LECTURE

A Brief History of the Mortality and Immortality of Cultured Cells

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Abstract. During the first half of this century it was believed that because cultured normal cells were immortal, aging must be caused by extracellular events. Thirty-five years ago we overthrough this dogma when we discovered that normal cells do have a limited capacity to divide and that aging occurs intracellularly. We also observed that only cancer cells are immortal. Normal cells are mortal because telomeres shorten at each division. Immortal cancer cells express the enzyme telomerase that prevents shortening. Recently, it was discovered that the telomerase gene when inserted into normal cells immortalizes them. There appears to be a relationship between these findings and aging, longevity determination and cancer. After performing the miracles that take us from conception to birth, and then to sexual maturation and adulthood, natural selection was unable to favor the development of a more elementary mechanism that would simply maintain those earlier miracles forever. This failure is called aging. Because few feral animals age, evolution could not have favored animals exhibiting age changes. Natural selection favors animals that are most likely to become reproductively successful by developing greater survival skills and reserve capacity in vital systems to better survive predation, disease, accidents and environmental extremes. Natural selection diminishes after sexual maturation because the species will not benefit from members favored for greater development of physiological reserve. A species betters its chances of survival by investing its resources and energy in increasing opportunities for reproductive success rather than on post-reproductive longevity. The level of physiological reserve remaining after reproductive maturity determines potential longevity and evolves incidental to the selection process that acts on earlier developmental events. Physiological reserve does not renew at the same rate that it incurs losses because molecular disorder increases. These age changes increase vulnerability to predation, accidents or disease. (Keio J Med 47 (3): 174–182, September 1998)

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The Coming of Age of Aging Research

It is almost forty years since I first realized that our finding that cultured normal human cells have a limited capacity to divide and that this might be telling us something about aging.[1] At that time only a few dozen intrepid people in the world did research in biogerontology because the stigma of working in a field recognized for centuries as a black art commanded little scientific respect.

It is only within the past twenty years that the field of research in aging has emerged as a legitimate area for scientific inquiry.

Those using cell cultures in biogerontological research forty years ago were doubly damned because cell culture itself was just emerging from condemnation as a black art.

Although, today, the science of biogerontology is flourishing, it still has far to go before it emerges completely from what has been analogous to alchemy in the middle ages where the main goal was to turn base metals into gold. The popular belief by many non-gerontologists that the goal of gerontology is to make us all immortal is equivalent to the belief that the goal

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of modern chemistry is the transmutation of the elements.

In 1958 I began research on the possible viral etiology of human cancer. At that time several oncogenic viruses had been isolated from rodents and other animals so it seemed reasonable that humans should not be an exception. My research plan was to grow a variety of human tumor cell cultures and normal human cell cultures. The naive idea was to determine whether extracts or supernatant fluids from the cultured cancer cell populations would have some effect on the cultured normal cells. An effect, if found, might be evidence for the presence of a cancer virus. At that time the concept of transforming normal cells to cancer cells *in vitro* was just emerging as a credible concept.

Although it was not difficult to obtain human cancer tissue what was difficult was acquiring normal human tissue – especially from fetuses. We wanted fetal tissue because of the belief that adult human tissue would have a greater likelihood of containing unwanted garden variety viruses that would confound our work.

The Central Dogma

The central dogma in cell culture at that time was that all cultured cells have the potential to replicate indefinitely in culture and when they fail to do so, it was simply a reflection of our ignorance of how best to cultivate them. Thus, it was no surprise when I found that the fibroblast cell cultures that we grew from human fetal tissues were luxuriating for some months and then died. They died, I was taught, because no one knew what culture conditions were required to grow cells indefinitely.

Ever since the development of cell culture techniques at the turn of this century by Ross Harrison,[2] with one exception, every culture of normal cells ever set ultimately died. I will return to the notable exception later.

It was not until Wilton Earle isolated the abnormal mouse L cell line in 1943,[3] that the first authentic immortal cell line was discovered.

By 1960 there were dozens of papers in the literature that described the mortality of cultured cells with the assumption that they died because of our inability to understand how to grow them. And, even culturing presumably normal human cells already had been done. It required no special tricks. For example, Enders, Weller and Robbins did this in 1949[4] when they showed that the poliomyelitis virus could replicate in non-neural cells *in vitro*. The cells they used were fibroblasts, presumably normal, and grown from human neonatal foreskins. That discovery made it clear that the basis for making a poliomyelitis vaccine was established. Enders, Weller and Robbins shared a Nobel Prize for this work a few years later.

Normal Cells Are Mortal

Our finding, reported in 1961[1] that cultured normal human cells are, in fact, mortal, resulted from my observation that of the dozen or so human fetal fibroblast cell populations that were set at random times, only those that had been subcultivated longest had stopped dividing. Cultures only stopped dividing after about fifty population doublings regardless of when in time they were initiated. This was the major clue that diverted my attention from the cancer work and encouraged me to make an excursion into the question of why the cells were dying only after fifty population doublings despite the fact that the same technician, pool of media and group of glassware was being used.

It seemed to me odd that only those cultures that had undergone between 40 and 60 population doublings ceased dividing while younger cultures grown under identical conditions were luxuriating.

What I thought would be a little side excursion lasted for thirty years. I never did return to the cancer project.

The Details

I called the first or primary culture, Phase I, the ten or so months of luxurious growth was called Phase II and the period in which cell replication diminished and ultimately stopped, Phase III.

My curiosity having been piqued, I began a series of experiments with my colleague, Paul Moorhead, a talented cytogeneticist, in an effort to show whether or not our observation was caused by a cultural artifact or a simple error. Paul Moorhead made the fundamental and unique observation that our cell strains were cytogenetically normal. Because inadequate culture conditions had been invoked for the previous sixty years as the reason why all cells, alleged to be immortal, could not be proven to be immortal, our major concern was to prove that our growth conditions were adequate and that the phenomenon we had discovered was the result of an intracellular counter.

Of the dozens of experiments that were done, I will sketch only two. Paul and I designed an experiment that came closest to proving that a finite lifetime is a fundamental property of normal cells. Fortunately, at that time it had just become possible for cytogeneticists to distinguish between cultured male and female cells. One of the most definitive experiments that we undertook was one in which we mixed the same number of male cells at the fortieth doubling with an equal number of female cells at the twentieth doubling. After twenty more doublings of the mixture we found that the only cells present were female. Unmixed control cul-

tures stopped dividing at the anticipated times. The older male cells in the mixture stopped dividing and were overgrown by the younger and still flourishing female cells. Clearly, any virus, toxic factor, nutrient deficiency or cultural condition that might have eliminated the male cells could not be expected to spare the younger female cells. Nor were the young female cells capable of rescuing the older male cells. In fact, any artifact that might be suggested to cause the elimination of the male cell component in the mixture must be one that could discriminate between male and female cells, – a possibility that we considered to be near zero.

Overcoming Intimidation by Dogma

Yet, so intimidated were we by the dogma, that we were still fearful of publishing the results. Paul and I were both fresh out of post doctoral positions and to make a mistake while challenging a central dogma in the entire field of cell biology would have torpedoed any hope of a successful scientific career. We decided to do one more experiment. It does not appear in the methods section of the paper but it had a simple and effective design. We decided to send cultures to three or four of the leaders in the field of cell culture who had expressed grave doubts about our work and cautioned us not to publish our results.

We reasoned that if these experts could culture the cells indefinitely under their superior culture conditions, we would know that we had made a serious error. Cultures at an early population doubling level were sent to several leaders in the field with instructions to call us six months hence when we predicted that the flourishing cultures would cease replicating. All of the recipients called at the predicted time and we decided then to publish. We concluded that if we had made a mistake then so had the leaders in the field.

A final consideration that persuaded us to publish our results was our studies on the behavior of our normal cell strains when inoculated into the hamster cheek pouch, – an immunologically privileged site. It was then known that abnormal immortal cell lines like the L cell or HeLa would proliferate in the hamster cheek pouch and that normal cells would not. Our normal cell strains did not proliferate when inoculated into the hamster cheek pouch. Furthermore, it was legal at that time to inoculate our normal human cells into one of the forearms of terminal human cancer patients and HeLa cells into the other forearm as a control. As expected, the HeLa cells proliferated to form a nodule that was excised but the normal human cell strains did not proliferate. Thus, in addition to karyological results we had another major criterion for distinguishing mortal normal human cell strains from immortal abnormal cancer cell lines.

Finally, we pointed out that normal human or animal cells do not grow in suspension culture.[1] Only abnormal cancer cell lines do.

Our results[1] led us to several conclusions one of the most important of which is that there are two classes of cultured cells, – mortal and immortal and that these two classes of cells have an *in vivo* counterpart.

This relationship was published in 1965[5] and appeared as follows:

Heteroploid Cell : Transplantable		= Diploid Cell : Normal Somatic	
Lines	Tumors	Strains	Tissue
(*in vitro*)	(*in vivo*)	(*in vitro*)	(*in vivo*)
1. Heteroploid		1. Diploid	
2. Cancer Cells		2. Normal Cells	
3. Indefinite growth		3. Finite growth	

This was the first attempt to show that cell populations could be classified into two distinct categories characterized chiefly by whether they are mortal or immortal. Derivative of this relationship is the present interest in the mechanisms that lead to the immortalization of normal mortal cells. Had it not been shown that normal cells are mortal, the concept of immortalization would never have been appreciated. The acquisition of the property of immortality by normal cells is thought by many to be a fundamental underpinning of both aging and cancer research.

The Idea of a Counter

We concluded also that our normal human cells must contain some kind of counting mechanism because of the repeatablity of our finding that fibroblasts from human embryonic tissue underwent a number of population doublings that ended within a narrow range between 40 and 60.

Additional evidence for our belief that the cells must contain a counting mechanism was results of our studies on the cryopreservation of normal human diploid cell strains. This showed that cells frozen at any population doubling level from one to fifty retained memory of their doubling level until reconstitution so that the total number of population doublings traversed both before and after freezing totaled fifty. This observation was also reported in our first paper in 1961[1] and led us to postulate for the first time that an intracellular clock or event counter must be present in normal cells. Thus was born the idea of a putative clock present in normal cells. Today, thirty-five years later, the coalescence of several diverse fields of research has resulted in an explosion of fascinating information that has identified the nature of that clock.

I might add that the ability of WI-38 to remember at what population doubling it is when frozen is as accurate today as it was when I first developed that strain in 1962. After 36 years of cryopreservation WI-38's

memory is retained without loss. This is the longest period of time that viable normal human cells have ever been frozen (Hayflick, unpublished results).

It should be noted that the ability of a normal mortal human cell strain to undergo fifty population doublings does not result in a trivial number of cells. Fifty population doublings will result in the production of twenty million metric tons of cells.[1]

After making the observations that I have just described we decided to publish our results but we needed to offer an interpretation of our findings.

One interpretation was that cultured normal cells are unable to make *in vitro* an essential molecule that can only be made or found *in vivo*. The result would be, that as normal cells divide in culture, they would deplete this hypothetical molecule, much as an automobile might run out of gas, and then the cell would stop dividing. This idea was discounted because if the essential molecule was even as small as hydrogen, then the cells could not reach the fiftieth population doubling without weighing three times as much as we knew they weighed and be composed entirely of hydrogen at the first population doubling. Thus, simple dilution of an essential molecule was ruled out as an explanation for the Phase III Phenomenon.

Is It Aging?

One attractive hypothesis remained. Because the central dogma demanded that all cultured cells are potentially immortal, biogerontologists had quite properly concluded that the ultimate causes of aging did not have an intracellular origin. This was clear because when normal human cells are cultured in the absence of normal control mechanisms of the entire body, and if these same cells when cultured are allegedly immortal then aging cannot be the result of intracellular events. It was for this reason that the focus of attention on what little fundamental work was done in biogerontology during the previous sixty years was diverted to such possible extracellular causes of age changes as radiation, changes in the extracellular matrix, stress, and many other putative non-cellular causes.

Because we had proven that cultured normal human cells were, in fact, mortal, we suggested that the Phase III Phenomenon was a manifestation of aging at the cell level. I expected that this idea would be disproved quickly but of the thousands of papers published in the last 35 years this field (which I subsequently named cytogerontology), none have disproved this suggestion.

In a second paper that I published in 1965,[5] I showed that cell strains derived from older human donors underwent fewer doublings than cultures set from fetal tissue. This lent more weight to the association of the Phase III Phenomenon with aging.

A Practical Use

In addition to our interpretation that the Phase III Phenomenon might be a manifestation of aging at the cell level, our 1961 paper also described that the human diploid cell strains had the broadest and most sensitive human virus spectrum of any cell population then known. WI-38 quickly became a standard cell culture in all virus diagnostic laboratories for the detection of viruses from human clinical specimens. Several new common cold virus strains were quickly isolated using our normal human cells.[6] We also suggested that these normal cells would be a better and safer substrate for human virus vaccine preparation than the then existing and dangerous primary monkey kidney cells.[7] After a ten year struggle with the Division of Biologics Standards, now a part of the FDA, WI-38 became the first normal human diploid cell strain to be used for human virus vaccine production.[8] Today, there are many licensed vaccines produced in WI-38 or similar strains. These include poliomyelitis, adenovirus types 4 and 7, rubella, rubeola, varicella and rabies. All of the rubella vaccine used in the Western Hemisphere today is produced in WI-38. Hundreds of millions of people throughout the world have been inoculated or fed vaccines produced in WI-38 and other human diploid cell strains with no reports of untoward effects traceable to the cell substrate itself.

What Was Found

In Our 1961 Paper We Made Six Major Points

First, unlike any of our predecessors, we proved the cells to be normal in every respect. Second, we demonstrated that loss of replicative capacity was not an artifact of culture conditions that had been believed for the previous sixty years. Third, we interpreted the phenomenon to be a manifestation of aging at the cell level. Fourth we reported that two broad classes of cells existed, – normal mortal cell strains and abnormal or immortal cancer cell lines. Fifth, we suggested that a counting mechanism must exist and fifth, we described the exquisite sensitivity of normal human cell strains to human viruses and proposed that they be used for the manufacture of human virus vaccines.

The Worlds Biggest Rooster

You may recall that earlier I had mentioned that ever since the development of cell culture techniques at the turn of this century by Ross Harrison, with one exception, every culture of normal cells ever set ultimately died. I want to describe this alleged exception.

Interest in vertebrate cell immortality reached its

zenith in the early part of this century when, Alexis Carrel, a noted French cell culturist, surgeon and Nobel Laureate, described experiments purporting to show that fibroblasts derived from chick heart tissue could be cultured serially indefinitely. This work was done by Carrel at the Rockefeller Institute in New York City. The allegedly immortal chick cell strain was voluntarily terminated after 34 years in continuous culture.[9,10] Albert Ebeling, who cultured the cells for most of the 34 years discarded them in 1946, two years after Carrels' death. Ebeling remarked that the mass of cells accumulated since their first establishment in culture would be greater than that of the sun. In 1921 a newspaper, The World, wrote that the cell mass would have formed a "rooster ... big enough today to cross the Atlantic in a stride;... so monstrous that when perched on this mundane sphere, the world, it would look like a weathercock".

The New York World Telegram inquired after the health of the cells at the beginning of each New Year.[11] They even published a premature obituary of the cells in 1940. The Carrel-Ebeling experiment was of enormous importance to biogerontologists because, if true, it implied that cells released from *in vivo* controls could divide and function normally for a period of time greater than the lifespan of the species.

Carrel's results and their interpretation were of vital concern to biogerontologists because they strongly suggested that because isolated cells are immortal, aging must not be the result of events occurring within individual cells.

I have suggested that, although probably unknown to them, Carrel and Ebeling made a serious technical error. The alleged immortal chick heart cell culture was fed in those years with an extract of chick embryo tissue prepared daily and extracted under conditions that permitted the addition of fresh living cells to the alleged immortal culture at each feeding. It had to be assumed that Carrel's chick culture consisted of normal cells. This is so because, until quite recently, no one has ever found an immortal chick fibroblast population. One of these immortal cell lines was produced by my former post-doctoral student Masayoshi Namba, who exposed chick cells to the carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (NMNG) and the other arose spontaneously. In both cases the immortal cells were shown to be abnormal and to produce retroviruses. Thus, they are both abnormal cell lines.

An historian of science has published a series of papers in support of his belief that Carrel may have known about this error but never admitted it.[11-13] Even if this explanation is untrue it is important to note that, despite many efforts, no one has ever confirmed Carrel's work even to the extent of keeping normal chick cells proliferating for as short period of time as two or three years. Since confirmation is lacking and the scientific method demands this, Carrel's studies are invalid.

Rejection

Despite what we thought were several worthwhile contributions, our 1961 paper was not easy to have published. The dogma that we thought we had overturned was so well entrenched that the original manuscript was rejected in 1960 by The Journal of Experimental Medicine. The editor rejected the paper with the statement that "The largest fact to have come out from tissue culture in the last fifty years is that cells inherently capable of multiplying will do so indefinitely if supplied with the right milieu *in vitro*." That belief was tantamount to the belief that, given the right milieu *in vivo*, human beings also will live forever. Ponce de Leon called the right milieu *in vivo* "The Fountain of Youth" and those who believe that cultured normal cells must be immortal, if only the right medium can be found are, like Ponce de Leon, still searching for that fountain of youth after 37 years of trying.

The editor who rejected the paper was Peyton Rous, who, a few years later, was awarded a Nobel Prize for his discovery of the first oncogenic virus.

Paul Moorhead and I were crushed when we learned that the paper was rejected. Fortunately our chief at the Wistar Institute, Hillary Koprowski, knew well an editor of Experimental Cell Research, – George Klein. The paper was sent to him and within two months it was accepted for publication without change. According to the Institute for Scientific Information's Current Contents, the paper[1] was one of the 200 most cited papers in the world for the 21 year period from 1961 to 1982 when the total number of citations reached 1,560. Today, the citations are well over 3,000.

The Telomere Replicometer

As I had reported in 1965[5] cell mortality and immortality are inextricably linked to aging and cancer. Consequently, the importance of identifying the putative counter that we proposed existed would be difficult to exaggerate.

I believe that the counting mechanism should not be called a clock or chronometer because these are devices that measure the passage of time. Because the replicative limit of normal cells is only indirectly related to the passage of time but directly related to the number of cell doublings, or more precisely DNA replications, the putative mechanism should be more properly referred to as an event counter. A device that measures quantity or counts events is called a meter, which would justify the suggestion that the term "replicometer" be used to

designate the putative molecular event counter. We and others have searched for this event counter since 1961 without much success.

In early efforts to determine the location of the replicometer, experimental results were reported in 1975 by my doctoral student Woodring Wright and me, in which the nuclei of old and young cultured cells were fused to the enucleated cytoplasms of opposite aged cytoplasts. The results revealed that the replicometer was located in the nucleus.[14,15]

But, more progress has been made in locating and describing the replicometer in the last ten years than was made in the previous twenty-five years thanks to a remarkable confluence of observations made in several diverse biological fields. (For recent reviews of this rapidly developing field see 16–19).

It had been known at least since a lecture given by Hermann Muller in 1938[20] and the work of Barbara McClintock[21] that the tips of chromosomes contain discrete structures called telomeres. However, the precise role that these structures played in cell replication was unclear. There was some evidence that telomeres prevented chromosomes from fusing to each other end to end and that they permitted the attachment of chromosome ends to the nuclear envelope.

In the early 1970's it was observed that the properties of DNA polymerase prevent it from fully replicating the linear ends of DNA.[22–25] This has been called the "end-replication problem." The problem is the inability of DNA polymerase to completely replicate the 3′ end of linear duplex DNA.

In the late 1960's my Russian colleague, Dr. Alexey Olovnikov, who had just heard a lecture in which my work was discussed, wondered how normal cells might have a limited capacity to replicate as he entered a Moscow subway station.[24] When the train stopped at the station he had a remarkable flash of insight. Olovnikov saw an analogy between the train which represented the DNA polymerase and the track which represented the DNA. If the train engine was imagined to be the polymerase that replicated the DNA track, the first segment of DNA would not be replicated because it was underneath the engine at the start. This was analogous to the "end-replication problem." Olovnikov realized that this repeated shortening of the DNA molecule at each round of DNA replication would shorten the DNA molecule and might be the explanation for my finding that normal cells can only replicate a specific number of times.[24]

Because the loss of DNA that contained vital genetic information at each division seemed unlikely, Olovnikov reasoned that the telomeres might consist of some repeated nucleotide sequences that did not contain any genetic information but behaved much like a buffer. At each round of DNA replication the buffer would simply loose what portion of the DNA molecule was not copied (the telomeric ends) and thus protect the downstream genes. The length of the buffer would thus determine the number of rounds possible for DNA replication.

Olovnikov's imaginative solution to the "end replication problem", although published in both Russian[22] and English,[23] languished in the literature until several discoveries commencing in the late 1970's began to support his armchair speculations and in the past decade proved them substantially to be correct.

Telomere Structure Discovered

In 1978 Elizabeth Blackburn, working with the ciliated protozoan, *Tetrahymena*, found that the telomeres consisted of a simple sequence of hexameric repeats of the nucleotides TTGGGG.[26] It was later found that the telomere repeat sequence in human cells was TTAGGG.[27] Like other eukaryotic organisms the telomeres in human cells consist of thousands of repeats of the sequence TTAGGG. It is now known that this sequence is highly conserved and is identical from the slime mold to humans.[28]

Calvin Harley, who had worked for several years with my system of senescent human cells, had a fortuitous discussion with Carol Greider and both decided to explore the possibility that the limited proliferative capacity of cultured normal cells might be explained by diminishing telomere length. They made the surprising finding that the mean telomere length decreased by 2 to 3 kilobase pairs (kbp) during the entire *in vitro* lifetime of several strains of cultured normal human diploid fibroblasts.[29]

In 1992[30] they reported that the decrease was progressive and averaged fifty base pairs for each population doubling. The telomere shortening seen in aging normal human fibroblasts also occurs in many other normal cultured cell types. It is not an artifact of cultured cells because it is also manifest *in vivo* in skin epidermal cells,[31] peripheral blood leukocytes, colon mucosa epithelia[32] and many other normal cell types.

Allsopp *et al.*[32] reported that after analyzing the cultured normal fibroblasts from 31 human donors, aged from several months to 93 years, a striking correlation, valid over the entire age range, was found between replicative capacity and initial telomere length. Thus, cell strains with shorter telomeres underwent significantly fewer doublings than those with longer telomeres. The authors suggested that telomere length was a biomarker of somatic cell aging in humans and that this is consistent with a causal role for telomere loss in aging. They also reported that fibroblasts from Hutchinson-Gilford progeria donors had short telomeres consistent with their reduced division potential *in*

vitro. Telomeres from sperm DNA did not decrease with donor age suggesting that a mechanism for maintaining telomere length may be active in the germ line.

Telomeric shortening, which occurs in several classes of dividing normal somatic cells, may be the replicometer that determines the number of times that a normal cell is able to divide. Once a critical or threshold number of telomeric $(TTAGGG)_n$ repeats is reached, downstream events are presumably triggered that signal the cells to stop dividing. An alternative explanation of how telomere shortening acts as a biological clock has been offered by Wright and Shay.[33] Their telomere positional effect explanation of cell senescence is based on a novel two-stage model.

Achieving Immortality

The essential remaining question in this fascinating story is this: How does that class of cells that we identified as immortal[5] avoid telomere shortening that, if it occurs, would lead to their demise?

The answer to this critical question originated in studies with *Tetrahymena* by Greider and Blackburn,[34] who discovered the ribonucleoprotein enzyme terminal transferase called telomerase. They found that telomeres are synthesized de novo by telomerase, a ribonucleoprotein enzyme that extends the 3′ end of telomeres and thus elongates them. This ribonucleoprotein complex contains a reverse transcriptase and RNA template for the synthesis of the repeated sequence.[35] It was simultaneously reported that cancer cells have shorter telomeres than do adjacent normal cells[36,37] thus providing the first link for the role of telomeres in cancer biology.

Telomerase was later found to occur in extracts of immortal human cell lines[38,39] and in about 90% of all human tumors studied.[18] The telomerase RNA component was cloned a few years ago[40] and subsequently the catalytic portion of the enzyme was cloned.[41] This enzyme is the only known reverse transcriptase that is necessary for normal cell activity.

Unlike normal mortal cultured cell strains, immortal cultured abnormal cell lines, produce telomerase. Thus, the telomeres of immortal cells do not shorten with serial passage *in vitro*.[29]

In recent years telomerase has also been found to be expressed in several classes of normal cells. These include fetal tissue, normal bone marrow stem cells, testes, peripheral blood lymphocytes, skin epidermis and intestinal crypt cells (For references, see 18). All of these cells have high turnover rates or are in a continuously replicating pool of differentiating cells. It is important to note that the level of telomerase activity found in these normal cell populations is significantly less per cell than that found in cancer cell populations.[18]

The observation that telomeres shorten as normal cells divide, provides the first evidence for the putative replicometer.[29] This, in combination with the discovery of the enzyme telomerase,[34] has gone very far in explaining why most normal somatic cells have a finite capacity to replicate *in vivo* and *in vitro* and how immortal cancer cells might circumvent this inevitability.

Early this year it was reported that normal, mortal, human cell strains could be immortalized with retention of their normal properties by transfecting them with vectors encoding the human telomerase catalytic subunit.[42] Thus, the normal longevity determination mechanism of telomere shortening in normal human cells has been circumvented. This has provided direct evidence proving the role of telomere shortening in cell senescence and telomerase expression in cell immortality.

This discovery has profound theoretical and practical implications that include the immortalization of highly differentiated normal human cell types for the production of medically important proteins.

Because exquisitely sensitive methods exist for the detection of telomerase in a single cell, this procedure will likely be exploited as a sensitive diagnostic tool to detect the presence of cancer cells in clinical specimens. Other researchers are exploring the possibility that telomerase inhibitors might be found that could be used therapeutically in the treatment of cancer.

Telomeres As Longevity Determinators

I would like to suggest an alternative hypothesis for the role of telomeres in aging. I propose that telomere shortening may be the molecular equivalent of longevity determination which is different from aging and is defined as follows:

Because few feral animals age, evolution could not have favored a genetic program for age changes. Natural selection favors animals that are most likely to become reproductively successful by developing greater survival skills and reserve capacity in vital systems to better survive predation, disease, accidents and environmental extremes. Natural selection diminishes after reproductive success because the species will not benefit from members favored for greater longevity.

The level of physiological reserve remaining after reproductive maturity determines longevity and evolves incidental to the selection process that acts on earlier developmental events. Physiological reserve does not renew at the same rate that it incurs losses because after reproductive success, molecular disorder increases at a rate greater than does capacity for repair. This increase in molecular disorder is aging and increases

vulnerability to predation, accidents or disease (For review see 43).

As has been reported over the past thirty-five years, hundreds of biological changes occur in normal cells as they replicate *in vitro*.[44] These changes are the result of increasing molecular disorder and all compromise the internal milieu ultimately leading to loss of cell function. Thus, the number of population doublings that a normal cell is capable of undergoing and that is determined by telomere length may be the *in vitro* expression of maximum potential longevity. The molecular disorders that herald the approaching loss of replicative capacity, and diminution of telomere length, are age changes. When this same molecular disorder occurs in cells *in vivo*, these age changes lead to an increase in vulnerability to disease or pathology which results in death well before maximum longevity is reached.

Struggles

When I reflect on the events surrounding my work over the past forty years I am forced to observe that it has been a continuous struggle with naysayers. From the time that our paper describing the finite lifetime of cultured normal cells was first rejected in 1960, nothing reported in that paper was ever immediately embraced. Decades passed before the fundamental observation was accepted (and there are still doubters), to say nothing about the controversy that was generated by our suggestion that the Phase III Phenomenon was telling us something about aging. Our observation that the immortalization of normal cells was now possible was not appreciated until recent years and our suggestion that normal human cells be used for human virus vaccine production resulted in a controversy that was finally won after ten years of formidable struggles with several national control authorities.

Even my assertion that I had intellectual property rights in WI-38 resulted in the necessity that I file a law suit against the NIH. After seven years of litigation I won that suit and WI-38, which was confiscated from my laboratory by several kindly public servants from the NIH, was returned to me and the principle of title to self-duplicating systems established. This resulted in a substantial contribution to the birth of the biotechnology industry in which it was now legal to use self duplicating biological materials developed in whole or in part with federal funds for commercial exploitation. Until our suit was won that would have been a criminal act.[8,45,46]

Upon reflection on these events it seems that they have resulted in a better understanding of several aspects of cell behavior, a greater knowledge of the aging process and a practical benefit for human health. I could not have hoped for more than this.

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