the review by Downes must raise serious questions about the judgement and competence of the Editor.

My concerns are these:

1. Dr Downes states that '*It starts with articles published over 30 years ago, concerning the proliferation of human diploid fibroblasts in culture.*' (See refs 1, 2.)

These references are inappropriate. Swim did not publish articles on '*the proliferation of human diploid fibroblasts*' as Downes states for a remarkably simple reason: Swim and Parker did no cytogenetic studies on the cell populations that they described! In fact, we stated in our original paper[3]: '*Swim and Parker arrived at a similar conclusion, with cell strains assumed by us to be diploid.*' (Emphasis added.) The correct human diploid number was not reported until 1956.

Moorhead and I were the first to show that the finite lifetime of human cultured cells depended on the fact that they were normal and diploid (or, more accurately, that they had the chromosome constitution of the tissue of origin). Swim and Parker reported nothing fundamentally new. They were two of the hundreds of cell culturists who, for more than fifty years before us, observed that most cells in culture were mortal, as they themselves state. And, like all who preceded them, Swim and Parker believed that an improved medium would render the cells immortal. We showed that, unlike immortal cancer cells, normal cells are intrinsically mortal and that media manipulations will not confer immortality upon them.

As a result of the erroneous report of Alexis Carrel that he had developed an immortal chick cell line in the early 1990s and the authentic discovery of immortal mammalian cell lines in the 1940s, the dogma in this field prior to our work was this: All cultured cells capable of dividing will divide indefinitely if provided with the correct *in vitro* conditions. (In fact, this was the reason given for rejection of our paper[3] by *The Journal of Experimental Medicine*, to whom it was first submitted.) The fact that most cultures set from primary tissue between 1900 and 1961 were found to be mortal, also acknowledged by Swim and Parker[1], was interpreted by the cell biology community to occur because the proper culture conditions to provide immortality were unknown.

Our work overturned this dogma of 50 years duration because, unlike workers before us, including Swim and Parker, we showed that: (a) finite cultures consist of normal cells, determined by several criteria including diploidy; (b) the only cells that are immortal are those that are abnormal (cancer-like) in several respects, including karyotype; and (c) the finite lifetime of cultured normal cells is not due to an artifact of cultivation or media composition but is an intrinsic property of all normal cells[3].

2. Downes states that '*The three phases of fibroblast culture – initial lag, proliferative phase and eventual senescence – are now familiar to most mammalian cell biologists. What they may not find familiar is the name of the author of the seminal papers, H. Earle Swim.*'

Swim and Parker describe no phases, make no interpretation of senescence and provide nothing that could be characterized as 'seminal' information. Their work was substantially an iteration of many previous reports. Moorhead and I were the first to: (a) describe the three phases (but not 'lag' as quoted, rather, 'primary culture'); (b) prove that cell mortality is an inherent property of normal cells; and (c) interpret the finite lifetime of cultured normal cells to be senescence, *in vitro*.

3. Downes states, '*For the concepts of cellular senescence* in vitro *and of the finite lifespan of fibroblasts, are generally credited to Leonard Hayflick[3], who from 1961 onwards described his findings (essentially identical with Swim's) and gained general acceptance for them.*'

Downes' statement that my work was '*essentially identical with Swim's*' is an atrocious distortion of the truth because our principle findings (see 1. and 2. above), were never reported by Swim and Parker. I challenge Downes or the Gavrilovs to prove that Swim and Parker made the key observations that we reported.

4. Downes quotes the Gavrilovs to the effect that they attributed Hayflick's success to '*vigorous propaganda*'.

If the Gavrilovs interpret our peer-reviewed publications to be 'vigorous propaganda', then I plead guilty, as must

every other scientist who has ever published the results of their work, including the Gavrilovs themselves.

5. Downes states, '*Hayflick, nevertheless, read and appreciated that paper; at least he cited it in 1961, though never again.*'

This statement is demonstrably false. See, for example, refs 4, 5 and 6.

6. Downes states '*His* (Hayflick's) *publications likewise ignore Weismann.*'

When our paper was written in 1961 we had no knowledge of Weismann's speculation, published in 1898, that somatic cells might be mortal. Are the Gavrilovs (and by implication, Downes) accusing us of being ignorant of everything that was written in the scientific literature in the nineteenth century? If so, I plead guilty as most present-day scientists will, except of course, the Gavrilovs and Downes. In point of fact this speculation by Weismann was not brought to the attention of the modern gerontological community until 1982[7]. Consequently, I have referred to Weismann's speculation in my subsequent publications several times since that date. And why not? I am flattered to learn that our key finding was anticipated by so famous a biologist as August Weismann. What possible reason would we have had not to cite Weismann's speculation, had we known about it in 1961?

7. Downes states that '*The Gavrilovs themselves, and independently E. Bell and co-workers, have observed that fibroblasts in the final "senescent" phase do not in fact die, but simply cease growing; attempts to subculture them, by detaching them from their culture vessels with trypsin, will kill them, but left undisturbed they will live happily for months.*'

Contrary to this quotation, fibroblasts, in the '*final "senescent" phase*' do in fact die. The truth that they may survive for many months in a non-dividing state – a fact known for decades – should not obscure the critical fact that they do eventually die.

8. Downes says '*In appropriate media, indeed it* (senescence) *can be prevented altogether.*'[8]

The cells studied were not karyotypically normal; the *sine qua non* for the expression of senescence. Also, this work has not been repeated.

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From Leonid A. Gavrilov and Natalia S. Gavrilova

Sir,

Although the Weismann-Swim-Hayflick concept of proliferative limit was already discussed in great detail in our book[1] which has received a lot of attention from many scientific journals including *BioEssays*[2], we are very pleased to return to this discussion again by invitation of *BioEssays* and to reply to Dr Hayflick's comments. Since his comments have many very different declarations mixed together, and since not all the readers of *BioEssays* have read our book, we shall start our reply from the very beginning in a chronological manner (in an abridged form since all the details could be found in our book):

1. The idea that the limited lifespan of organisms is determined by the limited capacity of somatic cells for division was originally put forward not by Dr Hayflick in 1961, but by the famous German biologist August Weismann a century ago[3]. Moreover, it was Weismann who postulated that the differences in the longevity of animal species are caused by the different number of generations that the somatic cells of each species can produce (thus, the cells of long-lived species are capable of completing more divisions).

Weismann's idea has received a lot of attention in our century too. For example, the Nobel Prize winner and the founder of gerontology (and the father of the term 'gerontology') Ilya Mechnikov devoted a special chapter for discussing and criticising the Weismann theory of cell division limit in his famous book *Essais optimistes*[4]. Since this book was reprinted many times (at least in 1907, 1908, 1913, 1964, 1987), any scientist interested in aging research had an opportunity to read about Weismann's theory of cell division limit.

2. The author of the first convincing experimental evidence and clear conclusion that animal cells in culture cannot be propagated indefinitely was not Dr Hayflick but another American scientist, Dr H. Earle Swim from Western Reserve University School of Medicine in Cleveland, Ohio, together with his co-authors, Dr Robert F. Parker and Dr R. F. Haff. In 1959 after analysing results from 336 publications, including the results of his own experiments on the serial cultivation of 23 strains of fibroblasts derived from normal tissues of the rabbit and chick embryo[5], as well as 51 strains of human

fibroblasts derived from foreskin, placenta, testicle, uterus and embryonic tissues[6], Dr Swim came to the following fundamentally important conclusions: '*...in most instances where growth occurs the cells eventually undergo nonspecific degeneration* (ref. 7, see p. 145). *The common experience of many investigators indicates that the early cultivation of cells usually follows a characteristic course which can be conveniently divided into three phases. In phase I the cells proliferate rapidly after an initial lag and usually can be transferred serially without difficulty. Phase II is characterized by a decrease in multiplication to a point where it usually ceases and the cells are eventually lost as a result of nonspecific degeneration. This was accompanied at first by an increase in the number of granules in the cytoplasm of the cells; later, degenerating cells were observed and their numbers increased progressively until the bottoms of the flasks were covered with a dense layer of cellular debris...*'[5]. The important conclusion was also drawn that when cells stop multiplying this is not a methodological artifact caused by such factors as inoculum size, toxic media, or their inability of proliferate on glass[6]. Finally, Dr Swim notes that, infrequently, a third stage is recognized by the appearance of actively proliferating cells in phase II cultures (ref. 7, p. 159). It should be emphasized that phases I and II represent the usual pattern, while phase III is a relatively rare event (ref. 7, p. 160). Dr Swim[7] also noted that in these rare cases of reappearance of active proliferation, the new proliferating cells often differ from the original cells both in morphology and growth pattern (cell transformation).

3. In view of the above mentioned quotations from Dr Swim, it is clear that such of Dr Hayflick's statements as: '*Swim...describe no phases... and Moorhead and I were the first to... describe the three phases*' are absolutely wrong.

It is true, however, that Dr Hayflick was the first to declare that limited proliferative capacity is the property of all diploid cells and diploid cells only. Unfortunately, there are problems with this declaration.

Firstly, there are a lot of aneuploid and polyploid cells in 'old' cultures, thus the lack of diploid karyotype *per se* is not sufficient for unlimited proliferation.

Secondly, virtually unlimited proliferation could be observed for normal diploid cells too (if these cells do not embark on terminal differentiation). For example, it turns out that normal diploid mouse embryo cells, which under standard conditions manifest a growth crisis after 7-10 population doublings, may be successfully cultivated without any sign of an approaching growth crisis for at least 200 population doublings. All that is necessary is to change the composition of the culture medium (excluding blood serum and adding a number of ingredients, including the epidermal growth factor). In this case the cells, which are apparently capable of unlimited multiplication, remain diploid and nontumorigenic[8].

Certain normal diploid cells show a practically inexhaustible capability for proliferation not only *in vitro*, but also *in vivo*. For example, it is well known that normal cells of *Drosophila* imaginal discs can proliferate indefinitely if their differentiation inductors are absent[9]. It is also well established that there is not any intrinsic limit to the proliferation of normal hemopoietic stem cells[9]. Thus, the declaration of Dr Hayflick that proliferation limit is an intrinsic property of all normal cells cannot be true.

4. We would agree with Dr Hayflick that cells do eventually die. The only problem is that this declaration means nothing. For example, the atoms of radioactive elements also do eventually die, but they do not age (their 'rate of dying' is constant and does not increase with age). The same is true for cell cultures: there is no evidence for real aging, i.e. age-dependent increase in cellular mortality rates. Instead, the cellular cultures are surprisingly claimed to be senescent and dead simply because they have stopped active proliferation. This is definitely an unacceptable definition of death since according to it all of us have dead brains! It is clear that decrease in proliferation rates is not necessarily a manifestation of cell deterioration and aging; instead, it might be a consequence of 'healthy' cell differentiation (see our book for details). For this reason the so-called 'aging' in cell cultures may not have any relation to the problems of real cellular aging.

5. Finally, we would like to emphasize that Dr Hayflick has made a significant contribution to the promotion of Weismann's ideas, reproducing Dr Swim's experimental results as well as their further development. For this reason in our book we called this scientific approach the Weismann-Swim-Hayflick concept, in historical order. We propose that Dr Hayflick might wish to organize a scientific meeting in 1997 to celebrate the 40th anniversary of Dr Swim's discovery at the School of Medicine at Cleveland where Dr Swim worked. This meeting might be sponsored by the American Federation for Aging Research where Dr Hayflick is a key person, and we would be happy to take part in such a meeting, together with Dr Hayflick and Dr Downes, to discuss the issues of mutual interest. Such a meeting might be interesting to many readers of *BioEssays* too.

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Leonard Hayflick replies

Sir,

1. I am pleased to have never stated that '*the limited lifespan of organisms is determined by the limited capacity of somatic cells for division*' because functional losses in non-dividing cells are equally important contributors to age changes.

2. We never claimed that we were first to report '*that animal cells in culture cannot be propagated indefinitely*' - a fact known since at least the early 1900s[1]. What we did prove experimentally for the first time was (a) contrary to what was believed since the turn of the century, demonstrably normal cells are not inherently immortal if only we could find the proper *in vitro* conditions; (b) that their finite lifetime is not an artifact of culture conditions; and (c) to interpret this phenomenon as aging at the cell level.

Swim and Parker did not prove (a) that their cells were normal; (b) that the failure of normal cells to divide indefinitely is not an artifact of culture conditions; (c) never described three phases; and (d) did not interpret what they observed as a manifestation of aging at the cell level. For the Gavrilovs to argue otherwise, is to prove the obvious – that English is not their first language. They are unable to quote from the references they give in support of their beliefs because to make their points they must put their own revisionist spin on what the authors have actually written.

3. Aneuploidy, and the hundreds of other age-associated changes found to occur in normal cells as they approach Phase III[2], in no way compromises the fact that originally normal diploid cells have a finite lifetime.

The alleged immortal normal mouse cells referred to by the Gavrilovs are karyologically abnormal and the experiment has never been repeated. Drosophila imaginal discs have never been shown to '*proliferate indefinitely*' because transplantations ended after six years and the Gavrilovs fail to understand that it is not elapsed time but number of population doublings that is significant. That number was not determined for the disc cells which undoubtedly underwent few doublings. As for the hemopoietic cells, transplants failed after four grafts, numbers of population doublings were not determined, and the cells were not proven to be normal.

4. By any definition of aging applicable to whole animals, aging occurs in their cultured normal cells. There is, indeed, an age dependent increase in mortality rates in cultured normal cells (e.g. ref. 3). Also, many of the several hundred biological changes that occur in cultured cells as they approach the end of their *in vitro* lifetime are identical to those found in whole animals as they age[2].

5. The scientific meeting that the Gavrilovs propose to be held in 1997 was already held in 1991 in celebration of the 30th anniversary of the publication of our paper[4] and the proceedings published[5]. The Gavrilovs were not invited to that meeting because they do not work in this field, a fact that should be clear from what appears above.

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From Robin Holliday

Sir,

This controversy was sparked off by Stephen Downe's review of Gavrilov and Gavrilova's book in *BioEssays*, in which he wrote '*they have an interesting and important story to tell, which is not as widely known as it should be*'. The story relates to the discovery that human diploid cells have finite proliferative potential in culture. The history of scientific discovery is nearly always of interest, *provided* serious account is taken of the facts.

Everyone knows that August Weismann made a clear distinction between the immortality of the germ line and the mortality of the soma, but the Gavrilovs are incorrect in stating that his prediction of the limited proliferation of somatic cells was also well known. How many have read Metchnikoff's book? It was only when Kirkwood and Cremer (1982) wrote their scholarly review: '*Cytogerontology since 1881: a reappraisal of August Weismann and a review of modern progress*'[1] that his prediction was 'rediscovered', and that is when Leonard Hayflick, myself and many others first learned about it.

When cells were first grown *in vitro*, Alexis Carrel became famous for the continuous propagation of chick cells. He claimed that they had grown for over 30 years, which is longer than the lifetime of the chicken. Later on, human and mouse cells, such as the HeLa and L cell lines, were shown to grow indefinitely. It was therefore not surprising that it became generally accepted, that avian and mammalian cells were 'immortal' in culture. As Hayflick says, many attempts to grow primary diploid cells in culture were unsuccessful, but this was invariably attributed to faulty technique, inadequate media, and so on. This is quite clear from Swim and Parker's 1957 paper[2]. Their aim was to obtain permanent lines from primary tissue, but in this they failed because all their fibroblast cultures died out. They do indeed document their finite proliferation, but much of the paper describes the variations in the media, serum, or addition of nutrients, by which means they hoped to perpetuate growth. They did not attribute the cessation of growth to cellular ageing, but believed that continued growth depended on the interaction, or symbiosis, between different 'physiological types of cells', and that selection of the fastest growing cells lead to the loss of the necessary interaction. They concluded that '*it seems reasonable to assume that with improved media and sufficient persistence, permanent lines of morphologically*

unaltered fibroblasts could be derived from the normal human tissue employed in this study'. This was undoubtedly the prevailing view at the time amongst cell biologists.

Hayflick and Moorhead[3] and Hayflick[4] repeated and confirmed the basic observation, but they also carried out a large number of additional experiments. These showed, amongst other things, that the life span of human cells depended on the number of cumulative divisions, not chronological time, that mycoplasma or other contamination was not responsible, and that cells stored in liquid nitrogen retained their remaining proliferative capacity. They were the first to suggest that the terminal stage of growth, designated Phase III, was a manifestation of senescence at the cellular level. In this connection, Hayflick also showed that foetal fibroblasts grew for more population doublings than those from adult tissue. Since that time innumerable laboratories have confirmed the finite growth of both human and chick fibroblasts, and also that neither became spontaneously immortalised. In addition, at least nine other types of dividing human or bovine somatic cells have been shown to have finite lifespan *in vitro*[5]. What then are we to make of Carrel's early results? Careful historical research by Witkowski[6] indicates that the procedures were defective; in effect, Carrel's assistants made sure, by illicit means, that the cultures kept on growing.

Gavrilov and Gavrilova raise several other points about cellular ageing. They refer to the report that normal mouse cells grow indefinitely in defined media. As Hayflick correctly states, the diploidy of these cells was not established, and it is well known that mouse primary cells can immortalise without changing their phenotype (e.g. contact inhibited 3T3 cells). Human diploid cells have never been shown to grow indefinitely in defined media. They also claim that Swim[7] recognised the significance of three phases of growth (I, II & III), but Swim's phase III is *immortalisation*, not senescence. With regard to ageing *in vivo*, it has been very clearly shown that antibody secreting memory cells have finite proliferation[8], and also that successive transplantation of mammary tissue between isogenic strains of mice cannot be sustained[9]. Similarly, transplantation experiments have shown that haematopoietic cells have limited proliferative capacity *in vivo*[10,11]. The interpretation of this result is debatable, since the limit to growth may be due to the dilution out of immortal stem cells.

The reference to the lifespan of radioactive isotopes, is scarcely relevant to the debate. The significant observation is that diploid cells can divide many times whilst retaining an outwardly normal phenotype. Something is changing during their cumulative growth which leads ultimately to senescence, which is instantly recognisable to anyone who has worked with these cells. They are abnormal in shape and size, become increasingly granular and gradually detach to form debris in the medium. Cumulative change is the major characteristic of biological ageing. It is immaterial that other types of normal cell grow indefinitely. Embryonic Drosophila and mouse cells probably do so, and none of us would be here if germ cells were not potentially immortal. It is an interesting and attractive hypothesis that some dividing cells age because they have lost telomerase, whereas others retain the enzyme and preserve telomeric DNA[12]. This is only one of several proposals to explain the difference between mortal somatic cells and permanent lines.

The history of science is full of examples of valid observations, unaccompanied by any meaningful interpretation. Swim and Parker made the observations, but their interpretation was wrong. Hayflick and Moorhead[3] realised the significance of what they saw, and Hayflick[4] made clear – for the first time – the fundamental distinction between normal diploid somatic cells with finite growth, and transformed heteroploid cell lines. Cytogerontologists have accepted their conclusions with experiments documented in nearly 1000 papers which attempt, in one way or another, to uncover the secret of the 'Hayflick limit' to growth. Almost all correctly refer to the seminal studies, not to Swim and Parker (1957).

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This correspondence is now closed
Ed.