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Replicative Hayflick Limit

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Abstract

In the mid-20th century, Leonard Hayflick discovered that normal human cells have a limited number of divisions, which became a significant contribution to the study of replicative aging. Together with Paul Moorhead, he demonstrated that the replicative limit of cells is a fundamental biological feature. Their research led to the concept of the replicometer and the idea of telomeres as a mechanism that restricts cell divisions. Alexey Olovnikov proposed a hypothesis explaining the shortening of telomeres during each replication cycle. The discoveries of Hayflick, Olovnikov, and Blackburn laid the foundation for research into replicative immortality, which is of critical importance for understanding replicative aging and cancer. Research has identified the existence of two classes of cells—replicatively mortal and replicatively immortal. To this day, the replicometer remains to be discovered.

Keywords: Replicative Limit, Replicative Aging, Hayflick, Fibroblast Cultures, Telomeres.

In the mid-20th century, Leonard Hayflick discovered a phenomenon that revolutionized our understanding of cellular biology: normal human cells in culture have a limited capacity for division (Hayflick, L., & Moorhead, P. S., 1961). This groundbreaking observation provided crucial insights into the processes of aging. At the time, only a small number of researchers around the world ventured into the field of biogerontology, which was considered obscure and lacked prestige in the scientific community. The discipline, rooted in ancient tales such as the mystical practices of Aietes and Medea, was viewed with skepticism and often dismissed as unscientific.

The prevailing dogma in cell culture studies of that era held that all cells were inherently capable of infinite division. When cells ceased to proliferate, it was presumed to result from insufficient knowledge about optimal cultivation conditions. Therefore, Hayflick's observations initially did not attract much attention. His work with human embryonic fibroblast cell cultures revealed a pattern: the cells flourished robustly for months before they inevitably stopped dividing and perished. This cessation of proliferation was commonly attributed to unknown limitations in culture techniques or nutrient formulations.

Ever since Ross Harrison developed techniques for cell culture in the early 20th century (Harrison, R. G., 1907), every normal cell culture, with one notable exception, ultimately succumbed to death. This was accepted as a technical inevitability. However, in 1943, Wilton Earle successfully isolated the first immortalized cell line, known as L-cells, from mouse fibroblasts (Earle, 1943). This significant achievement marked a turning point in cellular research and set the stage for Hayflick's later discoveries.

In 1949, a group of researchers, including Enders, Weller, and Robbins, achieved a milestone in cell culture by demonstrating that the poliovirus could replicate in non-neuronal human embryonic cells (Enders et al., 1949). The cells they used were fibroblasts derived from the foreskin of a newborn, believed to be normal at the time. These experiments further popularized the cultivation of human cells, making it accessible and widely adopted.

Hayflick's keen observations revealed that human fibroblast cultures, initiated at different times, exhibited a remarkable consistency: they stopped dividing after approximately 50 population doublings, regardless of the cultivation conditions. He classified the stages of cell culture into three phases. Phase I represented the primary culture when cells were freshly isolated. Phase II encompassed a period of vigorous growth lasting several months. Phase III marked a decline in cell division, culminating in the cessation of proliferation and cell death. Motivated by these findings, Hayflick embarked on a series of experiments in collaboration with cytogeneticist Paul Moorhead. Their objective was to determine whether the observed limit on cell division was an artifact of culture conditions or a fundamental biological phenomenon. They conducted a pivotal experiment by mixing equal numbers of male cells (bearing XY chromosomes) at their 40th division with female cells (bearing XX chromosomes) at their 20th division. The mixed culture was monitored for 20 additional population doublings. Astonishingly, only the younger female cells persisted, while the older male cells ceased to divide. Control experiments confirmed this outcome: younger cells consistently outcompeted older ones, disproving hypotheses that culture conditions, toxins, or nutrient deficiencies were responsible.

This series of experiments provided a groundbreaking insight: older cells lose their proliferative capacity earlier than younger cells, even under identical conditions. It became evident that the cessation of division was not due to external factors but was instead an intrinsic property of normal cells. To further substantiate their findings, Hayflick and Moorhead sent samples of cell cultures to other leading experts in the field of cell culture, individuals who had initially expressed skepticism about their results. These samples, provided at early stages of division, came with instructions to monitor them for six months—the period during which Hayflick and Moorhead predicted that the cultures would stop dividing. As anticipated, every recipient reported that the cultures ceased proliferation, further validating the original observations. In another crucial experiment, human cells were transplanted into the cheek pouch of hamsters, a site that is immunologically privileged and does not provoke an immune response. Unlike abnormal, immortalized cell lines such as L-cells or HeLa cells,

which proliferated unchecked in this environment, normal human cells failed to grow. This result highlighted the profound differences between normal, replication-limited cells and immortalized, abnormal cells commonly associated with cancer.

Additionally, Hayflick and Moorhead tested normal human cells by inoculating them into terminally ill cancer patients. In these experiments, normal cells were transplanted into one arm of the patient, while immortal HeLa cells served as a control and were transplanted into the other arm. As expected, the HeLa cells proliferated and formed a nodule, while the normal human cells showed no evidence of growth. These findings, coupled with cytogenetic analyses, solidified the distinction between normal replicative mortality and the unchecked replication characteristic of cancer cells.

One of the most profound conclusions from Hayflick and Moorhead's work was the identification of two distinct classes of cells: replicatively mortal and replicatively immortal. Furthermore, they posited that these classes have corresponding counterparts in vivo, with replicative immortality often associated with cancer. Their work laid the foundation for subsequent research into the mechanisms that govern cellular aging and immortalization, two phenomena that are deeply intertwined with the broader processes of aging and oncogenesis (Hayflick, 1965). The discovery of the replicative limit introduced the concept of a hypothetical intracellular mechanism, later termed the "replicometer," which acts as a biological counter, limiting the number of population doublings. This concept was supported by observations in cryopreservation experiments. Human diploid cells frozen at different population doubling levels—from one to fifty—retained a "memory" of their replication history. Upon thawing, these cells resumed proliferation but ceased dividing once they reached the cumulative limit of 50 population doublings. This remarkable consistency strongly suggested the existence of an intrinsic "biological clock" governing cellular lifespan.

Hayflick's unpublished observations further reinforced the robustness of this mechanism. For instance, the WI-38 cell strain, developed by Hayflick in 1962, demonstrated its capacity to retain division memory after decades of cryopreservation. Even after 36 years of storage, WI-38 cells preserved their replication history without any loss, representing the longest known preservation of viable human cells in frozen conditions.

Alternative explanations for the cessation of cellular division were proposed but ultimately rejected. One hypothesis suggested that normal cells might deplete an essential molecule in culture that is only available in vivo. However, calculations demonstrated that such a molecule would have to be replenished rapidly, as cells continued dividing for up to 50 doublings. A depletion model akin to a car running out of fuel was deemed implausible.

This left one viable explanation: the limit on cellular division must be due to intrinsic molecular processes. For decades, the dominant view had been that cells in culture were immortal and that aging resulted from extracellular factors such as radiation, environmental stress, or changes in the extracellular matrix. Hayflick and Moorhead's findings challenged this

paradigm, proving that normal human cells were inherently limited in their replication potential. This revelation suggested that cellular aging, or "Phase III," could represent a manifestation of organismal aging at the cellular level.

Further experiments supported the link between cellular aging and the replicative limit. For example, it was observed that fibroblast cultures derived from older donors underwent fewer divisions compared to those derived from embryonic tissues. This correlation reinforced the hypothesis that the phenomenon of "Phase III" is intrinsically tied to aging processes.

The field of cellular immortality was not without its controversies. Early in the 20th century, Alexis Carrel, a Nobel Prize-winning surgeon, claimed that fibroblasts derived from chicken heart tissue could divide indefinitely in culture. Carrel's findings suggested that isolated cells were inherently immortal, implying that aging was not a result of intracellular processes. His experiments, however, were later called into question. It was revealed that his cultures were maintained using daily additions of embryonic chicken tissue extract, inadvertently introducing fresh cells into the culture. Despite repeated attempts, no other researcher was able to replicate Carrel's results, leading to the conclusion that his findings were invalid.

The revelations stemming from Hayflick and Moorhead's work on cellular aging spurred further investigations into the mechanisms underpinning replicative mortality and immortality, which were increasingly recognized as fundamental to understanding both aging and cancer. The critical importance of identifying the hypothetical replicometer proposed by Hayflick and Moorhead could not be overstated. The replicometer, they hypothesized, was a molecular mechanism that tracked the number of cellular divisions, dictating the replicative lifespan of normal cells.

At the same time, advancements in chromosomal biology offered tantalizing clues about the nature of this replicometer. Decades earlier, scientists such as Hermann Muller (Muller, 1962) and Barbara McClintock (McClintock, 1941) had described specialized structures at the ends of chromosomes, now known as telomeres. These regions were thought to prevent chromosomes from fusing end-to-end and to assist in their attachment to the nuclear envelope during cellular processes. However, the precise role of telomeres in regulating cell division remained unclear.

In the early 1970s, research revealed a critical problem in DNA replication: the so-called "end-replication problem." DNA polymerase, the enzyme responsible for copying genetic material, was unable to fully replicate the ends of linear DNA strands (Watson, 1972). This limitation meant that with each replication cycle, a small portion of DNA at the chromosome ends remained unreplicated, leading to progressive shortening of the telomeres.

Around the same time, Alexey Olovnikov independently hypothesized that this progressive shortening of telomeres might explain why normal cells have a limited capacity to divide (Olovnikov, 1996). As he traveled on the Moscow metro, Olovnikov imagined DNA polymerase as a train on a track, representing the DNA strand. The enzyme, like a locomotive, could not

replicate the segment of DNA beneath it at the track's starting point. Olovnikov proposed that telomeres function as a buffer zone of repetitive, non-coding sequences, which are gradually shortened with each replication cycle. Once the telomeres reach a critically short length, the cell can no longer divide, triggering senescence.

This groundbreaking idea was supported by later discoveries. In 1978, Elizabeth Blackburn and Joseph Gall, working with the ciliated protozoan Tetrahymena, identified that telomeres consisted of simple, repetitive sequences of six nucleotide pairs (TTGGGG) (Blackburn & Gall, 1978). In humans and other vertebrates, this sequence was later found to be TTAGGG, repeated thousands of times to form the telomeric regions of chromosomes. Notably, this sequence was highly conserved across species, underscoring its evolutionary importance (Henderson, 1995).

Further research revealed that telomere shortening occurred not only in cultured cells but also in vivo. Studies demonstrated that telomere length progressively decreased with age in various human cell types, including skin epidermal cells (Lindsey et al., 1991), peripheral blood leukocytes, and epithelial cells of the colon (Allsopp et al., 1992). These findings provided the first tangible evidence for the existence of a replicometer and its potential role in aging.

Allsopp et al. conducted a seminal study analyzing fibroblast cultures from 31 human donors ranging in age from a few months to 93 years. They found a striking correlation between the initial length of telomeres and the replicative capacity of the cells. Fibroblasts with shorter telomeres underwent fewer divisions compared to those with longer telomeres. The researchers concluded that telomere length serves as a biomarker of replicative aging in human somatic cells, lending support to the hypothesis that telomere shortening is causally linked to the aging process. Furthermore, fibroblasts derived from donors with Hutchinson-Gilford progeria syndrome—a condition characterized by accelerated aging—were found to have abnormally short telomeres and reduced replicative potential in vitro.

Interestingly, sperm cells, which play a critical role in reproduction, were found to maintain telomere length regardless of donor age. This observation hinted at the existence of a molecular mechanism capable of preserving telomere length in specific cell types.

The discovery of telomere shortening as a key driver of replicative aging was soon followed by the identification of telomerase, an enzyme capable of counteracting this process. In studies of Tetrahymena, Blackburn and Carol Greider identified telomerase as a ribonucleoprotein enzyme that synthesizes telomeric repeats de novo, effectively extending the length of telomeres (Greider & Blackburn, 1985). Telomerase contains two critical components: a reverse transcriptase enzyme and an RNA template used to synthesize the repetitive sequences (Shippen-Lentz & Blackburn, 1990).

Subsequent research revealed that telomerase activity was absent in most normal human somatic cells, which explained why telomeres shorten over successive divisions. However, telomerase was found to be active in certain cell types,

such as germ cells, stem cells, and cancer cells. This discovery established a direct link between telomerase activity and the phenomenon of replicative immortality. Indeed, telomerase was detected in approximately 90% of all human cancers studied, highlighting its critical role in tumorigenesis (Hastie et al., 1990; de Lange et al., 1990).

In a groundbreaking experiment, researchers demonstrated that introducing the catalytic subunit of human telomerase into normal human cells could extend their replicative lifespan without inducing malignant transformation (Bodnar et al., 1998). This finding provided definitive evidence that telomere shortening is a primary mechanism limiting cellular replication and that telomerase plays a pivotal role in bypassing this limitation.

These findings established telomerase as a key player in the regulation of cellular aging and opened new avenues for therapeutic interventions. The introduction of telomerase into normal cells not only extended their replicative lifespan but also provided critical insights into the mechanisms underlying aging and immortality at the cellular level. This discovery marked a turning point in our understanding of cellular biology, as it highlighted the central role of telomere dynamics in determining the lifespan and fate of cells.

While telomerase activity is typically absent in most normal somatic cells, its expression was found in several types of cells with high regenerative potential. These included fetal tissues, stem cells in the bone marrow, testicular tissues, peripheral blood lymphocytes, skin epidermal cells, and intestinal crypt cells. In these populations, telomerase plays a critical role in maintaining telomere length, allowing for continuous proliferation to support tissue regeneration and renewal. Importantly, however, the levels of telomerase activity observed in these normal cells were significantly lower than those found in cancer cells, suggesting a tightly regulated mechanism to prevent uncontrolled growth.

One of the most compelling experiments demonstrated that normal human cell lines could be rendered replicatively immortal by introducing vectors containing genes encoding the catalytic subunit of human telomerase (Bodnar et al., 1998). Remarkably, these cells retained their normal properties despite gaining the ability to divide indefinitely, providing direct evidence that telomere shortening is the primary mechanism limiting the replicative lifespan of normal cells. These experiments also suggested that cellular aging, driven by the replicative limit, may not directly underpin the aging of entire organisms.

Leonard Hayflick himself proposed an alternative hypothesis regarding the role of telomeres in aging. According to his theory, grounded in the second law of thermodynamics, the shortening of telomeres in replication-limited cells may represent a molecular equivalent of a pre-set lifespan for an organism, which differs from aging and is defined as follows:

In nature, very few wild animals experience aging, as they often succumb to predation, disease, accidents, or environmental extremes before reaching an advanced age. As a result, natural selection could not favor a genetic program designed specifically for aging. Instead, natural selection prioritizes

traits that maximize reproductive success and survival during the early stages of life. These traits include enhanced survival skills and sufficient physiological reserves to overcome external challenges. Once reproductive success is achieved, natural selection exerts less influence, as the survival of the species no longer depends on prolonged individual longevity.

According to Hayflick's view, the level of physiological reserve remaining after reproductive maturity determines the lifespan of an organism. This reserve develops as a byproduct of selection processes favoring early life traits. However, as organisms age, the rate of damage accumulation surpasses the body's repair capacity, leading to an increase in molecular disorder. This disorder manifests as the physiological decline associated with aging, ultimately increasing vulnerability to disease, predation, or environmental stress (Hayflick, 1994).

Hayflick argued that the accumulation of molecular disorder, and the processes that sustain it, lead to a gradual loss of cellular function. Consequently, the number of population doublings a normal cell can undergo—determined by telomere length—may represent the maximum potential lifespan of a culture. The molecular disruptions that signal the approach of replicative senescence and reduced telomere length are age-related changes that, when occurring in vivo, increase susceptibility to disease and pathology, leading to death before reaching the theoretical maximum lifespan.

Despite Hayflick's skepticism regarding the existence of a specific aging program, he acknowledged that the phenomenon of aging at the cellular level could have implications for understanding organismal aging. Over years of discussions, Hayflick came to consider that molecular entropy might accumulate selectively in the oldest centrioles of stem cells during asymmetric division. This selective accumulation, he believed, could represent a novel perspective on the aging process. However, Hayflick never fully embraced this hypothesis before his passing, leaving it as an unresolved question in the field.

The link between telomeres and the replicative lifespan of cells has been extensively studied and remains a cornerstone of modern cellular biology. Observations that telomere shortening correlates with cellular aging have driven efforts to manipulate telomere dynamics for therapeutic purposes. For example, it is now known that the critical shortening of telomeres triggers a series of cellular responses that signal the cessation of division. This mechanism protects against genomic instability but simultaneously limits the regenerative capacity of tissues.

Further studies have revealed a critical distinction between two cell types: those destined to perish due to telomere shortening and those that evade this fate. Cells involved in tissue regeneration, such as stem cells, and reproductive cells, such as spermatozoa and zygotes, exhibit mechanisms that preserve telomere length, allowing them to bypass replicative senescence. In their seminal studies with Tetrahymena, Greider and Blackburn provided direct evidence of the critical role telomerase plays in maintaining telomeres. This enzyme acts by synthesizing telomeric repeats at the chromosome ends, effectively counteracting telomere shortening (Greider

& Blackburn, 1985). The discovery that telomerase is a ribonucleoprotein complex containing reverse transcriptase and an RNA template revolutionized our understanding of how cellular immortality is achieved (Shippen-Lentz & Blackburn, 1990).

Additionally, studies have shown that cancer cells often exploit this mechanism to achieve uncontrolled proliferation. Telomerase activity has been detected in approximately 90% of human tumors, highlighting its central role in enabling cancer cells to evade the normal replicative limits imposed by telomeres (Hastie et al., 1990; de Lange et al., 1990).

The connection between telomere biology and cancer has profound implications for understanding tumorigenesis and developing potential therapies. While normal somatic cells experience telomere shortening and eventual replicative senescence, cancer cells evade this limit by reactivating telomerase. This discovery has catalyzed the exploration of telomerase inhibitors as therapeutic agents in oncology, aiming to suppress the proliferative capacity of malignant cells.

Importantly, the role of telomerase extends beyond its function in cancer cells. Telomerase activity has also been observed in certain normal cell types, particularly those with high turnover or critical regenerative roles. Examples include fetal tissues, stem cells, and cells within continuously renewing systems such as the skin and intestinal epithelium. However, the activity of telomerase in these normal cells is tightly regulated to prevent uncontrolled proliferation. The relatively low levels of telomerase activity in these cells, compared to cancer cells, underscore its essential role in maintaining homeostasis without predisposing to malignancy.

One of the pivotal advancements in telomere research was the demonstration that telomerase can be experimentally manipulated to extend the lifespan of normal cells. In a landmark study, researchers transfected normal human cells with vectors encoding the catalytic subunit of human telomerase, successfully immortalizing the cells without inducing malignant transformation (Bodnar et al., 1998). This experiment provided compelling evidence that telomere shortening is the primary determinant of the replicative lifespan in normal cells. Moreover, it underscored the potential for therapeutic interventions targeting telomerase to counteract cellular aging and enhance regenerative capacity.

However, the implications of telomerase manipulation in the context of organismal aging remain a subject of ongoing debate. While extending the replicative lifespan of cells may hold promise for regenerative medicine, the relationship between cellular aging and organismal aging is complex and multifaceted. Hayflick himself argued that telomere shortening, while crucial for understanding cellular aging, does not fully explain the aging of whole organisms. Instead, he proposed that molecular entropy—manifested as an accumulation of damage and loss of repair efficiency—plays a central role in determining lifespan. Hayflick's hypothesis aligns with observations that physiological reserves decline with age, leading to increased vulnerability to environmental stressors and disease. According to this view, aging is not governed by a specific genetic program but

is instead a consequence of the gradual loss of functional integrity at the molecular and cellular levels. The shortening of telomeres in replication-limited cells may act as a molecular clock, signaling the finite capacity of cells to divide, but the broader process of aging involves a complex interplay of genetic, epigenetic, and environmental factors.

Despite these complexities, the discovery of telomeres and telomerase has had profound implications for biogerontology and cancer research. Telomeres serve as critical regulators of cellular lifespan, providing a buffer that protects genomic integrity during replication. The shortening of telomeres with each division acts as a built-in mechanism to prevent uncontrolled proliferation, but it also limits the regenerative capacity of tissues.

In cancer cells, the reactivation of telomerase enables the circumvention of this replicative barrier, facilitating unchecked growth and immortality. This dual role of telomeres and telomerase—as guardians of genomic stability and enablers of cellular immortality—highlights their importance in both aging and disease.

The discovery of telomerase as a key regulator of telomere dynamics has also opened new avenues for therapeutic innovation. In cancer treatment, the inhibition of telomerase activity represents a promising strategy to target the immortality of malignant cells. Conversely, the activation of telomerase in normal cells could enhance regenerative potential, offering hope for interventions to counteract age-related tissue decline.

However, the manipulation of telomerase activity comes with significant challenges and risks. Prolonged activation of telomerase in normal cells could increase the risk of oncogenesis, while its inhibition in cancer cells must be carefully balanced to avoid detrimental effects on normal regenerative processes. These complexities underscore the need for precise and targeted approaches to harness the therapeutic potential of telomerase.

In addition to telomere dynamics, recent research has explored other mechanisms that contribute to cellular aging and replicative limits. For example, the accumulation of DNA damage, oxidative stress, and epigenetic alterations are all thought to play roles in the aging process. These factors interact with telomere shortening to influence cellular function and lifespan. The interplay between telomeres, telomerase, and other molecular mechanisms of aging highlights the intricate nature of the aging process. While the replicative limit represents a fundamental feature of cellular biology, the broader phenomenon of aging involves a network of interconnected pathways that regulate cellular homeostasis and organismal health.

Hayflick's contributions to the understanding of cellular aging continue to inspire research into the molecular mechanisms that govern lifespan. His insights into the replicative limit and the role of telomeres have shaped the field of biogerontology, providing a foundation for exploring new strategies to extend healthy lifespan and combat age-related diseases.

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77. Прангишвили, А. И., Гаситашвили, З. А., Мацаберидзе, М. И., Чхартишвили, Л. С., Чичинадзе, К. Н., Ткемаладзе, Д. В., ... & Азмайпарашвили, З. А. СИСТЕМНЫЕ СОСТАВЛЯЮЩИЕ ЗДРАВООХРАНЕНИЯ И ИННОВАЦИЙ ДЛЯ ОРГАНИЗАЦИИ ЕВРОПЕЙСКОЙ НАНО-БИОМЕДИЦИНСКОЙ ЕКОСИСТЕМНОЙ ТЕХНОЛОГИЧЕСКОЙ ПЛАТФОРМЫ. В научном издании представлены материалы Десятой международной научно-технической конфе-ренции «Управление развитием крупномасштабных систем (MLSD'2016)» по следующим направле-ниям:• Проблемы управления развитием крупномасштабных систем, включая ТНК, Госхолдин-ги и Гос-корпорации., 365.
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