Degeneration in Miniature

History of Cell Death and Aging Research in the Twentieth Century

by

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ABSTRACT

Once perceived as an unimportant occurrence in living organisms, cell degeneration was reconfigured as an important biological phenomenon in development, aging, health, and diseases in the twentieth century. This dissertation tells a twentieth-century history of scientific investigations on cell degeneration, including cell death and aging. By describing four central developments in cell degeneration research with the four major chapters, I trace the emergence of the degenerating cell as a scientific object, describe the generations of a variety of concepts, interpretations and usages associated with cell death and aging, and analyze the transforming influences of the rising cell degeneration research. Particularly, the four chapters show how the changing scientific practices about cellular life in embryology, cell culture, aging research, and molecular biology of *Caenorhabditis elegans* shaped the interpretations about cell degeneration in the twentieth-century as life-shaping, limit-setting, complex, yet regulated. These events created and consolidated important concepts in life sciences such as programmed cell death, the Hayflick limit, apoptosis, and death genes. These cases also transformed the material and epistemic practices about the end of cellular life subsequently and led to the formations of new research communities. The four cases together show the ways cell degeneration became a shared subject between molecular cell biology, developmental biology, gerontology, oncology, and pathology of degenerative diseases. These practices and perspectives created a special kind of interconnectivity between different fields and led to a level of interdisciplinarity within cell degeneration research by the early 1990s.

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Introduction

This dissertation tells a history of cell degeneration research in the twentieth century. It tracks the emergence, variance, and evolution of a scientific object, the degenerative cell, upon which the assumptions and styles of contemporaneous biology left unmistakable imprints. By describing four central developments in cell degeneration research, the major chapters present a variety of ways twentieth-century material and epistemic practices about cellular life interacted with research communities and their norms at the time to create concepts, interpretations, and usages of biological cell death and aging. These historical interactions of scientific practices, motivations, and norms left distinctive marks in the layers of meanings associated with a degenerative cell. Particularly, the four episodes of research on cell degeneration highlighted the functionality, limitedness, complexity, and (yet) regularity of a cellular life regarding its ending processes, which in certain ways transformed the scientific practices about cells by the end of the century. These developments also created a level of interdisciplinarity surrounding the subject of cell degeneration by prescribing new cellular explanations for development, aging, cancer, and other diseases, and by restructuring the relations between these previously separate fields.

In current biological sciences, naturally occurring degenerative processes of cells have borne many names, such as programmed cell death, cell senescence, apoptosis, and the Hayflick limit. These names generally point to the biologically active, well-regulated processes that halt cell division and dissolve the cell body, which I call cell degeneration generally (Hayflick and Moorhead 1961, Hayflick 1965b, Lockshin and Williams 1964b, Kerr, Wyllie, and Currie 1972).

The study of cell degeneration attracted an increasing number of researchers from diverse fields of the life sciences during the latter half of the twentieth century. In the 1990s, the development of the field gained great momentum so that cell degeneration became a central research area with exceptional productivity and popularity. On the one hand, molecular biologists brought to light molecular details involved in cell degeneration, highlighting the dynamic molecular complexity at the end of the cellular life. The surging interest led to formation of various societies, journals, and conferences focusing on cell degeneration, turning what had once been a marginalized topic into a research field of its own. On the other hand, different kinds of research programs about cell degeneration engaged attention and input from a number of fields, including developmental biology, cancer research, biogerontology, and regenerative medicine (Greider 1998b, Landecker 2003). This made cell degeneration the center of a complex network of fields, methodologies, and ontological commitments.

In the new century, two Nobel Prizes were awarded for achievements in molecular biology of cell degeneration, one for cell death research in *C. elegans* (2002), the other for the discovery of the telomere, the length of which regulates cell aging (2009). The Nobel Prizes attest to the significance of cell degeneration in the current life sciences. These awards might make a curious person want to understand more about cell degeneration. However, a careful reader of the literature on the subject would soon be mired in the multiplicity of terms and definitions for what seems like the same phenomenon within different fields and research programs.

These studies are presented as diagnostic hallmarks, for example, or as a gateway towards defeating cancer, a model of molecular pathways, and an indication of life's

finitude. Every week, among the scores of papers published about cell degeneration, one may find reports of statistics of cell death monitored in AIDS or Alzheimer's disease, with the assumption that cell death should be a diagnostic criterion. Molecular biologists studying yeast, *C. elegans*, or extracted cells from humans may report gene products newly found to increase or inhibit cell degeneration, placing another piece within the complex networks of death and aging pathways. Oncologists may report their preliminary but encouraging results about a recently contrived strategy of conquering cancer that uses special drugs to promote the benign, protective kind of cell death and aging to kill cancer cells. At the same time, biogerontologists view the limit of cellular life as a limiting factor for longevity, while researchers in regenerative medicine take pains to craft precise culture media to avoid cell death and to achieve the immortal life of pluripotent stem cells. In addition to tangled meanings within scientific circles, concepts of cell degeneration such as apoptosis also entered major dictionaries, poetry, films, and cyberspace, disseminating a conception of cell degeneration as either an altruistic, suicidal decision of the cells for their owner's survival and development, or a sad limitation of life (Friedman and Brunet 2005, Tierney 2005).

It is now taken for granted that degeneration at the cellular level is not simply a passive, erratic reaction to injuries, but rather is the manifestation of active, regulated biological processes with diverse implications for health, diseases, and life's own generation. In light of this received wisdom, it may be surprising to learn that naturally occurring cell degeneration was only recognized as an actual biological phenomenon around the mid-twentieth century, and its study only solidified into a field in the 1990s.

The short history of cell degeneration research has been taken as a story about the rapidity of scientific development (Garfield and Melino 1997, Brady 2007b). In contrast, this dissertation shows a more complex story about evolution of scientific ideas and techniques that make the apparently booming cell degeneration research possible. Because the studies about cells since the early twentieth century involved complex biological contexts and evolving technologies, cell degeneration phenomena emerged from a diversity of circumstances. This study locates the important times and laboratories in which major concepts, interpretations, and investigations about cell degeneration are situated, and opens the trajectory of cell degeneration research at these major joints through a thorough examination of representative case studies ranging from the 1930s to 1990s. These cases represented the overall evolution of research that brought about the many facets of cell degeneration as we now know it.

Before describing the four major chapters, I provide a review of the sporadic cell degeneration research before the period that the dissertation covers in order to contextualize the research focused on here. Almost squarely in contrast to the contemporary conception about naturally occurring cell degeneration, the prevailing impression that cell death or aging gave in the late nineteenth and early twentieth century was of their morbid, static, and passive nature. As early as just after Schleiden and Schwann formulated their cell theory, cell death began to be noticed as natural philosophers started to peek through the lens of the microscope to the deeper layers of tissues. One of the earliest observations came from Carl Vogt, who in a monograph published in 1842 described the notochord cells during the metamorphosis of the midwife toad as being “resorbed,” “destroyed,” or as “disappearing” (Clarke and Clarke 1996).

Other reports about cell death followed, often basing on histological observations in specimens of early embryos. Many embryologists, however, found it hard to accept that morbid death could happen in actively growing embryos and expressed philosophical objections.

American embryologist Charles Sedgwick Minot also took interest in the cellular senescence, attributing the senescence as a natural result of the outgrowth of the cell in his monograph about cell growth and death in 1908 (Minot 1908). About a decade later, American zoologist Charles Manning Child developed Minot's thesis and defined senescence as the part of life cycle after the stage of growth, a stage that represent "primarily a decrease in rate of dynamic processes" (Child 1915, 58-59). During the time at Rockefeller University, French surgeon Alexis Carrel convincingly created "permanent life" in tissue culture from chick heart tissues and claimed immortality as an intrinsic characteristic of cells, and that all aging must result from passive injuries (Landecker 2007, 68-106).

These early reports and studies had depicted cell degeneration as harmful, passive, and erratic. Perhaps because of such images, cell degeneration was not pursued collectively as a worthy scientific problem before the mid-twentieth century. Although the early twentieth century biology introduced observational tools such as time-lapse cinematography that transformed the conception of cells with the understanding that they were more dynamic than people had previously assumed, the supposition that cell degeneration was a dynamic, functional, and well-regulated process was counterintuitive and required more than simple visualization tools to revert the preconceived assumptions about them. The major goal of the dissertation was to examine what combinations of

practices about cellular life and other conditions in biological science of the twentieth century brought the inner active, nuanced "life" of cell aging and death in front of the scientists' investigative gaze and how these surprises and how the outcomes form these investigations transformed life science as well.

Four Chapters in the History of Cell Degeneration Research

The major four chapters of the dissertation trace four central developments in cell degeneration research from early 1930s to early 1990s. They established the four qualities of cell degeneration, namely, life-shaping, limit-setting, complexity, and regularity. These developments were made in different stages throughout the development of the field. The first two chapters concern with the ways cell degeneration was established as biological meaningful phenomenon and how they attracted initial research efforts, while the later two chapters examine the continuing developments of cell degeneration research made possible by the research momentum within its research community and by outsiders.

The first two chapters show how cell degeneration emerged as a scientifically meaningful entity around the mid-twentieth century. Particularly, I discuss the discovery of cell death during the development of chick central nervous system and the finding that human diploid cells age in well-conditioned cell cultures. The two chapters highlight how the ways cellular life was perceived as functional and utilized for virus production set up the ways that researchers framed the life-shaping and limit-setting characteristics of cell degeneration. I also review how these new understandings propelled researchers to incorporate new practices that pay attention to cellular death and their timings in studies of development, aging, and cancer.

The later two chapters present how the meanings of cell degeneration were expanded and refined after the phenomenon was established as an important biological problem. At the same time, they depict the expansion of the research programs from both inside and outside of the field of cell degeneration research, In the 1970s, cell aging study evolved into a field with a small research community. Chapter 3 shows how the research trajectory initially based on simple mechanism of cell aging led Robin Holliday to the realization about the complexity cell aging involved. The momentum of Holliday's research not only revealed the molecular complexity of cell degeneration, but also prepared a group of researchers to embrace the systems approach to aging three decades later. As the field of cell degeneration expanded, important contributions also came from the outside. Chapter 4 illustrates one such case. H. Robert Horvitz's research that culminated in the molecular illuminations of cell death in the nematode *C. elegans* originally started with no intention of studying cell death *per se*. Yet led by the regularities of cell death patterns found in the cell lineage study, Horvitz eventually tackled the problem, and established the high level of molecular regularity as a trait of programmed cell death.

Together, the four chapters offer a story of making and remaking meanings about cell degeneration. It is also at the same time a story of expanding practices and growing territories of cell degeneration research that transformed previous scientific practices about cells. Consequently, each chapter's narrative will first reconstruct the scientific process that revealed the particularly meaning of cell degeneration, highlighting material and epistemic preconditions that were important for such revelations. Then it will offer a survey of how the particular new meaning of cell degeneration made in an episode as

well as the meaning-making process itself transformed certain cellular practices in life sciences and established unique connectiveness between different fields that formed the special interdisciplinary landscape of cell degeneration research that we see today.

Here I provide more detailed synopses for the four chapters.

Chapter 1: The Death that Crafts Life

Viktor Hamburger and Rita Levi-Montalcini's discovery of cell death in chick central neuron development

Naturally occurring cell degeneration began to be accepted as an actual biological phenomenon around the mid-twentieth century. As cell death observed in developing embryos was reported frequently, embryologists started to reframe cell death as a differentiating means that cells utilize to generate specific structures. German-born embryologist Viktor Hamburger and Italian-born neurobiologist Rita Levi-Montalcini's co-investigation of cell death in the developing central nervous systems of chicks in the late 1940s was one instance that cell death was reinterpreted for development.

In 1932, funded by the Rockefeller Foundation, Hamburger traveled from Germany to Frank R. Lillie's laboratory at the University of Chicago to help transfer his expertise in microsurgical methods of manipulating embryos from amphibians to chicks, which had just started to become a new experimental model (Allen 2004). Investigating how the chick central neurons develop, Hamburger initially formed a hypothesis based on the induction concept of the Spemann school in Germany that regarded development as a process of progressive growth of cells (Hamburger 1934). Almost ten years later, a Jewish-Italian neurobiologist trained in pathology, Rita Levi-Montalcini, challenged Hamburger's view. Based on her work originally done in a bedroom, hidden from

Mussolini's anti-semitic program, Levi-Montalcini proposed that instead of "inductive growth," cell multiplication and subsequent *cell death* formed chick central neurons (Levi-Montalcini and Levi 1942). Hamburger and Levi-Montalcini eventually co-investigated the issue after WWII in Hamburger's laboratory at University of Washington, St. Louis. During their collaboration, Levi-Montalcini brought in a silver-impregnation staining technique that made dying cells visible. She also created a sensational representation of dying cells as novelties and compared them to dying soldiers on the battlefield (Levi-Montalcini 1988). Hamburger, on the other hand, carefully assessed these observations, and discussed what the anomaly of cell death meant to Spemann school's view of development (Hamburger and Levi-Montalcini 1949, Hamburger 1988).

Together, Hamburger and Levi-Montalcini interpreted the death of groups of cells in the nervous system under the microscope as the necessary sacrifice that cells make for the sake of the whole organism. They framed that dying cells performed a function for development (Hamburger and Levi-Montalcini 1949). Early twentieth century embryological research often depended on tracing cellular activities through staining the cells in embryos and observing them using microscopes. In the larger developmental scheme, cells were seen as agents to fulfill a function. Prevailing views of cells as functional agents in development propelled embryologists to interpret cell death as functional as well once found existent in the developing embryo. Others followed suit with similar interpretations that framed cell death as an active biological strategy to generate and modify forms in development. In a 1951 review, German embryologist Alfred Glücksmann listed 74 occurrences of cell death in vertebrate development, most

of which were assigned a developmental function (Glücksmann 1951a). In the 1960s, John W. Saunders carried out extensive research to map out patterns of cell death in chick wing development (Saunders 1966b). By the mid-1960s, cell death became a well-established phenomenon that embryologists learned to pay attention to during their investigations.

Hamburger and Levi-Montalcini's study highlighted the roles of visualizing technology and preexisting theories for establishing cell death as an actual phenomenon and meaningful concept in the larger practice about cellular life in embryology. At the same time, the discovery of cell death in development called into question Hamburger's preconception and revised Spemann's theories about organizer. The existence of cell death in development shaped the revised development scheme in which cells are not only "organized" to act, but were maintained not to die. This new view about development became the theoretical basis for the Nobel-winning discovery of the nerve growth factor, a peptide that prevents cell death, by Levi-Montalcini and Stanley Cohen in the 1950s.

Chapter 2. Aging Under Glass

Leonard Hayflick's discovery of cell aging in vitro and his proposal for using it for aging research

In the 1950s, a growing number of scientists used cell culture to study viruses, vaccines, cancer, and cell biology. These cultured cells were assumed to be intrinsically immortal, which meant that they could divide indefinitely if the culture medium was precisely tailored to the cells' needs, as the pioneer of tissue culture technique Alexis Carrel dictated two decades previously (Landecker 2007). Consequently, if cells stopped

dividing or died in culture, it was not seen as a true phenomenon of the cell, but as an error on the part of the cell culturist.

While managing the cell culture facility at the Wistar Institute in Philadelphia in the late 1950s, a junior American microbiologist and cell culturist Leonard Hayflick provided huge quantities of cell cultures to scientists studying cancer and vaccines at the same institute, and found that the normal diploid human cells he cultured would stop dividing and die about half a year after being cultured (Hayflick and Moorhead 1961). The large scale of cell cultures Hayflick was responsible of helped him to encounter cell aging repetitively and recognize an undeniable pattern so that he was certain that cell aging in an intrinsic characteristic. Hayflick interpreted the behavior of dying cells as a manifestation of aging at the cellular level and called for studying aging using cell cultures to establish a fundamental "cell theory of aging" (Hayflick 1965b). He also proposed that one difference between cancer cells and normal cells is that normal cells age, while cancer cells don't.

Hayflick's idea about the intrinsic limitedness of cellular life was initially scoffed and rejected within the cell culture community. Yet many others confirmed Hayflick's observations while working with WI-38 cells that Hayflick distributed for safe vaccine production. In addition, Hayflick's proposal of using cell culture to study aging attracted a small number of gerontologists who appreciated the convenience and the potential for well-controlled experiments that cell cultures offered. They turned cell culture from a technical practice about cellular immortality to a scientific practice for investigating cellular mortality. Instead of trying to make cells grow forever, these cytogerontologists cultured cells from various resources continuously until the cessation of cell division in

order to measure the lifespan of cells. They named the eventual end of cell divisions "the Hayflick limit." The small community of cell aging research, including Robin Holliday, Vincent Cristofalo, Woodring Wright, George Martin, and James Smith, developed the field considerably. They suggested what mechanisms control aging process and what were the possible differences between cancer cells and normal cells (e.g. Cristofalo 1972).

The chapter highlights the roles of the mass production of cellular life for vaccine and virus production and the assumption of its immortality in the 1950s to Hayflick's discovery. As cell cultures were framed as technical things with infinite proliferative power, once Hayflick encountered the end of cell divisions, the phenomenon was readily interpreted as an evidence for an antithesis for Carrel's view: it must be a sign of an intrinsic limit on cellular life.

The chapter also analyzes the ways that the discovery transformed the practices about aging through adding cell culture as a legitimate investigative tool. This transformation has been either exaggerated or ignored in previous studies. Historian Jan Witkowski has suggested that, in a Kuhnian sense, Hayflick's discovery constituted an anomaly against the existing research paradigm, and the subsequent formation of new study of cell aging *in vitro* was a scientific revolution happened in the field of aging in the 1960s (Witkowski 1987). At the same time, blinded by the recent telomere study, many contemporary reviews and reports completely neglected the contributions from cell aging research community before 1990s. This chapter challenged both treatments of this history. Hayflick's discovery and proposal of using cell culture to studying aging led to conceptual changes and the introduction of cell culture practice to aging research in the

1960s and 1970s. These developments made cell aging an established research subject so that telomere research was noticed and gained momentum quickly in the 1990s. However, these conceptual and practical changes did not completely displace the whole paradigm of aging research, but for the most part took place through adding to the existing research programs with experiments about aging using cell cultures.

Chapter 3. Causes of Aging Are Likely to be Many

Robin Holliday's path to study the complexity of molecular mechanisms of cell aging from testing the "protein error catastrophe hypothesis of aging" in the 1970s and 1980s

As gerontologists began to use cell cultures to study aging, the problem of aging in cultured cells began to be isolated from the biological contexts of multicellular organisms and became a scientific problem for its own sake. Robin Holliday's study was one instance among many pursuits of cell aging around the time that tried to tackle the molecular mechanism of aging through cultured cells. Particularly, Holliday tried to test whether accumulation of DNA mutations, or errors in proteins causes aging through manipulating the lifespan of cultured cells. Nuclear physicist Leo Szilard had suggested that DNA mutations caused aging, while chemist Leslie Orgel had suggested instead that protein errors induced the aging process (Szilard 1958, Orgel 1963). Gerontologists had been debating about which theory explains aging, Sizlard's or Orgel's, to which Holliday hoped to provide experimental contributions.

Working at the Genetics Division of the National Institute for Medical Research (NIMR) in Mill Hill, UK, Holliday started to test Orgel's protein catastrophe theory of aging in the early 1970s. Using a variety of fungus, bacterium, and cell cultures, Holliday manipulated the length of cellular life *in vitro*. With the help of his students and

collaborators at the NIMR, Holliday initially used a variety of molecular interventions to demonstrate, in favor of the protein catastrophe theory, that cytoplasmic factors were more important than nuclear mutations for aging (Holliday 1986, 1972). Other researchers on cell aging, especially those in the US, disagreed. Reflecting on the confusing data different laboratories offered, Holliday eventually developed a systems thinking that took account of a variety of information handling apparatuses in the cell and incorporate alternative molecular reasonings. Holliday's program later morphed into the program of systems biology of aging developed by Holliday's student, Thomas Kirkwood, in Newcastle University in the early 2000s.

Holliday's initial program was based on an assumption that a simple underlying mechanism exists as a molecular clock to control cell aging, an assumption that many cell aging researchers shared. The fast-developing technologies of molecular biology in the 1970s offered many tools that Holliday and others could employ to manipulate cellular life so that he could obtain data that revealed different molecular aspects of cell aging. The realization about the complexity of cell aging motivated Holliday to incorporate computational tools and the evolutionary perspective. Coupled with opposing theories and new molecular tools, the trajectory of cell aging research changed from a straightforward, hypothesis-driven science to incorporate systems approaches.

Chapter 4. A Wormy Kind of Death

H. Robert Horvitz's work on cell lineages and his hunt for genes that regulate cell death in the nematode C. elegans since the late 1970s

Chapter 4 traces how the humble beginning of mapping cell lineage using the nematode *Caenorhabditis elegans* and isolating mutated *C. elegans* beginning in the

1970s led to the molecular illumination of programmed cell death in the early 1980s. In 1974, molecular biologist H. Robert Horvitz's joined the effort of Sydney Brenner and John Sulston at British Medical Research Council's Laboratory for Molecular Biology to study the developmental path of *C. elegans* as a first step towards understanding molecular biology of complex organisms. Together with Sulston, Horvitz traced the cell lineage of the 959 cells in the adult hermaphrodite worm (Sulston and Horvitz 1977). Because of the thorough approach they took to trace individual cellular changes throughout the development, each of 131 occurrences of cell death during the development of *C. elegans* was highly individualized and at the same time was contextualized within the full history of previous lines of cell divisions. Viewing cell death as a kind of cell differentiation, Horvitz took up cell death as his major research focus after moving to the Massachusetts Institute of Technology in 1978. He designed strategies to produce mutant worms that are aberrant in cell death patterns and hoped to use them to isolate genes related to cell death (Horvitz 2003b).

In the early 1980s, Horvitz and his students isolated both genes that initiate cell death, *ced-3* and *ced-4*, and genes that protect cells from dying such as *ced-9*. The research group also engaged in using early protein sequence databases such as GenBank to search for molecular sequences that are homologous to these death genes. As the search results eventually showed that genes that related to programmed cell death were related to human diseases such as follicular lymphoma and inflammation, programmed cell death was eventually framed as a highly conserved process between the worm and humans, and a regulative path related to both development and cancer (Horvitz, Ellis, and Sternberg 1982).

The chapter highlights how the practice of mapping cell lineage was fundamental to Horvitz's discoveries. The cell lineage mapping practice gave duel epistemic roles to cell death. Cell death was seen as both a programmed, active cell fate that links to a universal pathway of molecules, and at the same time a context-dependent, controlled phenomenon that is particular to the temporal and spatial positions within the whole worm body. The acquired knowledge about each cellular unit that made up the mosaic worm body also facilitated Horvitz's strategies in searching for mutant worms aberrant in their patterns of programmed cell death (Horvitz 2003b). These epistemic and technological advantages mapping cell linage of *C. elegans* offered conditioned Horvitz's highly logical approach of studying cell death that facilitated further discoveries of the molecular regularity of cell death.

The molecular regularity in cell death offered the possibility of linking the study of cell death to other phenomena through shared or homologous molecules. The use of protein sequence databases, for example, linked the cell death phenomenon to human diseases and led Horvitz's research to greater visibility. With these discoveries that cell death were implicated in diseases, biomedical field was mobilized to investigate cell death (Jiang 2012). The high level of molecular regularity of cell death also offered biomedical researchers a sense of control towards cellular fate. In late 1990s, researchers started to design therapeutic methods to specifically enhance programmed cell death in hopes of curing cancer.

Cell Degeneration Research as a Story of Transforming Practice about Cellular Life in the Twentieth Century

As a whole, the dissertation traces the emergence, developments, and influences of cell degeneration research within the prevailing practices about cellular life in twentieth-century life sciences. The major arguments are two-fold. First, the changing material and epistemic practices about cellular life's developmental function and biological timing shaped the interpretations of cell degeneration as life-shaping, limit-setting, complex, yet having molecular mechanisms. Second, the processes and outcomes of cell degeneration research transformed the practice about cellular life by introducing new practices and conceptual frameworks about cell death and aging into a number of fields and by connecting developmental biology, biology of aging, cancer research through these shared cell degeneration research. Here I offer the general direction of these two arguments whose detailed analysis will be provided in the ending chapter, "Conclusion."

Historical Imprints in the Many Facets of Cell Degeneration

The dissertation overall highlights how the changing material and epistemic practices about cells in life sciences shaped the diverse ways cell degeneration was represented, manipulated, and interpreted. Before researchers started to notice or investigate cell degeneration, the cellular life had already had epistemic and practical values that structured researcher's motivations, attentions, and assumptions. These sets of *a priori* conditions are part and parcel of the changing epistemic style of dealing with cells in the life sciences and have attracted historical investigations.

After several decades of the dominance of "gene talk" in terms of visibility in both biology and its historical study, recent historical scholarship has provided illuminating works that depict cells as central epistemic and practical units to investigate

life in the twentieth century. In *Transforming Traditions in American Biology, 1880-1915*, Jane Maienschein made clear that functional approaches to the behavior of cells, such as investigating how cells divide and grow into individual neurons and embryos, characterized a new American style of biology that diverged from the older European morphological tradition (Maienschein 1991). Hannah Landecker, in her book *Culturing Life: How Cells Became Technologies*, told a history of cell cultures as biologically malleable materials in the twentieth century laboratories that deeply changed our conception about "individuality, immortality, and hybridity" (Landecker 2007). William Bechtel, while examining the formation of modern cell biology from interactions and collaborations of cytology and biochemistry, pointed out that the elucidation of mechanisms in cells often began with localizing and visualizing particles of the cells that contained pivotal mechanistic components (Bechtel 2006).

My work builds on this historical scholarship and describes the impacts of changing material practices about cells in the life sciences through the lens of a history of cell degeneration research. I ask what existing practices of studying cellular life led to the study of cell death and aging and how. As I show through the four chapters, how cellular life was managed, manipulated, and viewed greatly shaped the emergence of cell degeneration as an actual biological phenomenon and its interpretations.

Just to name several examples from the dissertation to show how the changing practice about cellular life shaped the interpretations and approaches to cell degeneration. The traditions of tracing cellular changes to study development led to interpreting cell death found within the context of a developing embryo as developmentally functional. Using cell cultures as immortal technical things triggered Hayflick's interpretation of cell

aging in human diploid cell culture as a manifestation of human aging and mortality. Assumptions of the underlying molecular mechanisms of cellular life led Holliday and others to hypothesize, explore, and manipulate about the lifespan of cells. The tracing of cell lineage during the development of *C. elegans* and the assumption about a molecular developmental program catalyzed Horvitz's study to depict cell death as well-regulated. These historical cases shaped our current understanding about cell degeneration and left imprints from the practices about cellular life in the long twentieth century.

Cellular Life Transformed, Biological Fields Intertwined

The second argument concerns the influence of cell degeneration research in making life sciences different. Deeply shaped by the twentieth-century practice about cellular life, cell degeneration research also transformed the material and epistemic practice about cellular life in return. One significant outcome of such transformation was that various disciplines created converging research programs that focus on cell degeneration, forming the highly interdisciplinary landscape of cell degeneration research.

First, cell degeneration research created new modes of studying cells that various fields in biological sciences adopted over time. New practices such as mapping cell death during development, measuring cellular lifespan and studying aging through cell culture emerged and took hold. They propelled researchers in developmental biology, gerontology, cell biology, and oncology to become sensitive and attentive to the ends of cellular life and their timings.

Second, taking cell degeneration seriously triggered researchers to reframe theories or interpretations about how cells contribute to development, aging, and cancer.

For example, due to the discovery of cell death in chick neuron development, Hamburger revised the original depiction from Spemann about what cells do during development. Considering why cancer cells do not age in cell cultures, Hayflick hypothesized about the relation between normal cells and cancer cells. The regulatory genes that Horvitz found that were both involved in controlling cell death during development and implicated in certain cancers led to new interpretations about how cancers developed over time. These theoretical changes further prompted cell degeneration to become an important concept in the twentieth century science, infiltrating into conference talks, scientific reports, and grant applications of various disciplines.

Interests in cell degeneration from different fields created a mosaic research landscape, a degree of interdisciplinarity within cell degeneration research that encompassed investigative efforts from other established biological fields. Such interdisciplinarity was not actively advocated by the practitioners, but was developed over time through contributions of the various disciplines. The practices and perspectives of cell death and aging created a special kind of interconnectivity of different fields in the twentieth-century life sciences.

Chapter 1: The Death that Crafts Life

Viktor Hamburger and Rita Levi-Montalcini's Research on Cell Degeneration during the Development of the Chick Central Nervous System

This chapter traces the research trajectory of the German-American embryologist Viktor Hamburger's research about the central-peripheral problems of nerve development, first in amphibians, and then in chick embryos. This led to his collaborative discovery with Italian neurobiologist Rita Levi-Montalcini of cell death in the development of the chick central nervous system. Their discovery was one of a number of observations of cell death in the early- and mid-twentieth century. The case shows how investigators' interests in experimental novelty and theoretical implication of cell death phenomena facilitated the establishment of cell death as a biologically meaningful scientific object.

In the early 1930s, Hamburger worked at the University of Chicago on how chick neurons develop. He had a technical goal of transferring the microsurgical technique from the Spemann school of experimental embryology from amphibian embryos to chick embryos. He did not find cell death then, and he interpreted the development of the chick nervous system in terms of induced progressive cellular activities: cell division, growth, and migration. The Jewish Italian biologist Levi-Montalcini, working in a provisional laboratory converted from her bedroom during WWII, reexamined Hamburger's work in the early 1940s. She suggested that cell death was involved in chick neuron development. After reading Levi-Montalcini's paper, Hamburger invited her to work on the issue together at his laboratory at Washington University in St. Louis. Around 1947 and 1948, they meticulously traced cellular activities during the development of the chick embryos

and confirmed that cell death did exist in the various processes of the neuron development.

Hamburger and Levi-Montalcini's study was not the first to identify cell death in developmental processes. From the mid-nineteenth century through the early twentieth century, scattered histological observations of cell death were reported in different developmental stages in a number of species. These early morphological descriptions often mentioned a kind of granule visible in the histological section as a sign of cell death. Yet the cell death distributions were not followed through time and space within the developmental scheme of a particular organism in these earlier cases.

Hamburger and Levi-Montalcini's study also did not involve excessive death metaphors that we often use today such as "executors," "killers," and "protectors" to describe the genes, enzymes, and signaling molecules that control cell death. Although Levi-Montalcini invoked her memory about the recent war in the Europe and compared the dying cells to the sacrificing soldiers on the battlefield, their research focus was not how and why individual cells die but whether cell death occurs in the developing process, and if so, what are the patterns of cell death in terms of collective activities of groups of cells in certain areas of the embryo.

Hamburger and Levi-Montalcini's case is representative of how cell death phenomena surprised scientists in the early twentieth century and how the phenomena often took meticulous experimental investigations and theoretical changes to be made biologically meaningful. In this case, two investigators took different approaches to make sense of dying cells in development. Levi-Montalcini focused on the novelty of the cell death phenomenon as incentive for further research. Hamburger, while acknowledging

the existence of cell death, focused on revising his original inductive explanations. For Hamburger, cell death mattered and needed to be investigated further not only because of its novelty, but also due to its theoretical implications. Both approaches enhanced the epistemic importance of cell death in biological research around the time.

Hamburger and Levi-Montalcini's case also represented a way in which discoveries of cell death in development brought about epistemological changes in understanding the role of cellular life in development. Since the late nineteenth century, with the maturation of descriptive histology, microscopic and preparative techniques, embryologists had studied development in terms of cells, in addition to germ layers. In 1900, Edmund Beecher Wilson explicitly developed on this general focus on cells in embryology in his famous monograph *The Cell in Development and Inheritance*. In these studies, cells were depicted as the building blocks that multiply to form structures. Their activities encompass a presumed function for the final forms of the organism. In development, cellular life was seen as growing and dividing according a preformed developmental plan or by interacting with its environment. In 1930s, observing the chick neuron developments without noticing cell death, Hamburger held the view that the cell numbers increased over time because cells were induced to divide and multiply during development. He was trained in the Spemann school of experimental embryology that supposed that development happens because inductive agents caused cells grow and self-organize into more complex structures. The inductive view reinforced the primacy of progressive cellular activities such as divisions, migrations in development because these activities were what cells were induced to do.

The discovery of cell death in chick neuron development changed Hamburger's and eventually many other embryologists' assumptions about cellular life during development. Cell death fulfilled a role in development, because cells not only act but also tinker. Multiplying cells sometimes provide extra materials, or build a scaffold, for other developing cells to use temporarily. These materials or scaffolds need a mechanism to be eliminated after their temporary functions are fulfilled. As a result, embryologists started to pay attention not only to the activities of cells during development, but also to their disappearance and how and why. The establishment of cell death as an actual biological phenomenon happened at about the time when Spemann's once popular organizer concept lost its importance. There are many reasons why this organizer concept lost its appeal. The existence of cell death in chicken neuron development was one piece of counterevidence that exposed the overly narrow scope of the inductive principles.

This chapter focuses on the research processes of investigating chick nerve development before and during the collaboration between Hamburger and Levi-Montalcini. It describes the research contexts of experimental embryology that made Hamburger miss cell death in the 1930s, and then the research processes that exposed cell death and made it important to two researchers in epistemologically different ways. The influence of studying cell death on the practices and interpretations of cellular life in development is reviewed through Hamburger and Levi-Montalcini's research trajectories as well as the practice of studying cell death in the 1950s and 1960s.

After a brief review of cell death research prior to 1940s, I describe the intellectual background of Hamburger and his research in Germany and the US that led to his study of chick neuron development that ignored cell death in the 1930s. Then, I depict

Levi-Montalcini's wartime study on chick neuron development that suggested the existence of cell death in chick neuron development. Their collaborations in the 1940s is carefully examined to reveal their dissimilar styles of approaches towards novel phenomenon. I also reflect on why their approaches differ and discuss the role of experimentation and theories in the establishment of cell death in development in references to Hans-Jörg Rheinberger's concept of experimental system and Frederic Lawrence Holmes' concept of investigative pathway. Finally, I will briefly review how the discovery of cell death led Hamburger and Levi-Montalcini to different research trajectories as well as how it modified the approaches to cells in embryology in general.

Scattered Observations of Cell Death before 1940s

From the mid-nineteenth century through the early twentieth century, scattered histological observations of cell death were reported in a variety of specimens and processes such as in developing organs, metamorphic insects, and ovarian follicles. These early morphological descriptions often mentioned a kind of granule visible in the histological section as a sign of cell death. The findings about cell death in development at times created great interests from individual researchers. However, not all embryologists agreed that cells could die in progressive development. Nor did cell death study create concerted efforts in any considerable scale. When Hamburger and Levi-Montalcini studied cell death in the 1940s, the condition of cell death research was not unlike one of the late nineteenth century: there were sporadic reports to take reference, and also philosophical and theoretical objections. To understand why embryologists were surprised that cell death occurred in evelopment, let us review the some of the scattered studies about cell death before 1940s and the reactions from embryologists.

It is not surprising that cell death was first found in developmental processes that seem to bring clear regressive changes and loss of features, such as with metamorphosis. Soon after botanist Matthias Schleiden and physiologist Theodor Schwann formulated the cell theory around 1839, German histologist Carl Vogt reported about cell death in a monograph that described his microscopic study of cellular activities throughout the life cycle of midwife toads. Talking about the process of amphibian metamorphosis when the anuran notochord began to be replaced with vertebrae, Vogt described the notochord cells as being "resorbed," "destroyed," or as "disappearing" (Clarke and Clarke 1996). Observations of cell death regressing structures during metamorphosis had been reported since then.

Cell death in developmental processes that were considered as progressive was also reported. Among others, in 1906, a neurobiologist R. G. Collin reported observations of cell death in the chick spinal ganglia, including condensation of chromatin into black balls that were ejected to the cytoplasm. Yet, Collin did not follow up on his one-time cell death research and moved on to study neuroendocrinology instead. In his 1944 monograph *L'organisation nerveuse,* Collin did not say one word about neuronal cell death (Clarke and Clarke 1996).

The isolated and undeveloped condition of the one-time cell death research of Collin's was a typical fate of cell death discoveries before the 1930s. One reason might be a conceptualization or communication problem. Since these reports were sporadic, authors often gave new names to the cell death phenomena they had seen. The variety of these names, such as "coagulation necrosis," "autolysis," "pyknosis," "karyolysis," and "chromatolysis," although descriptive, probably have prevented scientists from

recognizing that they were investigating similar or identical phenomena (Majno and Joris 1995).

More importantly, the technical difficulty of establishing the patterns of cell death as well as the philosophical conundrum to explain why degenerative death can happen in actively developing organs inhibited taking cell death seriously. The limited resolution of microscopes and histological stains of the nineteenth century could only reveal coarse granular patterns. It was tedious and difficult to reconstruct the whole sequence of morphological change involved in cell death from glimpses of histological sections in which structures of cells were often distorted. In fact, many embryologists regarded the granules as experimental artifacts generated from poor slide preparation. Others considered the granulated nuclei observed in the reported dying cells were actually "mitotic metabolites," a sign of proliferating cells. Philosophically, the notion that death might happen in these developing or living organisms just did not seem right, since life was conventionally defined as the absence or resistance of death. In addition, generating cells only to destroy them seems wasteful, going against a biological logic that should maximize efficiency. Some embryologists such as Karl Peter thus interpreted these signs of death through a "progressive" view of the embryo. (Glücksmann 1951b).[1]

Beginning in the 1940s, experimental embryologists often unexpectedly encountered cell death when investigating how cells divide and differentiate to form certain tissues and organs. With improved dyes that selectively stain certain types of cells, biologists eventually established cell death as a real biological phenomenon and

[1] A telling quote about the conception of life as the antithesis of death from the early twentieth century is from Xavier Bichat, "Life is the totality of those functions which resist death." (Bichat 1909)

started to ask questions about why and how cells die at various times during development. Hamburger and Levi-Montalcini's study was one independent discovery of cell death among the instances that accumulated in the 1940s and 1950s that propelled biologists and pathologists to conceptualized these cell death with unified concepts such as "programmed cell death" or "apoptosis" in the 1960s and 1970s. From being negligent of cell death to incorporating cell death as an important process for understanding development, Hamburger and Levi-Montalcini's cell death research represented a shared experience of embryologists around 1950s and 1960s.

Viktor Hamburger and Hans Spemann's Programs at the Zoological Institute

Like many other investigators of cell death in the first half of twentieth century, Hamburger had questions other than cell death in mind when he started his investigation about neuronal development. The question Hamburger was interested in were the central-peripheral relations during the development of the chick nervous system, which he encountered while studying at the Hans Spemann Lab at the Zoological Institute in Freiburg and continued the research for more than half a century. Hamburger's early appreciation of nature and philosophy, as well as Spemann school of experimental embryology had left great influence in Hamburger's approach to embryology. This section focuses on these formative experiences.

Born in 1900 in Landeshut, Silesia, Germany, Hamburger greatly enjoyed the landscape of the town from an early age, from which his interests in natural history emerged. Hamburger remembered that he used to be impressed by the developing frog and salamander embryos and the hatching of larvae in his teenager years. He used to bring home jelly masses home and rear them in aquaria. In a hand-written note,

Hamburger wrote a memory. “One day young salamanders had metamorphosed and escaped from the aquarium, and in the evening, when my parents had a party, crawled up the window curtains – I do not remember whether my mother and the guests were bewildered or amused. At any rate, my interest in developing animals was alive early” (Allen 2004).

Hamburger graduated from his gymnasium in 1918 and was sent to Breslau for basic military trainings. After being discharged one year later, in 1919, Hamburger went to University of Heidelberg and took classes there, including Hans Driesch’s philosophical seminar on a variety of topics including vitalism and Curt Herbst’s graduate seminar on developmental biology. It was in Heidelberg that Hamburger’s “interests became sharply focused on experimental embryology and developmental genetics” (Hamburger 1996). However, Hamburger was not particularly eager to follow Herbst’s experimental approaches in testing the effects of chemical components, such as calcium, lithium and other ions on the patterns of development of sea urchins. He found Herbst’s experiments rather reductionistic. In the 1920s, Hamburger went back to Breslau and took courses of zoology, botany, geology, and mathematics at the University of Breslau. At this point, Hamburger became clear that he would like to seek an academic career. Knowing about his plan, his parents encouraged him to obtain some experiences outside Silesia.

During a visit to the Black Forest area near Freiburg in 1920, Hamburger was deeply impressed by the area and learned that the prominent zoologist, Hans Spemann, directed the Zoological Institute nearby in Freiburg. In the same year, Hamburger became enrolled as a graduate student at Spemann’s laboratory, working at the Institute that was

founded in 1888 by another distinguished scientist August Weismann. There, Hamburger enjoyed great level of freedom and flexibility to learn from the curricula and from the laboratory. Hamburger became great friends with Johannes Holtfreter and Hilde Proescholdt. There, Hamburger continued to discuss science, art, and philosophy with his friends, colleagues, and professors, especially Hilde Proescholdt, who herself had intense interests in philosophy of art, phenomenology, esthetics, and the natural history of the Black Forest.

As historian Garland E. Allen has pointed out, Hamburger "displayed a clearly 'philosophical turn of mind,'" which needs to be understood in the context of the overriding philosophical and artistic atmosphere in the 1920s German universities. His philosophy, however, did not follow the older school of vitalism. When he took the biology course from Driesch that covered a hodge-podge of topics and centering on the teachings of vitalism in 1920, Hamburger rejected Driesch's vitalism. He was reportedly more influenced by Martin Heidegger's writings that emphasized the interconnections between parts and the irreducibility of parts in a whole entity. Allen rightly pointed out that Hamburger's early exposures and leanings to philosophical holism shaped Hamburger's more holistic scientific approach towards the embryo that always incorporated concerns of the embryo's integrity and complexity.

At Hans Spemann's laboratory, students were supposed to learn the intricate techniques of extirpation and transplantation, most of which the lab master Spemann himself invented to remove one part of the donor salamander embryo and transfer the part to another host embryo. Using glass needles, hair loops (a looping structure made from human hair to constrict embryos), and micropipettes, Spemann and his students worked

on amphibian embryos to constrict individual embryos, fuse them, or cut one part of an embryo and transfer it to a host embryo. The amphibian embryos are tiny and soft, demanding steady hands, patience, and focus. Spemann expected nothing but perfect operations from his students. Under such exact experimental training, Hamburger himself became a virtuoso in experimentation. Receiving a number of visitors year round, such as Oscar Schotté from Switzerland and Ross Granville Harrison from the US, the institute also hosted heated intellectual exchange on the cutting edge of embryology. Afternoon teas were held during which lively conversations took place (Hamburger 1996).

Around 1922, Hilde Mangold (Proescholdt recently married to Otto Mangold and changed her last name) was trying hard to test the effects of transplantation of blastopore lip between salamander embryos. She found that three days after the transplantation, nearly complete secondary embryos could be found at the sites of transplantation. Among 274 experiments, 29 embryos formed neural structures in response to the transplanted blastopore lips. The results were interpreted that the host embryo was induced to develop through the "organization center" of blastopore lips. Thus it was hypothesized that dorsal lips of the blastophore contain a special agent that can organize the whole structures of the future embryos, which Spemann called the organizer. Spemann and Mangold discussed Mangold's results for more than 50 hours, and produced a milestone paper in 1924 that demonstrated that the upper lip of the blastophore might act as an organizer in amphibians. However, Hilde Mangold died from a tragic accident before the publication of the paper, the work which won Spemann a Nobel Prize in 1935 (Spemann and Mangold 1924, Sander and Faessler 2001).

The success of demonstrating the organizer in the dorsal lip of the blastophore initiated a series of experiments at Spemann's laboratory develop on the project further. For example, Hamburger's friend, another student of Spemann's, Johannes Holtfreter, tested the nature of the presumed organizer by treating the dorsal lips by grounding them, boiling them, or mixing them with alcohol or acid, before testing them on the host embryos. As the artistic Holtfreter depicted in a cartoon, each student in Spemann's laboratory worked like a workhorse to pull along the carriage of the laboratory, often on some aspect of the organizer (Figure 1).

The 1924 proof-of-concept Spemann-Mangold experiment about the organizer excited the field of embryology and fueled other researchers to follow up as well. By 1929, Conrad Hal Waddington started to study the organizer in Cambridge. Starting from 1933, Joseph Needham visited Otto Mangold's laboratory several times to co-investigate the organizer. Research on the organizer continued well into the early 1930s (Horder 2001). As Hamburger summarized in his book about the Spemann school, "Experimental embryology attained a commanding position in the filed of Biology during the first half of this century. To biology students of my generation, it held the same fascination as molecular biology and neurobiology do today... the Spemann-Mangold organizer experiment of 1924 … was widely regarded as the crowning achievement of this period" (Hamburger 1988, vii).

The concept of induction and the organizer left a lasting influence on Hamburger. Although Spemann did not think much about cells when formulating the inductive principles, when Hamburger visited the US ten years later, his hypothesis about how neurons were triggered to grow and divide followed the logic of induction. Yet,

Hamburger's own doctoral research was not based on research about the organizer contained in blastopore lips but about how the amphibian central nervous system is developed.

Investigating the Central-Peripheral Problem in Amphibians

In 1923, three years into Hamburger's PhD study, Hamburger started to consider what topic to choose for his thesis. Spemann seemed to want him to choose an independent topic that was removed from the major focus of the laboratory, and suggested he study the relations between the peripheral organ development and the central neuron development, or what the neurobiologists often called "the central-peripheral problem."

The central-peripheral question asks how much the development of the central neurons is dependent on the existence of the peripheral structures such as developing limbs. The pioneer of experimental neuroembryology Ross Granville Harrison initially raised the question. In his 1907 experiment in which he cultivated neurons *in vitro*, Harrison showed that axons of frog embryonic nerve cells could grow and demonstrated that the nervous system is composed of cells. His experiment resolved the long-standing debates between Santiago Ramón y Cajal and Camillo Golgi about whether the nervous system is made of cells or not. It also raised other questions: What triggered the neurons to grow? What guide the direction of neuronal growth so that the central neurons know how to innervate the peripheral limbs? What are the relation between the limb development and the central neurons that innervate the limbs (central-peripheral problem)?

Harrison followed the question himself by transplanting limbs between embryos and observed the effects on the neuronal development. In the 1920s, Harrison himself also moved on to other problems but other embryologists followed. Around 1922, for example, Paul Weiss, an embryologist working at the University of Chicago found that transplanted extra limbs in amphibians could perform movements comparable to movement patterns of normal limbs. This suggested that the grafted limb buds somehow directed nerve cells to grow out to innervate the extra muscles. The reactivity of the central nerve cells to newly introduced limb buds indicated that the relation between central neuronal growth and peripheral organs is not static, but modifiable (Hamburger 1980).

For the doctoral project, Spemann intentionally would have liked Hamburger to study something different than "hanging at the dorsal lips of the blastopore." He suggested Hamburger investigate the central-peripheral problem. Although this direction would carry Hamburger's away from further studies of the organizer found in the blastopore lips, the interaction between the central neurons and peripheral organs was still be related to the general study about induction. Embryonic induction was defined as "the initiation of developmental processes in a particular region of the embryo by (presumably chemical) interaction with adjacent contiguous structures" (Hamburger 1980). Spemann himself had studied the interaction between the optic vesicle and lens formation in the early 1910s. His skillful operations that differentiated the lens induction processes from two species of amphibians *Rana fusca* and *R. esculenta* had earned him early fame (Spemann 1912, Sander and Faessler 2001).

Spemann informed Hamburger of the work done by Bernhard Dürken as a starting point to explore the role of the sensory organs in shaping the nervous system. In 1911, while at the University of Breslau, Dürken had showed that ablation of developing eye tissues from frog embryos triggers massive defects in nerve development and postpones the formation of legs. Spemann nevertheless doubted the accuracy of the results. Having studied the relation between the development of lens and the effect of ablations of the optic vesicle, Spemann suspected that Dürken's results were overstated.

Using the techniques he learned at Spemann's laboratory, Hamburger repeated Dürken's experiment and concluded that the defects caused by deleting frog eyes existed, but happened less extensively than Dürken had suggested. For example, ablation of the eyes did not result in clear defects in the leg development. He hypothesized that the innervations of the limbs did not affect the normal limb differentiation, and only influenced the functional development of the limbs. This research culminated in Hamburger's PhD dissertation finished in 1925 spring, "Über den Einfluss des Nervensystems auf die Entwicklung der Extremitäten von *Rana fusca*" (On the Influence of the Nervous System on the Development of the Limbs of *Rana fusca*). Although not ground-breaking, the delicately performed experiments and the novel hypothesis Hamburger achieved through his thesis project carried Hamburger to further study of the "central-peripheral problem," into the new chick development model, as well as the surprising finding of cell death during the development of the central nervous system.

After receiving his doctoral degree, Hamburger carried out research in Göttingen, Dahlem, and later back in Spemann's laboratory in Freiburg for a while. During his one-year stay at the Kaiser-Wilhelm Institute in Dahlem in 1926, Hamburger investigated

muscle development in frogs and whether central nerve development influences the development of muscles. He removed all of the nerves inside developing frog limbs to see what effect the lack of nerves had on the development of muscles. He found that a nerveless limb could still form normal musculatures, although they would eventually degenerate because of disuse. He concluded that this result eliminated any possibility that the development of the central nervous system could cause structural formation of the musculatures. This research complemented the line of inquiry started in Hamburger's thesis research by studying about the central-peripheral relations backwards and asking what influences the central neuron development has on the peripheral organs (Hamburger 1927).

Also at the Kaiser-Wilhelm Institute, Hamburger learned extensively about the emerging field of developmental genetics. The Otto Mangold's embryology department in which Hamburger worked was located on the first floor of the institute. Richard Goldschmidt's genetics department was just one floor above. There, Hamburger regularly attended the afternoon teas with the genetics group and became a close friend with the geneticist Curt Stern. Hamburger tried to study with Stern about fruit fly genetics for a short time. This began Hamburger's genetics study, which he resumed later in the US to investigate the genetic mutations in chickens that created abnormal motion behaviors that made these chickens seemed to creep.

Investigating Chick Central Neuron Development through Inductive Principles

In 1927, Hamburger returned to Freiburg to take up the post of privatdozent there. Over the five years there, his teaching was widely praised and his eventual promotion through the academic ranks in Freiburg seemed assured. In 1932, the Rockefeller

Foundation invited Spemann to nominate a candidate for a one-year postdoctoral fellowship to work at Frank Rattray Lillie's laboratory at the University of Chicago. Although Spemann would have selected his favorite student, Otto Mangold, Mangold exceeded the age limit of thirty-five stipulated by the foundation. Hamburger was eventually nominated instead and arrived at Chicago in 1932. The fellowship expected Hamburger to work on problems that facilitate the transfer of technology in experimental embryology from amphibians to chick embryos.

Upon arrival, Hamburger discussed with Lillie what scientific problem he would work on with chicks. Lillie specifically suggested that Hamburger use his surgical skills to repeat former experiments on chick embryos that had produced conflicting results. One of Lillie's graduate students, Elizabeth Shorey had shown in 1909 that destroying chick wing buds resulted in diminished cell population, hypoplasia, of motor nerve fibers in the spinal cord and the sensory ganglia. However, in 1919, one of Ross Harrison's students at Yale University, Samuel Randall Detwiler, observed that in salamanders the lack of forelimb buds negatively influenced only the sensory nerves, leaving the motor columns intact. It was suspected that the different results Shorey obtained might have been due to her electrocautery technique that eliminated the wing bud, since electrocautery was highly invasive and might have caused extensive damage not related to the removal of wing buds. To repeat Shorey's experiments using the miscrosurgical techniques developed in Spemann's lab seemed appropriate for Hamburger and was one of the "central-peripheral problems" that Hamburger was interested in.

This was the first time that Hamburger used chick embryos to investigate developmental problems. He began with learning a great deal from scientists at Lillie's

laboratory about how to handle chick embryos, especially from Benjamin Willier and Mary Rawles. Hamburger soon developed a set of techniques that utilized the glass needle and hair loop to reach the chick embryos through a small window cut into the eggshell, as he had done with amphibian embryos in Spemann's laboratory. (Figure 2)

After developing the basic techniques of extirpating chick wing buds, Hamburger started the major operations for the investigation. Most of the extirpations were done on 68-72-hour chick embryos. For each embryo, Hamburger cut out a small rectangular window on the shell to expose the embryo and prepared it for extirpation. After removing the shell membrane under the window, he used a micro glass needle to make a small hole in the amnion that covered the right wing bud. He then further pushed the glass needle to cut through the base of the bud. After lifting the severed wing bud out of the shell, he sealed the opened window on the shell with a thin layer of warm paraffin. Most of the embryos were allowed to develop for 4 to 6 days after extirpation. Hamburger then fixed the embryos in Bouin's fluid, a commonly used solution for fixation and preservation of biological material, and stained them with a blue dye called Heidenhain's iron hematoxylin. The embryos were then sectioned and examined.

Hamburger's results turned out to be in agreement with Shorey's: upon extirpation of the wing buds and muscle structures, both sensory and motor neurons dwindled in size and cell number. He noted that when the right wing bud of a chick embryo was removed, the right half of the spinal cord of the same individual was significantly smaller than the left. He also counted the motor neuron numbers in a series of sections of the anterior horn of the spinal cord and calculated that the cell number in the right anterior horn was 61% fewer than the cell number on the left half. The

hyperplasia was less significant in the posterior horns, which showed 22% reduction of the neuron number on the extirpated side. The median part of the spinal cord, however, was not reduced in volume or in the number of neurons. In addition, the spinal ganglia were also shown to shrink in size.

These results were reported in the paper "The Effects of Wing Bud Extirpation on the Development of the Central Nervous System in Chick Embryos" published in *The Journal of Experimental Zoölogy* in 1934 (Hamburger 1934). In the paper, Hamburger analyzed the common patterns and variations manifested in the nine cases he had experimented on. For all nine extirpated chick embryos, hypoplasia was observed in the anterior and posterior horns of the spinal cords, as well as in the spinal ganglia.

Based on careful operations and analysis, Hamburger hypothesized about ways in which the central nervous system interacts with peripheral signals. He found that extra limb buds transplanted into the flank area of the chick embryo increased the size of nerves. In addition, the hypoplasia of motor columns increased proportionally when more muscle mass was removed. When all the muscle structures were removed, however, motor columns still developed about 40% of their normal size. In the 1934 paper, Hamburger hypothesized that there was a level of constituent growth in motor columns that was intrinsically determined by central neurons. The peripheral signals generated by developing limbs induced extra cell growth and proliferation. In other words, neurons were seen as recruited by inductive influence provided by the developing limbs (recruitment hypothesis). Hamburger also postulated that stimulating substances released by peripheral tissues might travel along the innervating neurons back to the neural center (retrograde transportation) and further induce the central nerves to grow.

In the 1934 paper Hamburger also reflected on what cell processes and through what mechanisms the peripheral stimuli regulate the development of the central nervous system. Since Hamburger assumed that cells confined within the spinal cord first carry out mitosis, and then start differentiation and migration, he imagined that the stimuli from the peripheral structures might affect any of those three steps in neurons: proliferation, differentiation, and migration. The possibility of cell death was not mentioned. Regarding how the stimuli were transferred from the peripheral structures to the central neurons, Hamburger suggested that the nerve fibers serve to transport the peripheral stimuli centripetally to the undifferentiated (also called uncommitted) neurons in the center. With these hypotheses Hamburger suggested that the peripheral stimuli induce undifferentiated cells to join the central nervous system through division, movement, or differentiation. Hamburger's 1934 hypothesis was an articulation of the recruitment hypothesis in the language of Spemann school's induction concept.

In the end of 1934 publication, Hamburger also compared, analyzed, and synthesized others' results regarding central-peripheral relations in birds, mammals, and amphibians. By reviewing relevant literature, Hamburger reported that research about the disease abrachia suggests that the congenital lack of limbs in mammals correlates with diminished size of central nervous system, with patterns similar to that observed in birds. He estimated that the central-peripheral relations in mammals and humans resemble the characteristics demonstrated in chicks, linking his own discovery to diseases and phenomena in higher organisms.

Hamburger's research at Chicago definitely developed experimental techniques of extirpation for chick embryos. It also showed that the chick embryo is a system suitable

for studying the central-peripheral problem because the motor columns are clearly visible, while in amphibians, the motor neurons are not organized into distinct units to be recognized. The hypothesis Hamburger developed held the notion of induction *a priori.* Although Hamburger compared the neuron areas between embryos that had different amounts of limb bud muscle removed, he did not follow the change of cell numbers or the areas of central nervous systems by examining the embryos more frequently than a one-time test. The caveat in the frequency of examining the chick embryo became one difference between Hamburger's approach and that of Italian researcher, Rita Levi-Montalcini in the early 1940s. Hamburger and Levi-Montalcini eventually led a productive long-term collaboration, but that did not begin until the late 1940s when Hamburger was at St. Louis.

Although his Rockefeller fellowship was originally intended to last for one year, the Nazi's rise to power in Germany in February 1933 changed that. Repressive laws were enacted to dismiss all Jewish professionals from working for the government, including university professors. Hamburger, who came from a Jewish background, received a letter from the Rector of the University of Freiburg, philosopher Martin Heidegger, that he had been dismissed from his position. Hamburger became an exile from his homeland. Fortunately, his time at Lillie's lab was extended, and in 1935, Hamburger accepted an offer from Washington University in St. Louis and remained in the US. In 1936, Hamburger also became an instructor in the embryology course of Marine Biological Laboratory, Woods Hole, Massachusetts and became a frequent visitor and investigator there. After five years of participating as an instructor, Hamburger became the director of the MBL embryology course in 1941 and revised the previously

descriptive curricula to one that emphasized novel approaches that emphasized the dynamic and causal aspects of embryogenesis. With his continued research in the central-peripheral problem of the development of the central nervous system as well as his teaching experiences in St. Louis and Woods Hole, Hamburger became exposed to a variety of ideas, and became a broad-minded yet careful researcher who sought to cultivate younger researchers.

Glimpse of Cell Death by Levi-Montalcini in Italy

Six year after Hamburger's 1934 paper reporting about the impact of damaged wing buds for central neuron development in chick embryos, in 1940, the Jewish-Italian neurobiologist Rita Levi-Montalcini (1909-2012) read the paper. The paper intrigued her and eventually provided a topic for her to pursue at home when Levi-Montalcini was forbidden to work as a Jewish scientist in Mussolini's fascist Italy in the early 1940s. At her primitively equipped bedroom, Levi-Montalcini had glimpses of death of the chick neurons.

Rita Levi-Montalcini was born in 1909 in Turin to a cultured family. Although her father Adamo Levi, an electrical engineer and mathematician, initially planned that his daughters would not go to universities, Rita decided that she would not be able to adjust to a domestic feminine role and asked permission to engage in science as a profession. With permission granted, Rita studied medicine with Guiseppe Levi at the University of Turin. There, she mastered a silver-impregnation staining method derived from the design of Ramon y Cajal. Her fellow students included Salvador Luria and Renato Dulbecco. All three made extraordinary contributions to science and eventually won Nobel Prizes.

In 1936, Levi-Montalcini graduated from medical school with a summa cum laude degree in Medicine and Surgery. Then she was enrolled in a three-year specialization in neurology and psychiatry while working at Turin's Clinic for Nervous and Mental Diseases. There she started to engage in promising research using oscillographic probes to test the spontaneous activities of the nervous centers of chick embryos. Levi-Montalcini was hoping to study the differentiation of the neuron centers and circuits in charge of motility and reception of stimuli from examining the chick embryos' nervous system in serial sections under the microscope. Her silver-impregnation staining methods helped to make the cells visible. Chick nervous system has high affinities for silver-salt impregnation, and the developmental time from the beginning of the development to the hatching time is only three weeks, which helped researchers to obtain results quickly (Levi-Montalcini 1988, 84-85).

However, Levi-Montalcini's research could not continue for long. 1936 was the year that Mussolini began to issue various anti-Semitic laws that banned non-Aryan Italian scientists from academic and professional careers. One prepared article of Levi-Montalcini's project was rejected by an Italian journal because she was a Jewish. The article was only published one year later by a Swiss journal. In 1938, Levi-Montalcini had to spend two years as a guest in a neurological institute because she could no longer work professionally at an Italian institute.

It was during a trip in the spring of 1940 when she traveled from the city Turin to a village where her family decided to hide from the Aryan world that Levi-Montalcini encountered Hamburger's 1934 paper. The train that carried Levi-Montalcini was a cattle train that was usually used for the transportation of livestock, but was adapted during the

war for civilian passengers because many trains were taken over for military use. With her legs dangling over the side in the open air, and with the fragrance of the mountain villages along the way, Levi-Montalcini read the paper that Levi had given her two years before. The "limpid style and the rigor of his analysis" greatly impressed Levi-Montalcini and seemed to her to "cast new light on the problem" (Levi-Montalcini 1988).

With no official means to investigate scientific problems, Levi-Montalcini decided to set up a small laboratory in her own bedroom. Although she could not use advanced electrophysiological technology, she could rely on her expertise in microsurgery and silver-impregnation technique that selectively colors nervous tissues. Levi-Montalcini's favorite system, the chick embryo, was also readily available in countryside. With her mother's help, Levi-Montalcini obtained a small thermostat to make an incubator, assembled a microscope using watchmaker's forceps, ophthalmic microscissors and surgical instruments such as sewing needles. Having finished the basic set-up, Levi-Montalcini started to use the bedroom-laboratory to investigate the question raised in Hamburger's 1934 paper, how development of central neurons in chickens was affected by extirpations of the limb buds. She tested the chick embryos whose budding limbs had been excised in three-day specimens, and impregnated the neurons using the silver technique. Then she counted the motor neurons from the spinal column and from the sensory ganglia every three days until the end of the twenty-day incubation period. The major difference between her experiments and Hamburger's was that Levi-Montalcini examined the embryo from earlier stages and more often. When Levi-Montalcini needed more experimental materials, she would bike to hilly villages begging

farmers to sell her some chick eggs “for her babies” with the addition that fertilized eggs would be preferable because they were more nutritious (Levi-Montalcini 1988, 90-94).

In the winter and spring of 1942, Levi-Montalcini’s work gained considerable progress. Based on her observation, she realized that Hamburger’s hypothesis that the smaller central neurons were due to fewer cell divisions and growth caused by the lack of inductive factors secreted by the limb structure might not be true. Her own results showed that the embryos with excised limbs continued normal cell divisions and differentiations for some time. Some of the neurons generated from these normal developmental processes, however, started to degenerate after the fibers sprang out of the cord and ganglia and reached the stump of the amputated limbs. The limbs seemed not to be providing the inductive factors, but something that prevented the existing neurons from dying.

Levi-Montalcini was intrigued by the great flexibility of the ways that the nervous system was formed. She wrote the reminiscences years later, “Now the nervous system appeared to me in a different light from its description in textbooks of neuroanatomy, where its structure is described as rigid and unchangeable. Only by following, from hour to hour in different specimens, as in a cinematographic sequence, the development of nerve centers and circuits, did I come to realize how dynamic these processes are; how individual cells behave in a way similar to that of living beings; how plastic and malleable is the entire nervous system” (Levi-Montalcini 1988, 96-97). The sense of discovery that the nervous system was formed with counterintuitive processes such as cell death encouraged Levi-Montalcini to continue her research. She published the results with her mentor Levi in a Belgian journal in 1942 (Levi-Montalcini and Levi 1942). In

the paper, Levi-Montalcini proposed that chick nerves develop through initial overproduction of neurons in early embryogenesis, followed by a mass deletion of excess neurons. She suggested that the peripheral influences intervened through the process of cell deletion.

In 1945, Levi-Montalcini eventually returned to Turin to resume research and training after the war. During the previous turbulent years, her study of chick neuron development seemed to provide an outlet for Levi-Montalcini to isolate herself from the pains and stress from the war. The research also established her confidence in carrying out individual research. What she did not predict was that the research she carried out also brought in collaborative opportunities with Hamburger, as well as the starting research that led her to a future Nobel Prize.

Co-Investigating about Cell Degeneration: A Conversation between Experiments and Inductive Theory

Because of the embargo on German and Italian publications during the war, Hamburger did not get a chance to read the 1942 paper until 1946. The discrepancy with his own interpretation of the process of central neuron development captured Hamburger's attention. He soon wrote a letter to Levi to ask whether Levi-Montalcini would like to visit his laboratory for a year to co-investigate the issue, with the cost covered by the Rockefeller Foundation.

Rita Levi-Montalcini arrived at Hamburger's laboratory on the new continent one year later, together with Renato Dulbecco who was heading to Bloomington, Indiana. Levi-Montalcini appreciated the scientific environment in the US greatly, and plunged into experimentations that demonstrated her previous results to Hamburger. Together,

Hamburger and Levi-Montalcini targeted examination of all activities of cell division, cell differentiation, and possible cell degeneration, to investigate the questions when, where, and to what extent and effect these cellular activity occur during the development of the central nervous system.

The experiments lasted for more than one year and were very productive. They applied their specialty staining methods to detect a variety of cell activities in the chick embryos of the same stage. Hamburger's methods of staining neurons with Hematoxylin was particularly useful for counting how many cells were going through division, while Levi-Montalcini's silver-impregnation method showed differentiated neurons lucidly. The material was partly silver impregnated and partly stained in Hematoxylin. More importantly, by combining the two methods, the surprising phenomena of cell degeneration could be checked through both methods, excluding potential methodological bias. As their paper eventually published in 1949 stated, "both techniques have to be used if all aspects of nerve tissue development are to be considered," because "they demonstrate the importance of two components which had not been analyzed before, namely proliferation and degeneration, and they bring into focus the complex interplay of different components" (Hamburger and Levi-Montalcini 1949).

Hamburger and Levi-Montalcini took more frequent checks of cell numbers than they had in previous experiments. For example, in one set of experiments, after removing chick limb buds from 36–48-hour embryos, she recorded the neuron numbers every day until the end of the sixth day post extirpation. The cell counts in spinal cords declined over the six-day period in both the extirpated and intact chick embryos, with the former displaying more significant cell deaths. Levi-Montalcini thus demonstrated to

Hamburger's satisfaction that those neurons were first overproduced in the central nervous system, followed by a process of degeneration in the excessive neurons.

Their careful study yielded results that showed that both Hamburger's and Levi-Montalcini's interpretations captured one side of the story of the development of the central nervous system. They found that both mitotic activities and cell degeneration were influenced by the extirpation of the limbs. Especially, in the cervical and thoracic ganglia they saw a large-scale degeneration of early differentiated neuroblasts, but no such degeneration occurs in limb innvervating ganglia. They concluded that the difference in size of ganglia was brought about by "differences in mitotic activity and, in part, by selective degeneration" (Hamburger and Levi-Montalcini 1949).

The collaboration demonstrated to Hamburger that cell degeneration was one important process that shaped the development of central neurons, and their work together refined and completed Levi-Montalcini's original observation. Although the two went through the same series of experiments and analyzed the same sets of results, it seems that Hamburger and Levi-Montalcini conceived the epistemic role of their 1947 experiments differently. While Levi-Montalcini saw the experimental *productivity* and *novelty* as the primary force, Hamburger conceived experiments as a *dialogue* with living systems that could only be meaningful with careful analysis under preexisting or new theories.

From both researchers' later writings that recount the early collaboration, one gets the impression that Levi-Montalcini was doing most of the technical work: extirpation, silver permeation, tissue sectioning, cell counting, and result reporting. She also paid special attention to the novelty of the death phenomena, and conjured vivid war

metaphors to describe the "massive neuronal death" that was detected on both the operated and non-operated sides.

As Levi-Montalcini recounted later in her autobiography, *In Praise of Imperfection: My Life and Work*, she took great pleasure in the silver-impregnation method she was using, "While I was somewhat haphazardly examining, under the microscope, the latest series of silver-salt-impregnated chick-embryo sections, their coloring had come out perfectly: the nerve cells that had begun to differentiate in the cerebral vesicles and spinal cord – the embryos had been fixed between the third and the seventh days of incubation—stood out in their every detail with a deep brown-blackish hue on the golden yellow background of the nonimpregnated cord tissue formed of satellite cells and as yet undifferentiated nerve cells." These cells seemed to invoke the vivid memory of the recent war for Levi-Montalcini, so that for her, "the thoracic and sacral segments offered a spectacle not unlike that of the maneuvers of large armies on a battlefield" (Levi-Montalcini 1988)[2].

The degenerating cells continued to sustain Levi-Montalcini's imagination so that the memory about the observations was still vivid when Levi-Montalcini wrote his autobiography in the 1980s. "That day, however, as I peered through the microscope, nerve cells were acquiring a personality not usually attributed to them… At the cervical

[2] Levi-Montalcini became quite vocal about her own achievements after the 1986 Nobel Prize and was said to exaggerate her contribution to the discovery of the nerve growth factor at the expense of giving proper credit to Hamburger. Consequently, her narrative of the discovery of cell death should be taken with a grain of salt. However, since quoted paragraphs do not deal with scientific credit directly, and that both Hamburger and Levi-Montalcini had acknowledged the experimental contributions of Levi-Montalcini during their collaboration in 1940s, I use these quotations to analyze Levi-Montalcini's approaches to experiments.

level, I was witness to the disappearance of the membrane marking the boundaries of the nucleus, the retraction of the nerve fiber, and a decrease in the volume of the cell bodies. In immediately successive stages, the same cells acquired an appearance known, in histological terminology as 'picnotic,' or indicating the establishment of a process of irreversible degeneration. The impression was that of a battlefield covered with corpses. In embryos fixed at the stage immediately afterward – that is, ten to twenty-four hours later – the ventro-medial sector was invaded by macrophages, or cells able to ingest and destroy bacteria and the detritus of other, degenerated cells. The image that suggested itself to me was of corpses being removed from a battlefield by special crews trained and equipped for the purpose. A few hours later, all traces of cellular detritus had vanished; the column of cells still differentiating was vastly reduced in diameter, and an inspection of the fibers of the cells that comprised it revealed that they were innervating the muscles of the same segment of the trunk" (Levi-Montalcini 1988).

Levi-Montalcini remembered that she was fascinated by the phenomena. She knocked on Hamburger's office door that day and asked him to follow her back to witness the spectacle. "Viktor listened attentively, amused by my enthusiasm, and agreed that they were observations of extreme interest, which provided the key for the study of differentiative processes in the nervous system up till then practically ignored" (Levi-Montalcini 1988).

Hamburger, on the other hand, was not so much intrigued by the novelty of the cell death phenomenon as by its implications about how accurate the induction principles captured how the peripheral organs were innervated. Having studied embryology in Germany, Hamburger had heard about the existence of cell death from other

embryologists, such as E. Kallius, the director of the Anatomy Department of the University of Heidelberg and his students Max Ernst and Alfred Glücksmann. In an extensive investigation of cell death around 1926, Ernst recorded the occurrences of cell death in a variety of animal classes and noted that cell death share similar patterns in corresponding stages of development from different animal classes (Ernst 1926). However, these cell deaths reported by Ernst often seemed to be necessary steps to sculpting certain structures, such as tubes, vacuoles or folds – the cells inside of a hollow structure had to disintegrate for the structures to take shape. With such knowledge, for Hamburger, neuronal death was not an important discovery by itself. Its significance was that cell death happened during the development of the central nervous system, in which no particular hollow structures seem to require cell death, and that Spemann's inductive principles may not apply to the neuron development (Hamburger 1992).

In reminiscence of the collaboration with Levi-Montalcini, Hamburger noted, "To an experimental embryologist of the Spemann school, the idea that we might be dealing with the death of neurons would have been hardly conceivable. Embryonic induction, on the other hand, was a familiar theme, and I had formulated the recruitment hypothesis after the fashion of assimilative induction which plays a role in the activity of the organizer" (Hamburger 1992). The finding of the massive cell death as an important process in the nerve development led Hamburger to question whether inductive growth could explain innervation. If most neurons have default fates of death, Hamburger reasoned, then the survival neurons that innervate the peripheral organs must be maintained not to die. The developing peripheral organs may not provide factors to induce growth, but factors that inhibit the cell death activity to *maintain* the cellular

survival. Hamburger was puzzled that these important implications of cell death appeared nowhere in Levi-Montalcini and Levi's previous publication about cell death. In the 1949 paper resulting from the collaboration for which Hamburger did most of the writing, he raised the suggestion that instead of inducing growth, the periphery may simply be "necessary for the maintenance of sensory neurons" instead (Hamburger 1992). In fact, Hamburger took the existence of cell death of a particular cell type as the primary indicator that induction was not the whole story. Ten years later, he repeated the experiment once more to confirm that the degeneration of both differentiated and undifferentiated neurons happen, just so that "the recruitment hypothesis can be laid to eternal rest. (Hamburger 1958)"

Since Hamburger did most of the writing of the 1949 paper he published with Levi-Montalcini in the *Journal of Experimental Zoology*, the importance of the novelty of cell death phenomenon is downplayed. The emphasis was on the importance of in-depth analysis and synthesis of experimental results that revise existing explanations about embryo. The paper started with a statement that emphasized the importance of synthesis of experiments and analyses, "from combination of direct observation, on all levels, and of causal analysis will emerge a more adequate description of embryological phenomena than from experimental studies alone." The section "cellular degeneration" lacked any perceptible excitement about the novelty of the phenomenon, but instead carefully described why the authors believed what they had observed were really cell degeneration, but not other potentially differentiated or undifferentiated cells. In the end of the paper, cell death was depicted as one of the many other cell processes that played a role in the nuanced dynamics of central neuron development in diverse organisms: "Degenerating

cells are of widespread occurrence in embryonic tissues. One should distinguish between *sporadic* cell degeneration of individual cells, and large-scale, localized and *patterned* degeneration processes which result in morphogenetically significant changes. The formation of gill slits, of mouth and anus, the resorption of tail buds in birds and man and of the tadpole tail at metamorphosis are but a few examples of the latter category. The present instance is a special case within this group" (Hamburger and Levi-Montalcini 1949).[3]

The Appeals of Cell Death with its Experimental Novelty and Theoretical Import

With the same set of experiments and results, Levi-Montalcini and Hamburger nevertheless incorporated the newly discovered cell degeneration in disparate ways. While Levi-Montalcini viewed cell death as a novel phenomenon worthy of scientific investigations solely because of its novelty, Hamburger viewed cell death as important most because its existence refuted the induction as a primary cause in chick neuron development. Certainly, how one conceives and interprets a particular new phenomenon varies with the singularity and complexity of the individual scientist, and cannot be captured only through their general philosophy or style towards research. However, the differences between Hamburger's and Levi-Montalcini's approaches towards

[3] I took these quotes from the 1949 paper as representative of Hamburger's approach for several reasons. First, the analysis of the chick neuron development in the 1949 paper is distinctly representative of the approach of experimental embryology that emphasizes the interaction of embryonic parts and the causes of developmental change. Such approach is more akin to Hamburger than to Levi-Montalcini. Second, both Hamburger and Levi-Montalcini have indicated that Hamburger was responsible for writing the 1949 paper. Third, extensive theoretical analysis about cell death was absent from Levi-Montalcini's previous paper about chick neuron development published in 1942. Hamburger also expressed that Levi-Montalcini and Levi were not specific in envisaging the implication of cell death elsewhere (Hamburger 1958). Thus it is likely Hamburger was solely responsible for these quotes regarding theoretical importance of cell death.

experimental novelties seemed to align with debates between two different philosophical views about biological experimentation.

First of all, while Hamburger's training held the causal explanation as the highest ideal, Levi-Montalcini came from a scientific culture that value visual observation and novel phenomena. Hamburger came from the core of the classic experimental embryology. In Levi-Montalcini's words, he is "one of Hans Spemann's pupils." Spemann's concepts of organizer and induction were supposed to provide an overarching principle that explain development and guides further experiments (Hamburger 1988). In contrast, Levi-Montalcini studied with the eclectic anatomist Giuseppe Levi, who inherited the visual tradition of neurobiology originated from Golgi and Cajal. Not necessarily registered to explicit hypothesis, studying with the master Levi, Levi-Montalcini used to count cell numbers in numerous embryo sections just to get a sense of how nervous system changes over time (Hitchcock 2004).

There were intrinsic differences in how experimentations figured in scheme of research in the two traditions. From early 1920s to late 1930s, Spemann's laboratory continued to experiment about the nature of organizer and how it worked to induce structures in the embryo. In the neuro-anatomic school that Levi-Montalcini came from, however, to observe and record new phenomena was prized. Cajal himself had used Golgi's staining techniques and recorded a number of new phenomena in the neuronal system, which became the basis of his cellular theory of the nervous system. Levi-Montalcini's mentor Levi had himself directly studied with Cajal and transmitted the silver-impregnation technique to his students including Levi-Montalcini.

In this case, Hamburger's seniority seemed also to be a factor that influenced his view about cell death as an experimental novelty. Hamburger was nine years older than Levi-Montalcini. Coming from a celebrated tradition of experimental embryology in 1930s, by the end of the 1940s, he had already established a leading position in the field of embryology. He was confident that surgical methods from experimental embryology including extirpation and transplantation constitute, as he put it, "a direct dialogue with the embryo." Levi-Montalcini, however, was not so sure. She doubted whether the traditional surgical interventions could reveal the secrets of the nervous system. Out of the shadow of the WWII, she was eager to establish herself in the new country. In a time when molecular biology began to promise attractive opportunities, she had seriously considered about switching to microbiology because research on phage had contributed to molecular biology greatly. She confided her concerns to a former colleague Salvador Luria, a microbiologist at the University of Indiana. I did not hide … my distressing doubts about the validity of our approach… I believe his doubts were much greater than mine … He excluded the possibility of my successfully taking up microbiology, unprepared as I was in the field. (Levi-Montalcini 1988).

With the new success, Levi-Montalcini soon recovered from her distrust of the experimental system at hand. However, her obsession about experimental novelty persisted. To study one novel phenomenon Hamburger pointed out to her, that tumor extracts can induce neuron growth, Levi-Montalcini went to Rio de Janeiro in Brazil in the 1950s to take advantage of the tissue culture facility there. She called the pattern of outgrowth of neurons in *vitro* the "halo effect" and described that it revealed itself "in a theatrical and grand way, as if spurred by the bright atmosphere of … the Carnival in

Rio" (Levi-Montalcini 1982). Her work in Brazil proved pivotal for the later discovery of nerve growth factor.

For Hamburger, he had heard about the existence of cell death in Germany, and the phenomenon was not completely new to him. Although the existence of cell degeneration interested him, he was more interested in such questions as to what extent and through what processes these cell degeneration play out in shaping the nervous system. Already well-established and well-funded, Hamburger took time to investigate the nuances in the balances between different cellular activities during the development of the central nervous system.

Hamburger's and Levi-Montalcini's different emphases about experimentation remind us of some important historiographical arguments about the roles of experiments in modern biology. In a spirit similar to Levi-Montalcini, Hans-Jörg Rheinberger emphasizes the novelty-producing capability of an "experimental system" in leading science to new frontiers. According to Rheinberger, an experimental system is a "generator of surprises," a "vehicle for materializing questions." Through its power of generating differences, an experimental system can take a life of its own which leads the researcher to unforeseen directions. Rheinberger regards the virtuosity of an experimenter as consisting of an "acquired intuition," which also happens to be a characteristic other scientists assign to Levi-Montalcini (Rheinberger 1997).

Rheinberger's work inspired others to examine scientific practice through various experimental systems, but also incurred criticisms. Historian Frederic Holmes commented that Rheinberger seems to consciously marginalize the purposes, intentions, and thoughts of the scientists, so that the roles Rheinberger assigned to experiments are

exaggerated. In Holmes' conceptual framework "investigative pathway," "*creativity is purposeful work*." The investigator can choose not to follow the directions opened up in the experimental system, if it does not fit into his overall research directions cultivated from past experiences (Holmes 2004, 122-124). It seems that Hamburger's use of experiments to resolve a puzzle in the central-peripheral problem can be better characterized by Holmes' perspective.

Philosopher Marcel Weber showed that although a case in oxidative phosphorylation research can be represented purely through Rheinberger's notion of experimental system, in such rendering, important factors such as epistemic norms that can better explain certain historical occurrences are left out of the picture (Weber 2005). Along with Weber, I offer an important dimension about experimental investigation that is missing from a Rheinbergerian view. That is, one same course of experiments can be viewed differently from its investigators.

In this case, both views about cell death contributed to make cell death a biologically meaningful phenomenon. The multiple connections of cell death to researchers' interest for different reasons made cell death as a scientific object stable. In the preface of an anthology of articles each tracing the emergence of a distinct scientific object, Lorraine Daston noted that scientific objects are at the same time real entities out in the world and inventions constructed with certain devices and molded by historically and locally contingent imaginations and interpretations (Daston 2000, 2-3). In order to emerge as meaningful, a scientific object needs to have the potential of encompassing a variety of representations that connect the scientific object to local, material, and practical networks. To adopt what Daston called applied metaphysics, neuronal death in chick

development in this case only became real through techniques of staining neurons and scientists' interest in chick embryos. Its appeals of novelty and its connections to scientific theories both helped to stabilize cell death as a lasting concept in biology.

Expanding Research on Cell Death during Development after 1949

The collaborative study of cell death and their different scientific approaches had lasting resonances in both Hamburger's and Levi-Montalcini's scientific careers. After 1949, Hamburger continued to suggest ways that biochemical or physical signals from the peripheral organs may maintain the survival of central neurons. He also gave theoretical guidance to Levi-Montlacini and suggested that the nerve growth factor should not only be relevant to tumors but also to normal development that prevented cell death, which proved to be true. Hamburger served, in historian Garland Allen's words, "a bridge figure" between ideas and methodologies. After Levi-Montalcini won the Nobel Prize for the discovery of the nerve growth factor, her sensational presentations of phenomena irritated Hamburger. For Hamburger, these distorted stories were far from how their science actually worked.

After 1949, Levi-Montalcini continued to emphasize on the novelties of phenomena she was studying. The effects from tumor explants that kept extremely high number of neurons in the chick embryo became the next fascination of Levi-Montalcini, which led to her co-discovery of the nerve growth factor. Ironically, Levi-Montalcini did not do research explicitly on neuron death and its mechanism after 1950.

Although the novel aspects and the theoretical implications of cell death led Hamburger and Levi-Montalcini to different research paths, many research motivated by the novelty of cell death accumulated from 1940s to 1970s and coalesced to propel

researchers to ask the biological implications of about cell death. Both the interests in the cell death phenomenon itself and the inquiries into the significance and mechanism of cell death influenced the practice about cellular life in embryology by directing embryologists' attention to cell death.

Although the novelty of cell death phenomena made the discoveries rewarding, without scientifically worthwhile questions to follow up on the initial finding of cell death, the interests in researching cell death in the nineteenth-century usually faded away. In the twentieth century, however, with increasingly powerful techniques to visualize cellular activities, reports about cell death became more frequent, which gradually made cell death known to an increasing number of embryologists. The increasing amount of cell death research such as Hamburger and Levi-Montalcini's in turn triggered other embryologists to pay attention to the occurrences of cell death. Well-known cases include developmental biologist John W. Saunders' study of cell death in chick wing development using the vital dye Nile Blue and insect physiologist Richard A. Lockshin's study of cell death in the metamorphosis of silkworms in the 1960s (Saunders, Gasseling, and Saunders 1962, Lockshin and Williams 1964a).

Among the scientific reports about cell death, Hamburger and Levi-Montalcini's paper published in 1949 was not the most widely cited. It nevertheless for the first time incorporated cell degeneration into the major stream of neuroembryology, by pupils of Italian neuroanatomical school and German school of experimental embryology. Hamburger and Levi-Montalcini combined their individual strength in experimental skill and analytical rigor to make the paper balanced with delicate visual observations and analytical analysis. Perhaps this is why the German embryologist Alfred Glücksmann

took great care in describing the discovery of the Hamburger/Levi-Montalcini paper in his 1951 review (Glücksmann 1951a).

Others also began to think about cell death in terms of their biological functions and mechanisms after the observations of cell death accumulated. Probably the most telling about the effect of the accumulations of cell death studies such as Hamburger and Levi-Montalcini's was the appearance of review articles about cell death in the 1950s and 1960s. In a 1951 review article, Alfred Glücksmann listed 74 distinct observations of cell death during development. Perhaps because of the sheer number of cell death reports he cited, Glücksmann summarized the common cellular morphological changes observed in these cell death and speculated about the functions of cell death by classifying them into morphogenetic, histiogenetic and phylogenetic degenerations (Glücksmann 1951a). American developmental biologist John Saunders also wrote a review around 1964 with the title "Death in Embryonic Systems," suggesting the control of cell death can be tissue-environmental, hormonal, or genetic (Saunders 1966a). These reviews about cell death went beyond simple descriptions of cell death. They were speculative and theoretically inclined, an approach Hamburger had used in 1949.

Hamburger and Levi-Montalcini's discovery of cell death also challenged Spemann's induction principle and encouraged embryologists to adopt more flexible causal frameworks about development. To carefully investigate the patterns of cell death in neuron development, Hamburger returned to the topic through examining the exact patterns of cell death in the lateral motor columns in 1958 and 1975 (Hamburger 1958, 1975). He was explicit that the 1958 attempt to reconfirm the extent of neuron death was to put the recruitment hypothesis through induction to an "eternal rest" (Hamburger

1992). In the 1940s and 1950s, the induction concept was in crisis. Spemann died early during the war while most of his students (including Hamburger and Holtfreter) emigrated to the US and changed research focuses. Conrad Waddington and Joseph Needham, two major researchers about the organizer in England also moved on to other research questions. In 1948, Holtfreter noted, "the phenomenon of embryonic induction, which has been a focal point of interest to so many embryologists, has lately somewhat faded out of view" (Holtfreter 1948). Why exactly the embryonic induction faded was a larger historical question. In Hamburger's case, the discovery of the existence of cell death in the chick neuron development was a crucial turning point. The discovery of cell death led Hamburger and possibly other researchers once convinced about the general induction principle to a new view of development with an open mind receptive to new observations and complex developmental processes.

In summary, Hamburger and Levi-Montalcini's co-investigation established cell death as a functional in active developing area in the chick central nervous system. Because of the expectation of inductive growth of cells during development, cell death was surprising to Hamburger and changed his belief of induction. Levi-Montalcini, on the other hand, regarded the novel phenomenon itself appealing. Both epistemic approaches towards cell death helped to establish cell death as a biologically functional process and meaningful phenomenon in the 1950s and propelled contemporaneous embryologists pay more attention to cellular death while studying developmental processes.

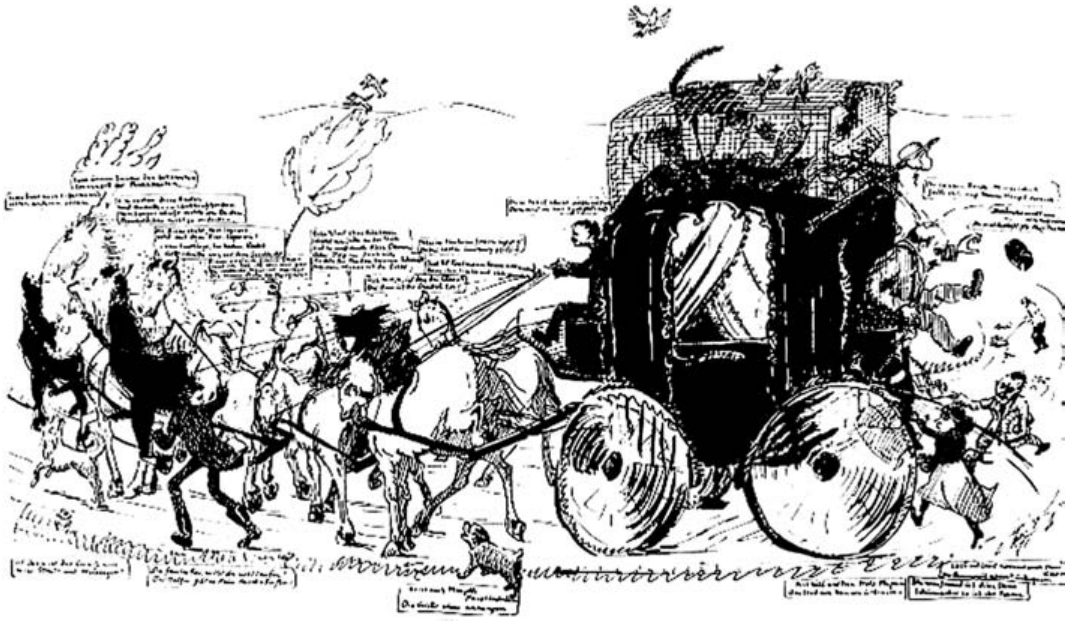
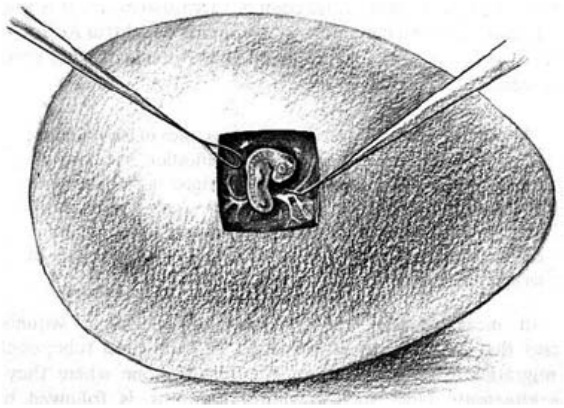


Figure 1. Drawing by Johannes Holtfreter in 1925 that depicts the scientists and staff at the Hans Spemann's laboratory. The horses represented students including Viktor Hamburger, Hilde Mangold, and Holtfreter himself, while Spemann is driving on the stagecoach (Hamburger 1996)



a.

b.

Figure 2 Microsurgical operations on chick embryos.

a. By opening a small window on the egg shell, Hamburger could operate on the chick embryos using a glass needle (right) to remove tissues and a hair loop (left) to transfer tissues. Afterwards, the window was sealed with a glass cover slip to facilitate further observation of the state of development (Hamburger 1977)

b. An embryologist working on surgically manipulate a chick embryo (Allen 2008)

Chapter 2: Aging Under Glass

The Discovery of the Hayflick Limit and the Evolution of the Cell Culture System for Aging Research

Acceptance of natural cell aging came later than the establishment of cell death as an acceptable phenomenon in development, and it seemed to arise more abruptly. In the early twentieth century, the French surgeon Alexis Carrel's idea that cells are intrinsically immortal held sway. When in the late 1950s American microbiologist Leonard Hayflick raised the issue that cells have a natural end to their proliferative life, his observations and ideas were initially rejected. As cell aging *in vitro* as a legitimate biological phenomenon became accepted in the late 1960s, the cell culture model of aging research attracted a community of cell aging researchers.

This chapter concerns the rise of aging of cultured cells as a scientific object and its transforming influences in aging research and other fields such as cancer research. It focuses on the research processes that led Hayflick to observe the cessation of proliferation in cultured cells and made him raise the issue about cell aging and its relation to cancer cells' immortality. The chapter also describes how, following Hayflick's interpretation about proliferative limit of cells, cell culture became a new experimental model for biology of aging research, and briefly how the emergence of cell aging as a natural phenomenon transformed the way virologists, cell biologists, cancer researchers, and gerontologists approached and interpreted the ends of cellular life.

Compared to the establishment of cell death, cell aging emerged more abruptly. Since the late nineteenth century, a number of observations of cell death had shown ways that advocators of cell death in development were trying to establish the phenomenon as

real and important more generally (Glücksmann 1951a). These recurring, though at times tenuous efforts to establish cell death research made the beginning of cell death research cumulative and consolidated (See Chapter 1). Such cumulative effects did not happen to the research of naturally occurring cell aging. Partly due to the less dramatic morphological changes involved in cell aging, and partly because the prominent French surgeon Alexis Carrel had boldly yet convincingly claimed that cells should be intrinsically immortal, relatively few before the mid-twentieth century believed that cells, left undisturbed, can undergo wear and tear by themselves (Witkowski 1980).

Then in the late 1950s, Hayflick found that human diploid cells cultured in perfect conditions consistently lost the capability of cell division after a while. Hayflick did not make the discovery because of intentional investigations on cellular life span. Instead, managing the cell culture facility at the Wistar Institute for Anatomy and Biology, Philadelphia, Hayflick's focus was on maintaining cultured cells with perfect technical conditions to meet the demand of cell cultures from virologists, cell biologists, and pathologists there. By 1960, Hayflick isolated 25 strains of cells from human fetal tissues and kept these cells growing. He found that strains of cells maintained in the cell culture would stop dividing about half a year after they were first isolated. The great numbers of cell strains and the consistency of the inception of "aging phenomenon" made the existence of cell aging undeniable. Hayflick soon published a report to show that human diploid cells age *in vitro* and it might be a manifestation of aging of the organism at the cellular level. He also suggested that an important difference between human normal cells and cancer cells is that normal cells age while cancer cells can divide forever (Hayflick and Moorhead 1961, Hayflick 1965b).

Going against the commonly held view about cellular immortality, Hayflick's suggestion that cells age in cell culture was initially rejected. Yet Hayflick confirmed his results carefully with other scientists whose work increasingly depended on the human diploid cell strains Hayflick isolated. As Hayflick gained confidence in his results, he called for more research on cell aging *in vitro* in order to study aging at the cellular level (Hayflick 1965b, Hayflick 1966). The prospect of cell culture as an experimental model more manageable than *in vivo* methods for studying human aging gradually attracted a small community of aging researchers. As they adopted cell culture to study the mechanism of cell aging, and to extract knowledge about the organism by testing lifespan of cells isolated from it, these gerontologists stabilized cell aging as an primary investigative subject in aging research. By the late 1980s, cell aging researchers had expanded their study into a legitimate research field by collaborating with each other, training students, publishing articles, monographs and edited volumes, holding conferences, and establishing new journals dedicated to cell aging itself (Macieira-Coelho 1988).

In addition to the work on biology of aging, in the 1970s and 1980s cell aging researchers also produced experiments, hypotheses, and new results in attempts to explain the why cancer cells divide indefinitely by comparing them with human normal cells that age. Suggestions were made that aging might be a process of terminal differentiation that cancer cells never go through, or that the loss of repetitive sequences of DNA led to cell aging, which did not happen in cancer cells (Pereira-Smith and Smith 1988, Goldstein and Reis 1984). These studies connected the cell aging research to

cancer research and posited cell aging as a concept and subject at the intersection of aging research and cancer research prior to the telomere research in the 1990s.

The rise of cell aging *in vitro* from a commonly perceived technical failure to a legitimate investigative problem transformed the practical and epistemic approaches to cellular life in cell cultures. In addition to the aforementioned changes of approach within aging and cancer research, biologists such as vaccine researchers, virologists, and later stem cell researchers, began to pay attention to the age and timing of the cells under investigation or production. The concept of the Hayflick limit, a term that Australian immunologist Macfarlane Burnett gave to the limitedness of human diploid cell division, changed the perceived timeframe of cells from the eternity to a finitude.

This chapter traces exactly how such limit-setting characteristic of cell degeneration emerged as well as its impacts. I first introduce Hayflick's training as a microbiologist, his cell culture work at the Wistar Institute, and the research processes and material contexts that led to his discovery. Then, I describe the immediate rejection of Hayflick's finding in the context of Carrel's dogma about the intrinsic immortality of cells and how that influenced the way cell culturists approached dying cells in culture in the early- and mid-twentieth century. After that, I describe Hayflick's proposal of using cell culture as a model for aging research and the gradual acceptance and use of the cell culture model by gerontologists. Then I show the influences of introducing cell culture model to aging research and cancer research by briefly describing the work of Hayflick's student Woodring Wright and his collaborator Jerry Shay in the 1980s. At the end of the chapter, I discuss the ways the mid-twentieth-century practices and views about immortal cell life structured the formation of the limit-setting characteristic of cell degeneration. I

also analyze the ways the rise of cell aging *in vitro* as both an actual biological phenomenon and an experimental model transformed biological practices about cellular time in cell culture in multiple fields. Part of this discussion offers a critique of the popular historiography about cell aging research that has exaggerated the historical significance of telomere research.

Hayflick and his Practice of Cell Culture as a Microbiologist

Leonard Hayflick was born in 1928 in Philadelphia. After finishing a bachelor' degree in microbiology and chemistry at the University of Pennsylvania in 1951, Hayflick had worked as a research assistant in bacteriology at Merck Sharp & Dohme for two years before he was convinced that his intellect was adequate for a PhD degree in biology. In 1953, Hayflick enrolled in the graduate program in Medical Microbiology and Chemistry at the University of Pennsylvania and became interested in the biology of mycoplasma, then known as pleuropneumonia-like organisms (PPLO). Originally aiming to study about PPLO with the prominent expert in the field Harry E. Morton, Hayflick was disappointed to find out that Morton would not accept more students since he already had too many. Stuart Mudd, then chairman of the Department of the Microbiology, suggested that Hayflick study with Warren Stinebring, a new assistant professor at the department. Hayflick accepted the offer and learned that Stinebring would allow him the academic freedom to pursue the PPLO research (Hayflick 2009).

Hayflick first studied about the pathogens of a widely spread middle-ear infection among the Wistar rats at the nearby Wistar Institute and showed that the pathogen causing the disease was a kind of PPLO. For the project, Hayflick worked with middle-ear tissues from dead albino rats and successfully isolated the PPLO from these tissues.

Hayflick's aptitude for microbiological operations greatly impressed Stinebring because he knew that Morton's students never isolated PPLO from real organisms themselves but obtained them secondarily. The project earned Hayflick a master degree in Medical Microbiology (Hayflick 2009).

For his PhD project, Hayflick planned to study PPLOs through transferring them between fertilized chick embryos because PPLO-associated diseases in chickens are of great economic and industrial importance. However, he was assigned an old room from the Department of Microbiology that need serious furnishing in order to provide the aseptic conditions needed for the project. With fellow student Frank Capral, Hayflick carried out the carpentry work that transformed the room to a laboratory suitable for asceptic work. As Hayflick finished the refurnishing and began to arrange new experiments about PPLO in 1954, his advisor Stinebring came back to the laboratory from a tissue culture course at Mary Imogene Bassett Hospital in Cooperstown, New York, exuding enthusiasm. Stinebring became convinced that tissue culture promised a great future for biology and tried to persuade Hayflick to carry out a project on PPLO that incorporated tissue culture as a method. Initially not sure, Hayflick was gradually convinced about the potential of tissue cultures and began to learn about related techniques (Hayflick 2009).

The aseptic laboratory that Hayflick and Capral set up proved to be conducive to tissue culture work as well. Actually, since the laboratory requirements between tissue culture and microbiology were similar, including sterile conditions, incubators, and microscopy, the scientists that the expanding practice of tissue culture attracted in the 1950s were predominantly microbiologists. In addition, the conceptual framework of

viewing the cell in tissue culture was not far removed from viewing the bacterium in a bacterial culture. The practical and conceptual affinity between cell culture and microbiology propelled many microbiologists to start to use tissue culture to study a variety of problems in the 1950s (Puck 1972).

First learning by himself and later being aided by a manual published by the Tissue Culture Course that Stinebring took, Hayflick soon familiarized himself with several methods to culture chick heart cells (Course 1955). He learned to produce double-slide culture that had a clot made of chick embryo extract and chick plasma that was used to contain chick heart explants. After a certain period of growth, the cells from the tissue migrated outside the clot and became ready to be transferred again. For monolayer chick cell culture, Hayflick adopted the use of trypsin that separates cells attached to each other. He also played with HeLa cells obtained from the Children's Hospital of Philadelphia and raised PPLO on them. Hayflick compared the different cytological changes after cells in different cultures were infected with PPLO, and recorded how some of them degenerate (Hayflick and Stinebring 1960). At this stage of research, however, Hayflick only needed a small number of cell cultures and he did not have the need to raise these cultures for long period of time.

Finishing his PhD in 1956, Hayflick did a year of postdoctoral research at the laboratory of the human cytogeneticist Charles Marc Pomerat at the University of Texas Medical Branch in Galveston, Texas. Pomerat had established cell cultures from humans, and was largely responsible for the development of tissue culture societies in Europe and Japan. He was enthusiastic about advocating what he called a "dynamic cytology," doing which means closely observing various changes happened in cultured cells and

sometimes filming them as record and as data (Goldblatt 1994). In Galveston, Hayflick joined Pomerat's work in testing the injury effects of irradiations on cultured HeLa cells (Pomerat et al. 1958). Using time-lapse phase-contrast cinematography, he also made a film recording how HeLa cells were nibbled away by simian virus 40. Although these cellular injuries being investigated were inflicted from microorganisms or radiations and not necessarily a natural course of cellular life, these years of studying the degeneration of cell cultures probably had cultivated Hayflick's view that cellular degeneration itself is a phenomenon worthy of scientific inquiry.

The Encounter of the Limit of Cell Proliferation *in vitro*

In 1958, Hayflick returned to Philadelphia and became an associate member at the Wistar Institute for Anatomy and Biology. During the late 1950s, the Wistar Institute was undergoing a reformation through which the Institute's manifold virus research programs were launched and began to flourish. Under the energetic and ambitious leadership of the Polish-born biologist Hilary Koprowski, most old and dusty specimens and skeletons the Wistar hoarded for roughly a century were removed and replaced with modern virus laboratories. Although on the first floor of the three-floored building of Wistar, the museum still retained Wistar's nineteenth-century heritage with a collection of Siamese twins, a cyclops, and other aberrant human embryos, in the middle of the second floor, a large cell culture facility was established to provide the cell cultures as substrates for the cutting-edge sciences about virus and vaccines (Hall 2003, 20).[4] Viruses could not grow

[4] In the ambitious remodeling plan of 1957, the second floor's "main wing was converted into seven laboratories, each equipped and desgned for its specific discipline, e.g., Biochemistry, Pathology, Virology, Biology, and Tissue culture. These laboratories are centrally air conditioned and serviced with electric, gas, compressed air and vacuum

by themselves and needed living cells to multiply. Hayflick's appointment was to manage the cell culture facility on the second floor and provide for the needs of cell culture from about thirty scientists at Wistar.

At Wistar's cell culture facility, Hayflick's daily job not only involved the isolating new cell types and maintaining a large number of cell cultures, but also entailed cleaning glassware and preparing growth media. Fulfilling the job's demands, Hayflick however did not want to simply be a supertechnician and started to plan a research project that would use these cell cultures to elucidate scientific questions. In 1958, both the needs of vaccine research of the institute and Hayflick's research agenda propelled Hayflick to isolate and culture human diploid cells. It was reported that the poliomyelitis vaccines commonly generated from monkey kidney cells were contaminated with strains of viruses and raised concerns about the safety of these vaccines. The director Koprowski became interested in obtaining human fetal cells that are usually clean from such infections. Hayflick himself also became interested in cancer biology and learned that a recent hypothesis suggested that certain virus strains cause cancer. He was designing a series of experiments in which he could transfer the materials from malignant tissues to human normal cells and test whether these materials, possibly containing oncogenic viruses, could transform normal cells to cancer cells (Hall 2003, 21-22, Hayflick 1998b).

Isolating clean human diploid cells required fetal tissues, which was difficult to obtain since non-medical abortions were illegal. Although medical abortions happened at times at the nearby University of Pennsylvania Hospital, they were not as frequent as

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lines. In addition to the laboratories a utility room to house equipment used in common as well as large cold and incubator rooms were built (Biology 1960, 9-10)."

Hayflick's task required. It happened that a polio researcher named Sven Gard from the Karolinska Institute in Stockholm, Sweden, where abortions were legal, was spending a sabbatical year at the Wistar Institute in 1958. Gard agreed to help Hayflick and send Hayflick fetal tissues after abortions occurred at the Karolinska Institute. In the next several years, Hayflick would receive packaged fetal tissues from lung, kidney, skin and other organs from airmail at erratic times due to the schedules of abortions happened across the Atlantic ocean (Wadman 2013, Hall 2003, 22-23).

Once Hayflick received one package of fetal tissues, he chopped the tissues or teased them apart into pieces of several millimeters, and digested away the connective tissues with trypsin. Then, Hayflick replanted portions of the digested tissues to new Blake flasks with fresh media and incubate them at 36 °C. If the isolation of cells was successful, the cells would sprawl the flat bottom of the flask after three days. They were then subcultivated by being divided to new flasks with fresh culture media. Due to the high demands of such human fetal cell cultures, Hayflick kept subcultivating these cultures every three or four days. By 1961, Hayflick had amassed 25 strains of cells from human fetal tissues, generated from a variety of organs such as from the lung, skin, muscle, kidney, heart, liver, thymus and thyroid (Hayflick and Moorhead 1961).

These human fetal cells proved to be clean and suitable for virus research and vaccine production. Virologists and vaccine researchers around the world started to send out requests to the Wistar Institute and ask for several ampules of these cells to be mailed to them. Answering these requests, Hayflick also enlisted a cytogeneticist Paul Moorhead to test whether the number and shape of the chromosomes, or so-called karyotypes of these cells were diploid and normal. They were. Hayflick now became prepared to

embark on his planned research to test whether supernatant from cancer cell cultures could transform the normal human fetal cells (Biology 1960, 24-26). Scientists at the Wistar Institute valued these cells so much so that the WIHL cells (Wistar Institute Human Lung) became the cover story of the Institute's 1958-1959 Biennial Report.

Having meticulously conformed to a strict regimen of cell culture maintenance, Hayflick believed he had provided "the most favorable conditions" for those cells (Hayflick 1975). However, after cycles of a relatively stable rate, some of the cell populations, Hayflick noticed, stopped dividing and eventually underwent decay and death. These cells had been responding to the each serial cultivation that provided fresh medium and diluted cell concentration with vigorous growth and cell divisions for about half a year, but stopped dividing and kept metabolizing for a while before they eventually died (Figure 3) (Hayflick and Moorhead 1961).

Hayflick at first dismissed the cessations of cellular proliferation as trivial accidents. However, the pattern of losing proliferative capability after continued culturing practice for about half a year was repeated by all the 25 strains Hayflick had isolated. He in time started to discern the consistent pattern of the declines of these cell cultures. The large number of the cell strains helped Hayflick to recognize the pattern. More importantly, since the timings of the abortions from which fetal tissues came from happened with erratic timings, the timings when Hayflick received these tissues and started cell cultures from them were accordingly staggered. The erratic timings each initial culture started made it quite implausible to explain the declined proliferation by the factors associated with laboratory errors of any particular period, such as contaminated batches of cell culture media. Hayflick realized that for all the cells being incubated in the

facility, “only those that had been subcultivated longest had stopped dividing”, while the more recently cultured were still “luxuriating” (Hayflick 1998b, Hall 2003, 24-27).

Having studied cell degeneration caused by viruses and irradiations, Hayflick was intrigued that cells could degenerate by themselves. Yet he wanted to make sure that the cessation of cell division observed was indeed a natural phenomenon and not an artifact caused by malpractice of his own. Hayflick and Moorhead were both junior scientists and were careful and thorough when they tested the various characteristics of the human diploid cells at hand as well as the validity of their own methods. Were their observations caused by any faulty medium or technique? Or were they a manifestation of those cells inherent features? How could they demonstrate either case through experiment? Could the phenomena of cessation of cellular proliferation an artifact of cell cultures in general? To probe some of the issues, they froze some cells in liquid nitrogen, and tested whether being frozen changed the life span of these cells. It did not. With Moorhead’s cytogenetic specialties, they also examined the number and shapes of chromosomes in cells of a later stage. They were mostly normal, though usually with more damaged chromosomes when the cells were older. All available evidence showed that the loss of capability of cell divisions was probably a real biological phenomenon (Hayflick and Moorhead 1961).

Hayflick and Moorhead were cautious also because they were preparing cells for future vaccine production. It was important to make sure that the cultured cells were susceptible to virus but were not contaminated before being inoculated by viruses. They also confirmed that these cells were not prone to generating tumors by planting them into hamster cheek pouches, and even to the forearms of terminal cancer patients to make sure these cells would not cause tumors *in vivo*.

In 1961, Hayflick and Moorhead published their results with a paper of 37 pages in the journal *Experimental Cell Research*, "The Serial Cultivation of Human Diploid Cell Strains." The paper was written with dual purposes: the authors took great care to describe technical aspects of these human diploid cells, while also embedded a message that these cells lost proliferative ability with regular patterns.

Hayflick was aware that cell culturists assumed that cellular life *in vitro* should be intrinsically immortal if the culture conditions were kept perfect. In the 1961 paper, Hayflick consciously revised such a view by dividing normal cellular life *in vitro* to three periods. While the prosperous growth and proliferation characterized the first two periods, visible deterioration occurred in the third period, Phase III (Figure 4). Since the fetal cells Hayflick isolated were derived from a variety of fetal tissues and had much variability in life span among them, Hayflick did not assign a fixed number to the upper limit divisions that cells could go though, but contended that "there does exist a finite limit to the cultivation period" (Hayflick and Moorhead 1961).

Although the message about the division limit of cells was stated vaguely and buried with other technical features of the human diploid cells, Hayflick made several important statements about the implications of the existence of such a limit to the categories of cell types. First, Hayflick postulated a demarcation of cell cultures into two types, one with proliferative limit such as cultures generated from fetal lung cells and the other without, such as HeLa cells. It was then known that cancer cells had aberrant chromosomal constitutions, thus were heteroploid. It had also been discovered that some normal cells would undergo "alteration," with changes in the chromosomes, and subsequently acquire malignant growth, which suggested association of growth pattern

with chromosomal type (Parker, Castor, and McCulloch 1957). Consistent with the possible association, Hayflick and Moorhead's human normal cells with finite life span had been shown to maintain their chromosomal diploidy throughout their lifetime. Hayflick and Moorhead thus surmised that cells that preserve the karyotype typical of original tissue should also retain a limited life span, whereas the alteration in chromosomal type would confer the cell with a malignant, ceaselessly proliferative life.

Hayflick consequently suggested that two theretofore interchangeable terms, "cell strain" and "cell line," should be designated with distinctions. "Cell strain" should be reserved specifically for diploid cells with a definite proliferative limit, and "cell line" should be dedicated to describe the ever-proliferating heteroploid cells. Hayflick also entertained the idea whether the cessation of cell division in cultures was related to organismic aging. At this stage, he was reluctant to make definitive claims yet pointed out that cellular aging *in vitro* could at least serve as "an operational concept" for future investigation. In terms of why these cells age in culture, Hayflick hypothesized a self-duplicating factor within the cell, which was essential to cell survival. If the rate of duplication of such factor lagged behind the rate of cell proliferation, he reasoned, then at a certain point, the concentration of the factor might drop to a threshold level and trigger the cell to deteriorate. He pointed to an analogous mechanism, the Kappa factor that determined the mode of survival in certain species of protozoa Paramecium as their reference (Hayflick and Moorhead 1961, Sonneborn 1959).

As a paper that reported a milestone discovery, the Hayflick/Moorhead paper in 1961 seemed understated. The hybrid purposes of the paper also eclipsed the importance of the discovery of the proliferative limit of cells *in vitro*. Yet the article did raise

skepticism to the suggestion that normal cells naturally age and die when the manuscript was being reviewed for the *Journal of Experimental Medicine*. The journal soon rejected the manuscript because the editors denied that cells could age if the conditions for the cell cultures were kept perfect.

Alexis Carrel, his Immortal Cell Cultures, and Rejections of the Hayflick Limit

The editors from the *Journal of Experimental Medicine* were also concerned about the appropriateness of the length and the topic. But their foremost concern was about Hayflick's suggestion that cells could age in cell culture. In the rejection letter, Peyton Rous, the prominent cancer researcher who used cell culture extensively himself, noted that "The inference that death of the cells in some of the uninfected cultures is due to 'senescence at the cellular level seems notably rash. 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*" (Rous 1961).

The "largest fact" that cells are capable of multiplying indefinitely Rous mentioned was established by the French surgeon Alexis Carrel. Having developed effective surgical methods for vascular suturing, Carrel won the Nobel Prize in 1912 and gained fame. After learning about Ross Harrison's experiment through which he invented tissue cultures and resolved a long-standing controversy about the composition of the nervous system, Alexis Carrel adapted Harrison's hanging-drop method to more convenient tissue culture techniques that used glassware, inventing the Carrel flask and other useful apparatus. Carrel's method helped to keep the animal cells alive longer *in vitro*. Especially, when he pumped chick embryonic juice into the culture media, Carrel noted that cells were "rejuvenated." Carrel suggested that these cells in culture could

have a “permanent life” if the media can be kept renewed. Indeed, Carrel and Albert Ebeling, his assistant at the Rockefeller Institute of Medical Research in New York, claimed that they raised an “immortal chick heart” tissue culture. The chick heart tissue culture lasted for more than thirty years before being discarded after Carrel’s death. For Carrel, cells were intrinsically immortal. It implied that for immortality to become a reality for human race, human cells needed to be rejuvenated with renewed plasma and body fluids. Carrel and the famous pilot Charles Lindbergh would embark on a project to design perfusion pump that aimed to rejuvenate organs in the 1930s (Friedman 2008, Maienschein 1978b, Landecker 2007, 68-106).

Although Carrel’s dream of human immortality research was truncated by his forced retirement and his unhappy life in the early 1940s, his notion of cellular immortality, along with his many inventions towards improving tissue culture left long lasting imprint to cell culture practice in the 1950s. Although during the late 1950s, the observation of declined proliferation of cells was rather a banal encounter among cell culturists, the phenomenon was dismissed as a sign of inadequate cell culture practice. If cells die *in vitro*, cell culturists would try harder to keep them alive, attempting to satisfy each cell type’s fastidious needs by tinkering the medium, or by changing temperature. Provided that cells were intrinsically immortal, the only causes of cell degeneration would be exterior factors.

For example, unknown to most cell culturists, two scientists working at Western Reserve University, H. Earle Swim and Robert F. Parker, carefully documented the patterns of proliferative cessations in human diploid cell cultures and published the results in 1957. Yet their conclusion was framed in an indecisive and strictly technical

way, as stated in the end of their report, "normal human fibroblasts will not proliferative indefinitely in the media used but may nevertheless yield permanent lines of cells as a result of infrequent alterations in their nutritional requirements" (Swim and Parker 1957, 242).

The dismissive attitude towards the natural declines of cell cultures more typical among cell culturists in the 1950s can be found in a 1957 paper published by the well-known cell biologist Theodore Puck. While trying to design a plating technique for single fibroblast, Puck also observed a series of declined cell cultures. He nevertheless regarded the observations as indicative of inadequate culture medium. Puck noted, "It is a common experience in tissue culture that freshly isolated human cells can be readily induced to multiply temporarily. After a period of weeks, the growth of such cultures usually declines and eventually stops …… This sequence of events strongly suggests an inadequacy of the nutrient medium due to the presence of toxic substances or to deficiencies in the required concentrations of nutrilites" (Puck, Cieciura, and Fisher 1957). In the question session during a plenary lecture given by Puck at the annual meeting of the Federation of American Societies for Experimental Biology in 1959, Hayflick actually asked Puck whether he had ever experienced that human cells stopped dividing. Puck answered affirmatively that it happened often but was not a problem since he could reconstitute new cultures from the cell stocks in the freezer. Puck's positive answer made Hayflick convinced that what he had observed was real (Hall 2003, 27-28).

With cell culturists' general dismissive attitudes towards the degenerative cell cultures, it was no surprise that Hayflick's suggestion about cell aging was not well received immediately. Besides the rejection letter from the *Journal of Experimental*

Medicine, Hayflick's hypothesis that cells do age *in vitro* was challenged in an open discussion during a conference on aging at the Salk Institute for Biological Studies, San Diego in 1965 (Hayflick 1965a, 117-123).

Hayflick himself also had had moments of doubts before 1961 and tried to confirm his results with various means. In addition to confirming the observation with Puck during the 1959 conference, Hayflick also sent out some of the human cells to a few respected cell culturists and virologists and asked them to test whether these cells would stop dividing after about half a year. All the feedback on the request was positive: they found that the cells would stop dividing just as Hayflick predicted (Hall 2003, 28). These confirmations made Hayflick confident about his findings in the early 1960s. After all, he had by then mastered a variety of cell culture techniques and witnessed the new cell culture maneuvers such as using antibiotics, roller tube method, and the single cell plating techniques invented in the 1950s that empowered cell culture to be applied to a wider range of cell types. For him, it seemed absurd to assume some cells could not keep dividing only because the cell culture technique was not adequate.

Eventually, *Experimental Cell Research*, a somewhat less prestigious journal, accepted Hayflick and Moorhead's paper. As Hayflick began to dig into the problem of cell aging deeper, his freezer in the basement of the Wistar Institute broke down by accident in the spring of 1961. All of the 25 strains of human diploid cells were destroyed. This accident devastated Hayflick and his colleagues at Wistar because many virologists' research at Wistar and around the world depended on these cells. Hayflick soon isolated WI-26 human cell strain but its cultures were quickly exhausted by high demands from scientists. The WI-27, that Hayflick isolated afterwards turned out to be

abnormal in its karyotype. Eventually, in June 1962, Hayflick obtained a cell strain to his satisfaction, WI-38, from the lung tissues of a female human fetus. WI-38 proved to be clean and had great susceptibility to a variety of viruses. Vaccines produced from WI-38 would immunize hundreds of millions of people against infectious diseases such as rubella, rabies, and polio.[5] The cell strain also became the substrate that Hayflick used to investigate cell aging *in vitro* deeper, and to connect the problem of cell aging *in vitro* to organismic aging (Wadman 2013, Hayflick 1965b).

Framing the Hayflick Limit for Aging Research

In early 1960s, Hayflick was not only busy with isolating, cultivating new human fetal cell strains, demonstrating their safety and productivities for vaccines (Hayflick et al. 1962), he also became interested again in the biology of PPLO. In a collaborated work with Robert Chanock from the National Institutes of Health, Hayflick isolated and cultured the etiological agent of primary atypical pneumonia using agar media and demonstrated this pathogen is PPLO. The study resolved a long-standing debate about the nature of the pathogen for atypical pneumonia and Hayflick did a series of work to continue the project for a while (Chanock, Hayflick, and Barile 1962). It was not until mid-1960s that Hayflick found time to return to studying the problem of cell aging.

Hayflick was interested in what are the parameters that gauge the lifespan of cell strains. Because storing cells in liquid nitrogen or not letting them grow for a while did not alter the period during which these cells were actively dividing, it was unlikely that the ages of cells were determined by absolute calendar time. Hayflick experimented on

[5] Hayflick prepared 800 glass ampules of WI-38 cells and preserved them in liquid nitrogen in July 1962, so that they can be retried when needed (Wadman 2013).

the effect of different dilution ratio in subcultivations on the durations of cell cultures and found they had no discernable influences either. After experimenting and calculating the times of cell divisions of three cell strains, WI-26, WI-38, and WI-44 before they stopped dividing, Hayflick figured that the total numbers of cell divisions each cells went through were roughly the same. Although different cell strains had varied maximal cell division times, they were roughly about 50. Hayflick called the number of cell divisions an individual cell went through before the onset of Phase III the number of cell doublings and defined that the number of potential cell doublings as the lifespan of individual cells. Hayflick reported this finding in *Experimental Cell Research* in 1965 with a paper titled "The Limited *in vitro* Lifetime of Human Diploid Cell Strains." The lifespan of cells was hence defined in terms of the number of cell doublings, a factor independent of specific experimental settings but dependent on the cell's intrinsic clock (Hayflick 1965b).

In addition to providing an explicit definition of the cellular lifespan, Hayflick also investigated whether the proliferative limit of cells could be a manifestation of aging at the cellular level. If dividing cells in culture share similar mechanisms with cellular aging *in vivo*, Hayflick reasoned that normal human cells should at least consume some of their proliferative potentials *in vivo* during the individual's lifetime. When cells in older individual were isolated, they should go through fewer cell doublings and reach the degenerative phase of cellular life, Phase III, earlier than fetal cells. To examine whether this was the case, Hayflick derived 8 strains of adult human cells and compared their number of cell doublings *in vitro* with that of fetal strains. He could not find direct correlations between donor age and cell doubling potential, but did find that the average number of cell doublings of adult cells, 20, was significantly lower than that of fetal cells,

48. Hayflick also learned that in repeated transplantation of the skin or mammary tissues from one host to another, researchers had found that at certain point, these grafts would fail to grow. The time they kept growing seemed to be inversely related to the age of animal donors, but unrelated to the age of hosts (Krohn 1962). Hayflick saw his experiments and Korhn's paper both providing evidence that the cell aging in humans was at least in certain respects similar to the cell aging *in vitro* (Hayflick 1965b).

In the 1965 paper, Hayflick emphatically called for more research about "a general cellular theory of aging" that was made possible because of his discovery of the natural limit of cell divisions in the human diploid cells. He again reiterated the demarcation of two types of cell: the diploid normal cells that had a limited lifespan, and the heteroploid cells, which are usually cancer cells, that could proliferate indefinitely. Hayflick also pointed out that aberrant chromosomes can be found in both aging cells and cancer cells and was puzzled why cancer cells do not age. He suggested, "any satisfactory theory of senescence at the cellular level …… must include, as a corollary, an explanation for the apparent lack of senescent-like changes in transplantable tumors *in vivo* and in heteroploid cell lines *in vitro*" (Hayflick 1965b).

Although Hayflick acknowledged that the mechanisms that lead cells to the degenerative mode, Phase III, still needed to be elucidated, Hayflick suggested Leo Szilard's somatic mutation hypothesis of aging as a possible explanation. In 1958, the nuclear-physicist-turned biologist Leo Szilard hypothesized that the mutational effects accumulated in DNA molecules from the cosmic irradiation might be the reason why organisms age (Szilard 1958). Szilard suggested that if one mutation caused by radiation is counted as one hit, there may be a threshold of the maximal number of mutational hits

that a cell can tolerate. More than that would cause the inactivation of the cell. One reason Hayflick favored Szilard's hypothesis was that the survival curves of human diploid cell strains over time were in similar shapes to the survival curves of cells or bacteria under irradiation experiments: for normal cell aging, cell number declined with time in Phase III; for radiation experiments, cell number declined with the increasing dose of radiation (Witkin 1946) (Figure 5). Extending on Szilard's hypothesis, Hayflick conjectured there might be either "n targets" to be mutated or "a single target which must sustain n hits before the target is inactivated" in a cell prior to the onset of Phase III (Hayflick 1965b). Hayflick kept holding this hypothesis as a plausible mechanism for cell aging well into the 1970s and 1980s.

In November 1965, Hayflick was invited to a symposium at the Salk Institute for Biological Studies, San Diego on "Topics in the Biology of Aging" dedicated to Leo Szilard who had recently passed away. The conference invited leading researchers of aging from a variety of fields and was funded by the Aging Program of the National Institute of Child Health and Human Development, the predecessor of National Institute on Aging. Regarding the cell degeneration and its relation to aging in organisms, both cell death and aging were included.[6] In the talk titled "Cell Culture and the Aging Phenomenon," Hayflick's suggestion of study cell aging *in vitro* became explicit and was presented to the attending experts in aging research. During the talk, Hayflick briefly introduced the experimental results from his previous publications and prescribed

[6] Notably, John W. Saunders, a leading researcher on the programmed cell death in chick wings working at the Marquette University gave a talk titled "Cell Death in Embryos: Accelerated Senescence?" (Krohn 1966)

questions that should be addressed in the study of aging in the light of the existence cell aging in cultures. First, the varied capability of cell division of cells from different species may provide clues to explain the difference of life spans in a variety of species. Second, how are the paces of stem cell division organized in organisms, so that a life-long population of dividing stem cells could be maintained. Third, with the experimentation on aging in tissue cultures, the cellular aging of multicellular organisms manifested in tissue cultures could be easily compared to the observations in the study of aging in protozoa, which may help redress the nature of cell division and its influence on the process of aging (Hayflick 1966). Steeped in the existing literature on aging research, Hayflick had encountered the diverse hypotheses and approaches in the field. He regarded that his model of researching aging in tissue culture could make the complex hypothesis testing in cellular aging more feasible and should be prioritized.

Hayflick noted, "The purpose of this presentation is not to develop another theory of aging but merely to reconsider, in light of newer knowledge, the question of the finite lifetime of cells cultured outside the animal body and what bearing this should have on current hypotheses. It is now possible that the powerful technique of cell cultivation may be exploited in investigating problems of senescence. Once the myth of the unlimited proliferation of normal cells *in vitro* is laid to rest, more emphasis should be placed on the notion that senescence results from the greater expression of events at the cellular level than at the tissue, organ, or organism level." Hayflick also emphasized the simplicity of cells in culture than studying the whole organism, "On the basis of current evidence the finite lifetime of normal cells *in vitro* may not only be a model for aging in

the whole organism, but indeed, might be the same phenomenon reduced to a lesser degree of complexity" (Hayflick 1966).

After Hayflick's talk and in the same session, Theodore Puck presented his work on prolonged cultures of mammal cells. Puck was skeptical towards Hayflick's suggestion of studying aging using cell cultures. He raised six questions that he considered necessary to be addressed before using cell cultures to study aging. These questions include "what are the biochemical mechanisms responsible for cessation of growth in diploid cells when such arrest occurs after a prolonged period of healthy multiplication under presumably *constant* environmental conditions," "what specific genetic determinants present in some or all diploid but not in some heteroploid cells underlie the biochemical action which terminates growth," and whether "this growth cessation involved in the mammalian aging process" (Krohn 1966, 101-123).

After the Hayflick's and Puck's talks, prominent gerontologists and biologists interested in problem of aging participated in heated discussions. They included gerontologists Bernard L. Strehler from the Veterans Administration Hostpital, Baltimore, Howard J. Curtis from Brookhaven National Laboratories, biologists Peter Medawar from National Institute of Medical Research, England, Leslie Orgel and Renato Dulbecco from the Salk Institute. They expressed both excitement and concerns about using cell cultures for aging research.

Certainly, Hayflick also went to others' talks and participated in the discussions during the 1965 conference. By doing so, Hayflick not only spread the message of using cell cultures to study aging, but also began to know the people in the field of gerontology. The conference became the starting point of Hayflick's long-term engagement with the

field of biology of aging as well as making gerontologists interested in cell cultures (Krohn 1966).

Studying Aging under Glass

When Hayflick made his call for a reconsideration of theories on cell aging around 1965, the biology of aging was a small yet expanding field. The fresh notion that cells can age *in vitro* and thus can be studied *in vitro* soon attracted a few biogerontologists. After all, tracing cellular changes inside higher organisms was difficult and time-consuming, if not downright impossible. The cell culture, in contrast, was a much more feasible platform for experimentation.

The development of the biology of aging in the US had a lot to do with the Canadian-born biologist Edmund Vincent Cowdry. Holding symposia about aging at the Marine Biological Laboratory, Woods Hole, Massachusetts for years and editing a series of volumes titled *Problems of Ageing* in the 1930s, Cowdry inculcated a synthetic and comprehensive style of doing aging research. Cowdry's style of leadership led to the highly interdisciplinary settings of gerontological research in the mid- and late-twentieth century. He was also a cytologist and was deeply influenced by Carrel's tissue culture experiments, probably through both being a colleague of Carrel's in the 1930s and their correspondence after 30s (Park 2008). Not surprisingly, Cowdry's article "Ageing of Individual Cells", published in the second edition of his edited volume, *Problems of Ageing* had the premise that cell aging is determined by environmental factors. It entailed that in the tissue culture cells should keep dividing permanently when the right media are provided. As such, using tissue culture to study aging phenomena meant to trace the life conditions of individual cells and analyze how they were injured before they die. The

duration of the whole cell population was irrelevant. To monitor individual cells, however, was difficult in the 1940s. As Cowdry noted, "to measure the length of life of individual cells is a task of almost insurmountable difficulty." The task involved tracing the cells that "wander about, or are washed from place to place," yet "individual cells can be followed in tissue cultures for hours, perhaps for days, but not for months or years" (Cowdry 1942, 630).

Instead of measuring individual cell's duration, Hayflick's suggestion of studying cell aging focused on the number of cell divisions, which provided unmatched experimental feasibility that appealed to biologists. During the 1965 meeting in San Diego, although Hayflick's proposal of using cell culture to study aging met the classical cell culturist's opposition from Puck, other audience at the Salk Institute was excited. During heated discussion after the session, new experiments to test the implications of the proliferative cessation of somatic cells to cancer biology were suggested. In one occasion, Curtis indicated that an accumulation of mutations might account for such cessation in proliferation, which could be tested experimentally. Although others raised concerns that aging in cell cultures might not be most relevant because only a portion of cells in mammalian bodies keep dividing for the organism's life time, the conference became the first occasion that many gerontologists heard about Hayflick's discovery and started to pursue researching aging using cell cultures (Krohn 1966, 101-123).

With the maturation of cell culture techniques in the 1950s and 60s, being able to make clones of single cells attested to the great potential of tissue cultures in diagnostic, pathological, and immunological research. Some important discoveries about the cells, such as pinocytosis, came from direct microscopic observations of cells in the culture.

Gerontologists were aware of the sea change of research tools in the biomedical fields and felt confined by the animal models that often took years to obtain meaningful results for aging research.

Aging research also went through extensive expansions during the 1950s and was ready to adopt new experimental tools into its frontiers. The 1965 conference in San Diego invited a number of biologists who researched about aging but was not trained in gerontology, such as Leslie Orgel and Hayflick himself. As historian W. Andrew Achenbaum observed, the 1960s and 1970s were times when a lot of scientists trained to be specialists or professionals in other fields were attracted to gerontology. Once they committed to the study of aging, they increasingly collaborated with people from different disciplines who were interested in aging and gradually identified themselves as partially gerontologists (Achenbaum 1995, 119-124). Particularly, with the perceived triumphs of the double helix in raising the status of molecular biology during the 1950s, biologists were proud of their tools and tried to offer gerontology with fresh biological research agenda, a motive that the 1965 San Diego meeting was predicated on.

As gerontologists and biologists interested in aging started to find tools to biologize gerontology, some of them were attracted to the experimental simplicity Hayflick's cell culture offered for studying the complex process of aging. One such researcher was the biochemist Vincent J. Cristofalo. Working as a postdoctoral fellow at Temple University in the early 1960s, Cristofalo heard about Hayflick's study of cellular aging. At the time Cristofalo was frustrated with the messiness of using tissue samples while studying liver tumors. His usual experimental rituals included tedious procedures such as grinding up tissues, fractionating the cell extracts, and trying to infiltrate

knowledge about cancer through various chemical analyses on these samples. Hayflick's cell culture system struck Cristofalo as a way to study single cells in environments that the researcher could control and "ask questions of the cell in its normal state" (Rattan 2001). In 1963, Cristofalo accepted a faculty appointment at the Wistar Institute to study cell aging biochemically. There, Cristofalo made a long-term career choice to study gerontological problems through investigating the chemical changes of the cells in culture throughout their lifetime. Cristofalo's choice of research topic paid off well. The National Institute on Aging funded Cristofalo's aging research based on cell culture for more than three decades. Along with Jay Roberts and George Baker, Cristofalo was dubbed as one of "the Philadelphia Mafia" in the gerontologic research circle (Adelman et al. 2001).

Some medical researchers of aging and age-related diseases also started to adopt Hayflick's cell culture experimental platform. One such researcher was Samuel Goldstein. Having trained as a medical doctor in Canada, in the early 1960s, Goldstein was taking his residency training and later as a research fellow in Harvard Medical School. His topic was the relationship between diabetes and the premature onset of several features of aging. After learning about Hayflick's research, Goldstein adopted the cell culture model of studying aging and started to test whether cells isolated from diabetic patients divide fewer times than normal cells, or whether these cells had a different glucose metabolic pattern. Goldstein called these undertakings of studying disease through cells *in vitro* "cell epidemiology." Although Goldstein did not find significant differences in cell doubling numbers between the cells from diabetes-prone individuals and the cells from the control group, Goldstein nevertheless found that cells

from the diabetes-prone group had much lower "plating efficiency," which meant that these cells divide much slower than the cells isolated from the control group. He proposed that such "plating efficiency" was probably a more important measure to gauge "physiological age" than the cell doubling potentials (Goldstein, Littlefield, and Soeldner 1969).

Goldstein also brought the cell culture system to the studies about other age-related issues. In a short report to *Lancet*, Goldstein described that cells from a 9-year-old boy with typical progeria displayed fewer mitoses in cell cultures and acclaimed that Hayflick's cellular aging model was "an excellent model for the study of ageing" (Goldstein 1969). He later showed that this *in vitro* model of aging was also useful in studying etiology of progeria and distinguishing normal aging from the processes in progeria. It had been shown that xeroderma pigmentosum, a progeric disease characterized by extreme sensitivity to ultra-violet light and natural sunlight, is due to compromised DNA repair system. At the same time, the gradual decline in DNA repair activity was proposed as a cause of normal aging. Goldstein designed open-end experiments to address the connection of DNA repair to etiology of xeroderma pigmentosum and the mechanisms of normal aging, in which he applied various dose of UV light to fibroblasts from both patients and normal individuals and tested the effect in the plating efficiency and DNA repair activity. The results published in 1971 showed that for UV-exposed cells derived from xeroderma pigmentosum patients, the plating efficiency declined and the DNA repair was much lower than the normal cells, which confirmed the pathology of xeroderma pigmentosum previously proposed (Goldstein 1971).

Hayflick's original proposal of using cell culture to study aging was thus expanded to diseases that were age-related or resembling accelerated aging. The cell culture that was put back to a limited timeframe provided a way to compare between normal cells and diseased cells, not only through their morphologies but also through their biological durations in the culture. Hayflick did not propose explicitly in the beginning to use cell cultures to study these diseases. This is one of the many ways that cell aging *in vitro* gained increasing research meanings and usages that Hayflick's proposal helped to inspire.

Hayflick's cell culture system of aging drastically changed scores of other researchers' methods about aging and related issues in the 1960s and 1970s. The ones that established lasting projects focusing on cellular aging in cell culture includes George M. Martin working at the University of Washington, James Smith from Baylor College of Medicine in Houston, Álvaro Macieira-Coelho and Woodring Wright, both studied with Hayflick in the 1960s, and Robin Holliday working at the MRC's National Institute for Medical Research in England, whose work will be the focus of the next chapter. These researchers' investigations created various interpretations, meanings, and uses of cell degeneration, which stabilized cell aging *in vitro* as a lasting scientific object around which a small research community began to form in the 1970s. The researchers of cell aging *in vitro* debated about whether cell aging was controlled by genetic factors or cytoplasmic factors, tested what the cellular fate will be if one fuses a cancer cell with an aging cell, measured how deterministic or stochastic the lifespan of a cell really is, and negotiated whether proliferative half of a cell really represents aging, or a kind of terminal differentiation (Holliday 1986, Macieira-Coelho 1988, Stein et al. 1982). These

researchers were aware the formation of the small community surrounding cell aging *in vitro* and further strengthened the development by writing review articles, holding conferences, even essay contests about the topic (Cristofalo 1972).

As cell aging *in vitro* became solidified as a research subject, the field of gerontology and oncology increasingly recognized Hayflick's work and credited him as an insider, even a leader. Hayflick played a major role in planning for the 1971 White House Conference on Aging in California, and served various panels for the National Cancer Institute. He was also appointed as a founding member of the National Advisory Council of the National Institute on Aging in 1975, and served as the president of the Gerontological Society in 1983. In 1994, Hayflick published a well-received book targeted to general readers, *How and Why we Age* (Hayflick 1994). As Hayflick's credentials increased over time, Hayflick started to write a number of reviews or historical accounts about his discovery. In these accounts, Hayflick depicted himself as originally doubtful about his discovery, yet he reconfirmed the results and challenged the dogma (Hayflick 1970). The implicated heroism of Hayflick's own narrative added a historical charm to studying *in vitro* cell aging and perhaps helped to attract more researchers towards the subject. Australia immunologist Sir Macfarlane Burnett's coining of "the Hayflick limit" in his 1974 book *Intrinsic Mutagenesis* particularly inaugurated the *in vitro* cell aging with a scientific lexicon and scientific importance during this period (Burnett 1974).

Studying Cell Immortalization though Aging Cells

By the mid-1970s, the limit of cell divisions as an intrinsic property of normal somatic cells was widely accepted. The small research community was established

around studying aging under glass during the time, which generated active research and training programs, a series of co-authored papers between its members, and most importantly, pushed its science forward. One of its scientific developments was to investigate the molecular connection between cancer and aging cells.

In the 1970s and early 1980s, researchers tried to investigate what genetic or biochemical differences existed between human normal cells and cancer cells that make the human normal cells age, while cancer cells do not. A notable strategy of such study was to fuse cancer cells with aging cells and test whether aging process or constant mitoses would take over. The results showed that aging was dominant and would make the hybrid cells stop dividing and eventually die. The dominance of the aging phenomenon raised the possibility that cell aging might be a well-regulated series of events that should not due to accumulations of recessive mutations. It also suggested the immortalization of cancer cells involved at least partial elimination of the aging process, making cell aging an important investigative subject and a potential therapeutic target to explore for cancer research (Pereira-Smith and Smith 1983, Stein et al. 1982).

These inklings about the relations between cancer cells and normal cells led to studies of immortalization of aging cells with simian virus 40 in the late 1980s and early 1990s that revealed important steps about how cell aging is related to cancer. Two major researchers in this area were molecular biologists Woodring Wright and Jerry Shay in the Department of Cell Biology at the University of Texas, Dallas. Wright had studied with Hayflick at Stanford University for his PhD project on the topic of how cells age *in vitro* in the early 1970s. They fused fibroblasts with enucleated cytoplasms and compared the lifespans of old/old, young/young, and old/young fusions. Their results suggested that the

cellular fates were in accord with the cells from which the nuclei came from. They thus concluded that the controlling factors of cell aging were probably located in the nuclei (Wright and Hayflick 1975).

Wright continued to investigate what such factors are in his new laboratory at the University of Texas. It was known that simian virus 40 (SV40) could immortalize normal cells and make them cancerous. In the 1970s, two proteins p53 and Rb were found to be the targets of SV40 and were thus called tumor suppressors. In the late 1980s, Wright and Shay investigated the relationship between cell aging and cell immortalization by transforming the cells already in Phase III with SV40. They found that some aging cells were transformed and divided for more times than normally aging cells. However, while Wright and Shay continued to culture these transformed cells, most of them eventually stopped dividing. Only a fraction of these cells seemed to reach true immortal states. Wright and Shay concluded that the immortalization of normal cells came with two stages, M1 and M2. M1 involved deactivating Rb and p53 proteins to prevent the cellular aging process observed in the normal cells, which SV40 would do. The M2, however, involved a process that was unknown and needed further investigation (Wright, Pereira-Smith, and Shay 1989, Wright and Shay 1992).

By the early 1990s, important clues about how cells age and how transformation of normal cells into cancer cells happens created renewed optimism in the field of cell aging research. There was a sense that after almost three decades of the discovery of the Hayflick limit, something big about the biology of cancer and aging would come out of the continued efforts in investigating the issue (Goldstein 1990, Cristofalo 1996). In a satellite meeting of the International Congress of Cell Biology with the topic “Control of

Cell Proliferation in Senescent Cells" held in Montreal, Canada, Hayflick, Cristofalo, Holliday, Wright and others reported recent results, giving the reporter from the National Institute on Aging a sense that this area of research would "be useful not only in understanding aging in vivo, but will also increase our understanding of cell transformation, wound healing, tissue regeneration, and abnormal cell growth" (Warner and Wang 1989).

During this period around 1990, the end structures of the DNA, telomeres, happened to be raised as a topic for aging research. Researchers showed that telomeres shorten with each cell division in the human diploid cells. Once the attritions of telomeres accumulated to reach a threshold, the cells could no longer divide further. Telomere shortening was thus provided as a reason why cells age (Harley, Futcher, and Greider 1990). Telomere research, originally from a rarified field focusing on the study of the *Tetrahymena* rDNA molecule, was soon being connected to cell aging and immortalization. The cell aging research community was particularly accommodating to the topic. Researchers such as Wright and Shay soon started their own programs on telomere. As the telomere research gained momentum and fame, however, other researchers pointed out that many valid mechanisms and hypotheses about cell aging raised before telomere research and worthy of further investigation, such as the role of p53 and Rb, were increasingly neglected (Sozou and Kirkwood 2001).

In 2009, three major scientists involved in telomere research were awarded the Nobel Prize. In the press release, the telomere was depicted as "an important piece in the puzzle" at the intersection of "human ageing, cancer, and stem cells" (Committee). The sexy field of telomere research also fueled pharmaceutical start-ups capitalizing on aims

ranging from cancer cures to immortality pills (Hall 2003). As a result, the current stories of cell aging research often portrayed the history as two steps: first, the discovery of the Hayflick limit which created a puzzle; then telomere came along to resolve the puzzle. What happened between 1961 and 1990 in the field of cell aging research, a period that solidified cell aging *in vitro* as an important scientific object at the crossroad of cancer and aging research, was mostly neglected in these narratives that are supposed to tell a history of cell aging research (Greider 1998a). One aim of this chapter is to problematize such historiographical approach of cell aging research in the twentieth century (See the next section).

The Creation of Aging Cell *in vitro* as a Scientific Object between Disciplines

The large-scale uses of cell cultures for vaccine production in the 1950s and in the Wistar Institute provided the material basis that cell aging became undeniable to Hayflick. Yet, the limit of cell division in the cell culture was not necessarily interpreted as a phenomenon related to science of aging or cancer. It could have been interpreted as purely a technical thing, as others did in the 1950s (Swim and Parker 1957, Puck, Cieciura, and Fisher 1957).

Hayflick's own ambition, previous views about cellular life *in vitro*, and disciplinary drive to move aging research along shaped the interpretation of cell degeneration as a limit-setting process of cellular life that worth scientific investigation. Hayflick's intention of transforming cell culture from a technical production system for vaccine production to a scientific system for his cancer research prepared his curious attitude towards novel phenomena. Carrel's dramatic interpretation about immortality of cell cultures, once proved wrong, shaped the oppositional interpretation of the cessation

of cell division *in vitro* as a sign for mortality and aging. The gerontologists' interests in adopting simpler experimental platforms for aging and the mobilization of biologists towards aging research in the 1960s converged in using cell cultures to study aging in the 1960s and 1970s. A small research community focusing on cell aging was formed gradually and studying cell aging *in vitro* with the ultimate aim of studying aging in *vivo* formed the core practice within the community. Though the associated research also extended to pathology of age-related diseases, cancer research, and discussions about stem cells.

If Viktor Hamburger and Rita Levi-Montalcini's discovery of cell death in the development of the chick central nervous system was a strong consolidation of a long series of findings about cell death occurring in development, Hayflick's discovery about cell aging in cell culture seemed more abrupt, overturning a long-standing notion about an intrinsic property of cells. Because of the abruptness of the discovery and the following transforming impacts, historian Jan Witkowski depicted the discovery of *in vitro* cell aging as a paradigm-changing event in a Kuhnian sense in the fields of cell culture and aging research (Witkowski 1987). Witkowski is correct that there were many dismissed "anomalies" that showed the degeneration of cells *in vitro* before Hayflick's discovery, and Hayflick's interpretation indeed changed the fundamental assumptions about the duration of cellular life. However, it is hard to identify what was the previous "paradigm" of aging research, and to detect any perceivable "scientific revolution" initiated by Hayflick's research.

This chapter, by describing the *processes* that led to Hayflick's discovery of *in vitro* cell aging and the cascade of events that enriched the meanings, interpretations, and

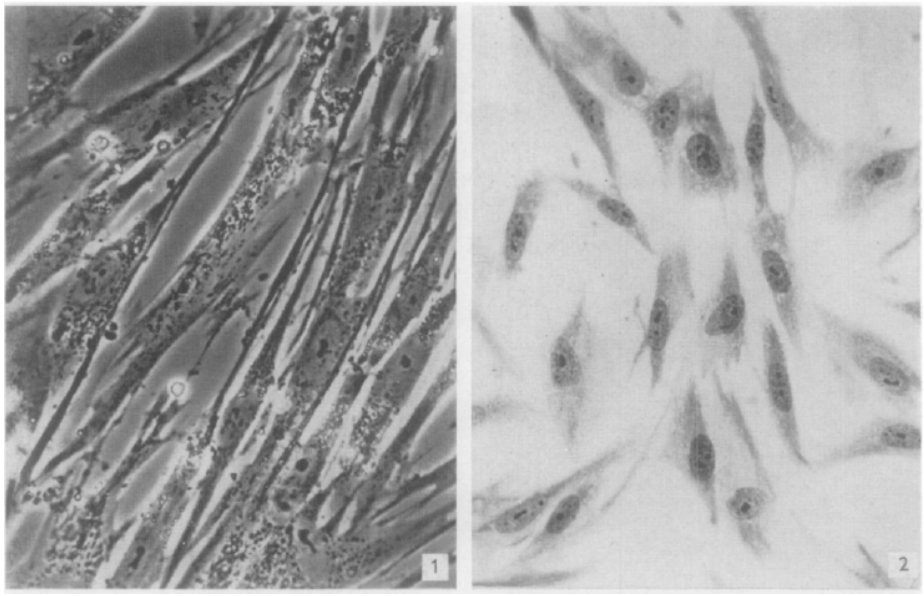
research usages of it, showed that the creation of cell degeneration *in vitro* as a scientific object was gradual. It transformed the practices of aging research not by replacing a whole set of practices but by adding cell culture practice to aging research's experimental repertoire. The cell culture system allowed researchers to ask questions about aging and associated diseases that were difficult to investigate with the whole organism. Yet the impacts of such addition were significant. As the community persisted the research on the aging phenomenon in cell culture, they touched questions about cancer, age-related disease, and mechanisms of cellular cycles, creating a kind of interdisciplinarity through the associated implications of cell aging.

Although Hayflick's discovery was probably no paradigm-shifting event, it is not justified to depict the cell aging research before 1990 simply as precursors of telomere research on aging, an approach contemporary science reviews, reports, and popular histories often take (Greider 1998a). Not surprisingly, glorifications of telomere research as such were often acts of self-galvanization from telomere researchers themselves and were often historically inaccurate. They omitted the fact that many clues about molecular mechanisms of cell aging and cancer were already elucidated in the field of cell aging in the 1980s and that the rise to fame of telomere research was largely due to the active adoption of telomeres into cell aging research by the cell aging research community already formed before 1990.

The view that there was no important discovery about cell aging in between 1961 and 1990 is not only historically inaccurate, but also poses a danger of creating homogenous and overly narrowed research programs in the field of cell aging research. In the 1990s, studying cell aging and immortalization through telomere was so dominant,

that other approaches to cell aging were often neglected (Sozou and Kirkwood 2001). Although it was realized that the homogenous approach of studying telomeres could not capture the complexity of cellular aging, and the systems biology of aging and cancer created a more encompassing approach in recent years, rationales of a lot of new research agenda seem to be a resonance from the 1970s and 1980s rather than being totally novel (See Chapter 3). Many experiments, hypotheses, and results from cell aging research documented in the literature between 1961 and 1990 could still provide a wide range of methods, inspirations, and historical data to enrich current cell aging research. The science on cell aging may run the danger of becoming anemic if the historical blood stream from its own origin is cut away.

a.



b.

Figure 3. Hayflick and his Cell Cultures

a. Hayflick examined a flask of cells in the 1980s (Wadman 2013).

b. Cell aging in cultures. Left: aging cells; right: cells in proliferation (Hayflick and Moorhead 1961).

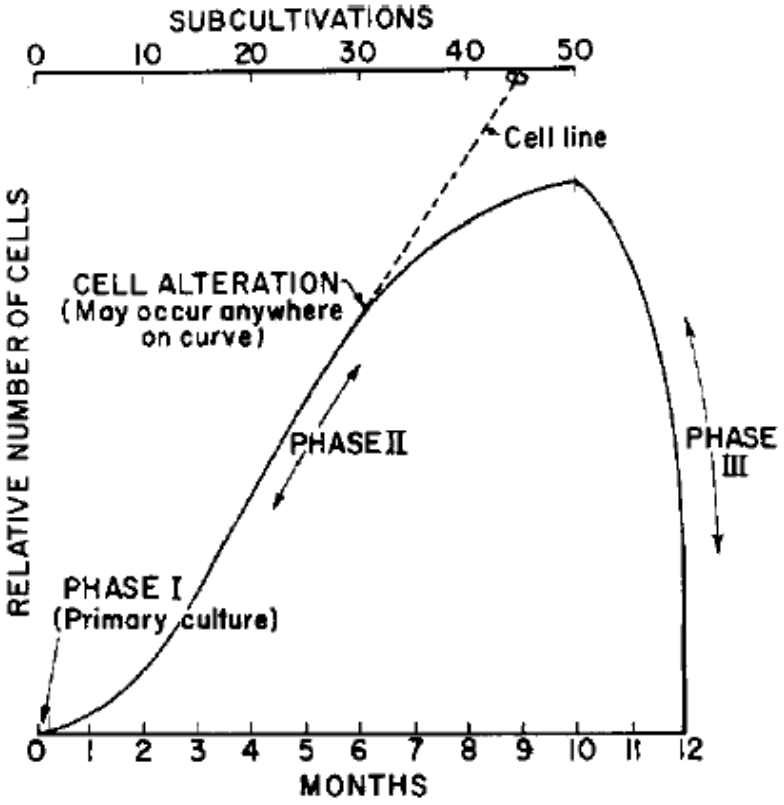


Figure 4. The Phase III Phenomenon

In 1961, Hayflick divided the life of cell cultures derived from human diploid cells to three phases. While during the first two phases, cells kept dividing and growth, in the third phase, cells stop dividing and eventually die (Hayflick and Moorhead 1961).

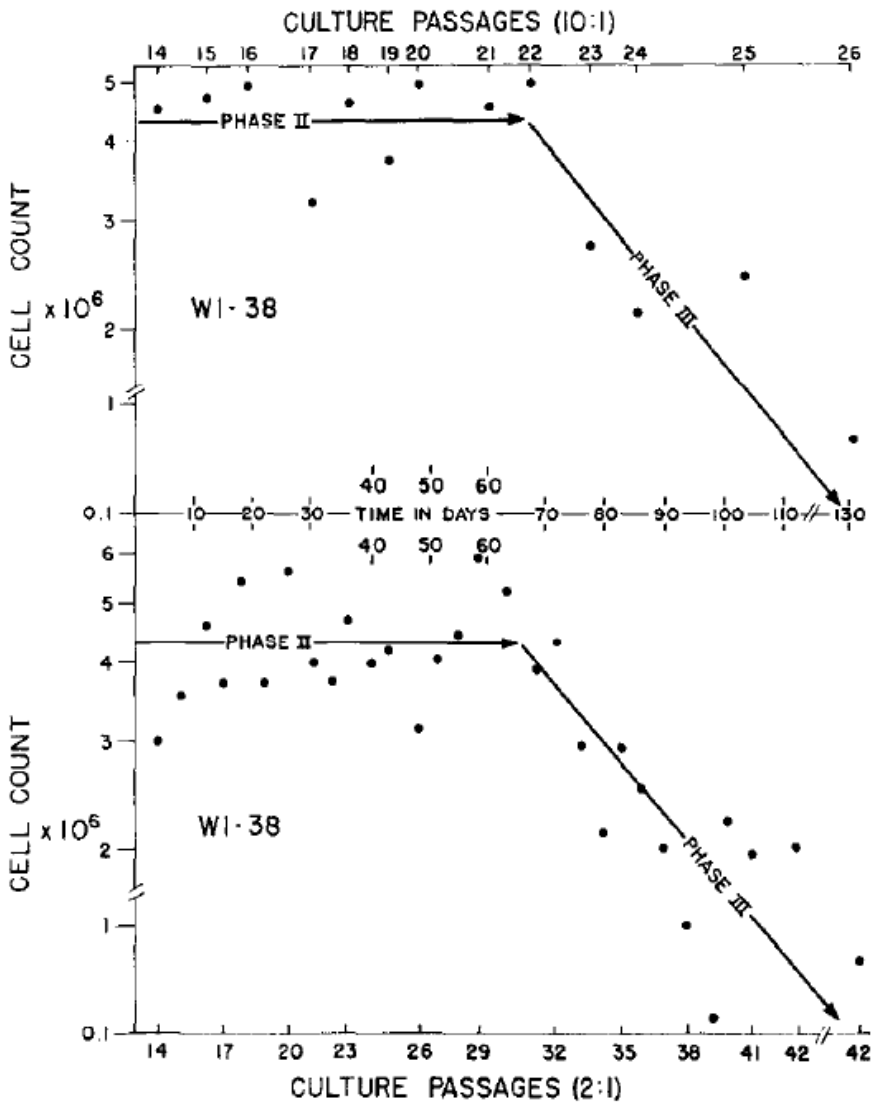


Figure 5. Survival Curves of Human Diploid Cell Cultures

From these survival curves, Hayflick found that the total number of cell divisions was not affected by the dilution ratio he used in subcultivations. He also noticed that the shape of these survival curves resembled the shape of survival curves of bacterial or cell culture in radiation studies (Hayflick 1965b).

Chapter 3: Causes of Aging Are Likely to be Many

Robin Holliday's Research on Cell Aging *in vitro* and Origin of an Integrative Approach in Molecular Biology

As the first two chapters show how cell degeneration emerged as a scientifically meaningful entity around the mid-twentieth century, the later two chapters present how the meanings of cell degeneration were expanded and led to new research trajectories such as systems biology of cell aging. As described in the previous chapter, in the 1970s cell aging study evolved into a field with a small research community. This chapter shows one research case among the many research programs generated within the cell aging research community, British molecular biologist Robin Holliday's research on cell aging. The chapter focuses on how the research trajectory initially based on looking at the simple mechanism of cell aging led Holliday to the realization about the complexity that cell aging involved. The momentum of Holliday's research not only revealed the molecular complexity of cell degeneration, but also prepared a group of researchers to embrace the systems approach to aging in the early 2000s.

As interested gerontologists began to use cell cultures to study aging, the problem of aging in cultured cells began to be isolated from its biological contexts and became a scientific problem for its own sake. Holliday's study was one instance among many pursuits of cell aging around the time that tried to tackle the molecular mechanism of aging through cultured cells. Particularly, Holliday tried to test whether accumulation of DNA mutations, or errors in proteins causes aging through manipulating the lifespan of cultured cells. Nuclear physicist Leo Szilard had suggested that DNA mutations caused aging, while chemist Leslie Orgel had suggested instead that protein errors induced the

aging process (Szilard 1958, Orgel 1963). Gerontologists had been debating about which theory explains aging, Sizlard's or Orgel's, to which Holliday hoped to provide experimental contributions.

Working at the Genetics Division of the National Institute for Medical Research (NIMR) in Mill Hill, UK, Holliday became aware of Hayflick's cell aging phenomenon and started to test Orgel's protein catastrophe theory of aging in the early 1970s. First using variety of fungus and bacterium, Holliday later used the human diploid cells MRC-5 and manipulated the length of their cellular life *in vitro*. With the help of his students and collaborators at the NIMR, Holliday initially used a variety of molecular interventions to demonstrate, in favor of the protein catastrophe theory, that cytoplasmic factors were more important than nuclear mutations for aging (Holliday 1986, 1972). Other researchers on cell aging, especially those in the US, disagreed. Reflecting on the confusing data that different laboratories offered, Holliday eventually developed a systems thinking that took account of a variety of information handling apparatuses in the cell and incorporated alternative molecular reasoning. Holliday's program later morphed into the program of systems biology of aging developed by Holliday's student, Thomas Kirkwood, in Newcastle University three decades later.

Holliday's initial program was based on an assumption that a simple underlying mechanism exists as a molecular clock to control cell aging, an assumption that many cell aging researchers shared. The fast-developing technologies of molecular biology in the 1970s offered many tools that Holliday and others could employ to manipulate cellular life so that he could obtain data that revealed different molecular aspects of cell aging. The realization about the complexity of cell aging motivated Holliday to incorporate

computational tools and evolutionary perspective. Coupled with opposing theories and new molecular tools, the trajectory of cell aging changed from a straightforward, hypothesis-driven science to a systems approach.

Holliday's investigative path involved a great deal of comparing experimental results with existing hypotheses to differentiate which can better explain cell aging. Consequently, at times, this chapter delves into discussions about hypotheses and theories that the experimental system of cell aging *in vitro* provided a platform to test. These discussions aim to describe ways the system of cell aging *in vitro* engaged with the expanding theoretical space about aging and redirected the theoretical discussions from focusing on distinguishing the correct simple mechanism to synthesizing a variety of mechanisms.

The chapter first introduces the theoretical debates in molecular biology of aging in the 1960s that Holliday thought he could use Hayflick's cell culture system to test. Then I describe Holliday's training in molecular biology and how he became interested in the problem of aging. After describing several initial experiments through which Holliday was convinced that protein errors cause aging, I discuss his later experiments and results from others that showed that the causes of aging are more complex than accumulations of protein errors. Unable to explain aging through any single molecular mechanism proposed for aging, Holliday started to explore alternatives. I describe particularly the evolutionary perspectives and epigenetic explanations of cell aging Holliday offered. At the end, I discuss how this episode of developing cell degeneration research relate to the subsequent development on systems biology of aging and challenge the current historiography of the origin of systems thinking in the twentieth-century biology.

Background: Thinking about Aging through Molecular Defects

Historians often note that molecular biology underwent rapid development after World War II, and by the early 1960s had started to expand beyond basic principles such as those involved in DNA replication and protein synthesis and into diverse biological and biomedical questions. After the breaking of the genetic code and articulation of a "central dogma" for protein synthesis, there was a sense among the pioneer molecular biologists that almost all fundamental problems in molecular biology were solved in principle, leaving behind only the tasks of filling in the details for puzzle solvers. After a discussion with Francis Crick, Sydney Brenner summarized the sentiment, "most of molecular biology had become inevitable," and it was time "to move on to other problems of biology which are new, mysterious and exciting" (de Chadarevian 1998). Responding to similar assessments of the field, many moved from studying molecular principles to investigating complex biological questions in high organisms, such as the development of nervous system and the nature of consciousness. The period from 1965 to 1972, as historian Michel Morange noted, is thus characterized with "an expansion of the molecular vision beyond its original field, leading to a take-over of other biological disciplines by molecular biologists" (Morange 1998, 177). The problem of aging emerged as one of these problems that molecular biologists hoped to elucidate through molecular approach.

To put mechanisms of aging into concrete, testable hypotheses, perhaps in molecular terms, seemed to be what was needed in the biology of aging, or as it is currently called biogerontology in the early 1960s. In the 1930s and 1940s, biogerontology in the United States had gone through active development with the

support of the Josiah Macy, Jr. Foundation and the skillful multidisciplinary leadership of the Canadian-American cytologist Edmund Vincent Cowdry (Park 2008). The eclectic approach to developing the field of gerontology, however, also encouraged the production of a plethora of theories generated from a variety of perspectives that did not necessarily target any fundamental cause of aging, nor did it have sound experimental evidence. The situation incurred criticism, and created a demand for experimentally testable hypotheses to explain aging.

Naturally, the ambition of early molecular biologists to transform an old biology through their new experimental and theoretical rigors was met with eagerness of the new generation of gerontologists who welcomed empirically sound, testable hypotheses. The condition of the field of aging, however, did not respond to the means of molecularization used in other fields. Exemplified by crystallographer Vernon Ingram's work in associating a point mutation on the hemoglobin gene to the defective protein that causes sickle cell anemia, a common goal for molecularization was to document correlations between a gene or a cluster of genes to a biological trait or disease. For another instance, it was envisioned in Brenner's project of studying development in the nematode *C. elegans* that identifying genes that participate in a developmental program will be feasible through generating mutants of worms and identifying their aberrant developmental path. In the field of aging research, however, the notion that there are genes coding for aging phenotype or an aging "program" had been widely criticized since the 1940s. Neo-Darwinians after the Modern Synthesis regarded the Weismannian idea that aging programs were naturally selected to protect species for resource exhaustion and to ensure the continuation of species as dubious. Evolutionary biologists in gerontology

such as Peter Medawar and Alex Comfort argued that only advantageous genes that express themselves before or during reproductive age could be preserved through natural selection in organisms. For them, it is unlikely that a gene coding for individual degeneration and presumably expressing itself after reproductive age can be selected and transmitted, rather than drifting randomly in the gene pool.[7] In the mid-century, theoretical preferences were given to those ideas that proposed some sort of defects as a basis for aging, such as the free radical theory and the cross-link theory, and those that suggested a genetic program for aging were deemed unfavorable. Molecular study of aging thus became a task with its special constraints: a molecular gerontologist could not simply go about finding genes that code for phenotypic aging.

As a consequence, hypotheses around the mid-century concerning aging in molecular terms often pointed to general defects of particular molecules. One of the well-known hypotheses conjectured that the mutational changes accumulated through time might cause aging. Leo Szilard, the nuclear physicist who advocated arms control after World War II and turned to studying biology around 1947, put forward the first formal articulation of somatic mutation hypothesis of aging in 1958 (Szilard 1958). Turning from the "science of death" to the "science of life," Szilard brought his style of constant brainstorming to studying the biological questions he found interesting at the moment. Convinced that "explanations existed for everything, and that therefore they could be found," Szilard offered his novel ideas as well as his confidence about the importance of cross-pollination of ideas to the community of molecular biologists in ways of

[7] August Weismann has summarized his theories about natural death and aging in (Weismann 1882) For the neo-Darwinian counter-arguments, see (Comfort 1956, 37-41).

questioning assumptions, articulating bold speculations, provoking new thoughts, and criticizing without reservation. In a post-war era in which the public concerns redirected from the catastrophic short-term effects of atomic bomb to long-term, low-dose radiation, Szilard himself studied mutation rates in bacteria, and suggested that mammalian aging might be due to the accumulated mutations caused by low dose of radiation.[8]

In the paper "On the Nature of the Aging Process" published in 1959, Szilard hypothesized that a certain amount of mutation on a chromosome constitute an "aging hit," that would inactivate the whole chromosome. Once a chromosome is destroyed, Szilard projected, the somatic cell that contains such chromosomes would become inadequate for certain physiological function, and eventually become unviable. In order for an individual to survive, a certain fraction of surviving cells needs to be maintained. When the "aging hits" accumulated to such a level that viable fraction of survival cells cannot be maintained, the individual dies. With these assumptions, Szilard worked out mathematical equations to suggest further parameters to measure in order to test his hypothesis. However, the experiments Szilard suggested, such as testing "graying of hair" or "the loss of accommodation of the eye" through studying irradiation effects on population of mice, were expensive and technically challenging (Szilard 1958).

Not all biologists, however, shared Szilard's emphasis on the role of mutation in aging. First of all, though important as hereditary material, DNA molecules were not seen as functioning directly in cells. As historian Robert Olby showed, the 1953 model of the "double helix" structure of DNA actually went through a rather "quiet debut," and was only recognized as important later after its role in protein synthesis became clear (Olby

[8] For a survey about the study of low-dose radiation, see (de Chadarevian 2006).

2003). Even in Crick's central dogma formulated in his 1958 paper "On Protein Synthesis," proteins rather than DNAs were said to be "uniquely important" in biology (Crick 1958). Second, for the problem of aging, the explosion of sudden declines and deteriorations in later life seemed inconsistent with the rather linear accumulation of mutations. If erroneous molecular changes were involved in aging, it should probably be a particular kind of errors that can significantly accelerate its rate of accumulation in later life.

The British chemist Leslie Orgel was one of the striving theoreticians on molecular biology who found Szilard's supposition dubious. Having studied transition metal chemistry in the early 1950s, Orgel became interested in biology under the influence of biologist Sidney Brenner and accepted a post in 1964 at the Salk Institute, La Jolla (Dunitz 1997). In 1963, Orgel worked out a hypothesis that proposed error accumulation in protein synthesis and synthesis-related apparatus as a potential cause of aging. In the paper "The Maintenance of the Accuracy of Protein Synthesis and Its Relevance to Ageing," Orgel specifically proposed his hypothesis as a counterargument to Szilard's (Orgel 1963). He noted, "the ability of a cell to produce its complement of functional proteins depends not only on the correct genetic specification of the various polypeptide sequences, but also on the competence of the protein-synthetic apparatus." Protein-synthetic apparatus, according to Orgel, descends at least partially through cytoplasmic inheritance: "A cell inherits, in addition to its genetic-DNA, the enzymes necessary for the transcription of that material into polypeptide sequences." Orgel reasoned that the errors in protein-synthetic machinery are probably more reactive catalytically so that they are more likely to initiate a catastrophic state of aging. He gave

three justifications for believing so. First of all, the error frequency in RNA and protein synthesis is likely to be higher than the error frequency in DNA replication. Second, each DNA mutation can only produce one type of defective protein. But if the protein-synthetic machinery is defective, it would influence all the proteins it produces. Defective protein synthesis could possibly produce errors in enzymes that are involved in DNA replication or in further protein synthesis. Orgel deemed that errors in these "information-handling enzymes" would propagate errors in other molecules quickly, culminating into a catastrophe leading to cell death (Orgel 1963).

Orgel apparently had known about the recent discovery of the American microbiology Leonard Hayflick that normal somatic cells kept in culture would reach a mitotic limit (Hayflick and Moorhead 1961). As many researchers started to adopt Hayflick's cell culture system to study cell aging, Orgel suggested using cell culture to test his error catastrophe hypothesis. In the 1963 paper, he suggested that his hypothesis be tested by supplementing amino acid analogues, such as *p* fluorophenylalanine and ethionine, to the culture medium of microorganisms or metazoan cells and examining whether the life span of these cells are shortened afterwards. Amino acid analogues can be incorporated into the newly synthesized protein molecules, but since the analogues have different side-chains than normal amino acids, the resulting protein molecules would have altered structures, thus producing the effects that would be expected from erroneously synthesized proteins. Orgel noted that this experimental approach had the advantage of "study the effect of errors in protein synthesis in the absence of complications due to *primary* [sic] nucleic acid changes" (Orgel 1963).

Orgel's hypothesis became later known as the error catastrophe hypothesis/theory of aging. Although Orgel's theory seems to diverge from a genocentric view that emphasizes DNA, it aligned well with the focus on the sequence and information propagation characteristic of molecular biology in the early 1960s. In fact, Orgel had worked closely with Francis Crick and other pioneer molecular biologists around Cambridge while he was working in Cambridge's chemistry department from 1955 to 1963 (Figure 6). He was among the first who saw the double helix model and a member of the "RNA Tie Club," which was formed to collectively explore the structure of RNA and the mechanism of protein synthesis. Orgel himself also worked to solve the problem about genetic codes, as documented in a 1957 paper he co-authored with Crick and John Stanley Griffith, "Codes without Commas" (Francis H. C. Crick, Griffith, and Orgel 1957).

Although the field of aging did not lack yet another hypothesis, compared to many other theories that made only vague experimental predictions, Orgel's hypothesis was potentially testable and sounded attractive to experimentalists. As Orgel put it, for testing his hypothesis, "there seems a fairly obvious experimental approach" (Orgel 1963). In fact, Russian biologist Zhores A. Medvedev had also proposed protein error as a potential cause of aging, but did not know about Hayflick's cell culture model of aging when he proposed it. At the same time, Hayflick and some pioneer investigators of cell aging in cultures already leaned towards Szilard's mutational explanation (Medvedev 1962, Hayflick and Moorhead 1961, Hayflick 1965b). If either the somatic mutation hypothesis or the error catastrophe hypothesis was true, they needed a critical test. Then

aging process would have a molecular, causal explanation. The field of aging would be like never before.

Robin Holliday, a British fungus geneticist who was visiting University of Washington, Seattle, to expand his research horizon, was intrigued by Orgel's paper in 1963. He soon discussed the paper in a journal club with his colleague in Seattle. Holliday was particularly interested in the problem because, from almost a decade of research on fungi, he knew that some fungus species would stop growing and die after a period of growth. A French researcher had suggested that the cause of death was located in the cytoplasm (Marcou 1961). The test Orgel suggested seemed to fit Holliday's expertise on fungus genetics, his interest on molecular biology, and the cytoplasmic cause of aging in fungi suggested by others. In February 1964, back at his home institution John Innes Institute at Hertford, Holliday wrote to Orgel, "I find the basic simplicity of your idea very appealing, and it seems to me that it could also be used to explain various cases of cytoplasmic inheritance. I wonder if you have considered this?"[9] This letter, in retrospect, initiated collaboration between Orgel and Holliday that lasted more than two decades.

Robin Holliday's Training in Molecular Biology and a Preliminary Test of Error Catastrophe

Robin Holliday (Figure 7) was born in 1932 in Palenstine into a British family. In 1952, already back in England for five years, Holliday entered Cambridge University to study Natural Science. In his third year, Holliday had the choice between botany and

[9] Robin Holliday to Leslie Orgel, February 5, 1964, Folder 14, Box 8, MSS 176, Leslie Orgel Papers, Mandeville Special Collections Library, University of California, San Diego.

biochemistry for specialized study of one year. He chose botany because he had one aunt and an elder brother who specialized on fungal biology.

In 1954, Holliday for the first time learned about Watson and Crick's structure of DNA and found it inspiring. The professor who introduced the Watson-Crick model to Holliday was Harold Whitehouse, a fungal geneticist who was interested in the mechanism of recombination. Holliday remembered the learning experience with Whitehouse "a defining moment in my career" (Holliday 2008, 72).

Holliday eventually secured a governmental grant for a three-year PhD project in fungus genetics with Whitehouse. Based on his work on smut fungus *Ustilago maydis*, a fungus parasite of maize, Holliday designed methods to quickly identify biochemical mutants from a batch of potential isolates (Holliday 1956). During these years, a group of undergraduate researchers became interested in collaborating with Holliday to test the radiation effects of cosmic rays accumulated through years using mice and *Ustilago maydis*. Initially positive about the project, Holliday became increasingly skeptical about whether the low radiation from cosmic rays can alter genetic components in any significant way, especially considering the low mutation rates from radiation using *U. maydis* (Holliday 2008, 103). As a result, he opted out of the project. It was not until late 1970s that fungus biologists figured out that *U. maydis* has extraordinary resistance to ionizing radiation, probably the most resistant among all eukaryotes that had been studied (Holliday 2004). These early doubts about the effects of low radiation might be related to his disbelief in Szilard's somatic mutation hypothesis of aging in the later years.

The late 1950s was an exciting time for molecular biology, especially around Cambridge. As Holliday was being inspired by the work presented in various lectures and

talks around the campus, his interests expanded to the study of linkage of gene and gene crossover structures during meiosis in *U. maydis*. Having consulted Francis Crick about the stability of DNA molecules and mismatched base pairs, Holliday continued this line of work beyond graduate school and proposed a chromosomal intermediate structure that facilitates gene exchanges in meiosis, which was later proved to be basically correct. The intermediate crossover structure of chromosomes he proposed is now known as the "Holliday Junction" (Holliday 1964, Liu and West 2004).

Holliday completed his PhD in genetics in 1959 and accepted a research post in the John Innes Institute at Hertford. There, he continued to study recombination and DNA repair, and to carry on other genetic work in *Ustilago*. It was then popular for junior British scientists to spend some time in the US to gain oversea experiences, and possibly "find new horizons." In 1962, Holliday obtained a Fulbright Scholarship and visited the laboratory of the yeast geneticist Herschel Roman at the University of Washington, Seattle for one year (Holliday 2008, 120-121).

When Robin Holliday read Orgel's paper in 1963 in Seattle, he became interested in testing Orgel's theory in fungal species. Back to England, Holliday's first work on testing Orgel's hypothesis was, however, not on fungi but on fruit flies. His colleague at the John Innes Institute, Brian Harrison, had worked for years on the aging of plant seeds in different conditions and sometimes on the aging of *Drosophila*. Upon Holliday's return, they decided to test Orgel's hypothesis on fruit flies together (Holliday 2008, 122-123). They exposed flies' larvae with amino acid analogues for 4 to 24 hours to introduce errors to protein synthesis, and found these developed flies often lived 5 to 10 days shorter than the flies without exposure to analogues. These results were consistent with

Orgel's biological prediction. However, Harrison and Holliday had not tested what really changed in the protein molecules in the adult flies. Therefore, they noted that it was still "necessary to test whether the biochemical basis of the induced senescence is also as predicted" (Harrison and Holliday 1967).

Optimisms: Tracing Cytoplasmic Catastrophe in Fungi and Human Cells

In July 1965, Holliday became a senior member of staff in the Microbiology Division at the National Institute for Medical Research (NIMR) in Mill Hill (Figure 8). The British evolutionary biologist and NIMR's director Peter Medawar had been influential for aging research since the 1950s.[10] Medawar had established a program on biology of aging at the University College London, having Alex Comfort, and later John Maynard Smith to investigate questions about aging.[11] At NIMR, Holliday started to request fungal strains from other fungal geneticists in hopes of testing Orgel's error catastrophe hypothesis in fungi.

In the 1950s, various fungal species, especially the *Neurospora crassa* had been important organisms for studies in biochemistry, nutrition, and genetics. The bread mold *Neurospora crassa* can be induced into mutants that can only live on medium supplemented with certain essential amino acids or vitamins, so that geneticists could isolate strains with certain genetic components by linking these components to their auxotroph markers. It was based on the genetic work based on such auxotroph mutants in

[10] For Peter Medawar's summary of the urgency and prospect of aging research, see (Medawar 1952, 4, 5)

[11] Email communication with Holliday, July 5, 2011.

Neurospora crassa that George Beadle and Edward Tatum proposed the famous one gene-one protein hypothesis, which led to their joint Nobel Prize in 1958.

The fungal trait that was more important for Holliday's aging research, however, was the fact that like human diploid cells, a lot of fungal species do not grow infinitely. After a period of steady growth, they would suddenly drop in growth rate and start to produce dark-colored pigments, showing distorted, swollen shapes in the hyphae fronts. The hyphae would eventually stop growing completely and die. Holliday called such aging phenomena in fungi "clonal senescence."[12] The growth rates in such fungi were also easy to measure. A technique called "race tube" confined the growth of fungi to one linear dimension along a tube so that researchers would tell how fast a strain grew from how long a hyphae extended along the tube in a certain period.

For most fungus geneticists, the aging phenomenon of fungal hyphae was a disadvantage compared to other microbes that can divide infinitely. After a period of constant fungal growth, the researchers were concerned that the aging culture with idiosyncratic physiology might compromise biochemical assays (Davis and Perkins 2002). Eventually, *E. coli* substituted for *N. crassa* as the dominant microorganism in genetic and biochemical research, partly due to the lack of conspicuous aging in *E. coli*. For Holliday, however, relatively short-lived fungi provided good biological material to measure and manipulate aging. Working on fungi, Holliday could also request mutants

[12] The cessation of growth in fungi was assigned different names in different fungus species. Holliday noted that in *Podospora anserine*, the growth cessation was called senescence and in the species *Aspergillus glaucus*, it was called vegetative death. He also noted "The phenotypes of the conditions in the two species are very similar and it would be surprising if the underlying cause was not the same." See (Holliday 1969)

that had altered life spans from other fungus geneticists who were in his established network.

By the end of the 1960s, Holliday had shown that the fungal strains raised in the medium supplemented with amino acid analogues have reduced life spans, as Orgel had predicted. Holliday called the early aging phenomenon "induced senescence" (Holliday 1969). Although these initial results were consistent with Orgel's hypothesis, by themselves they did not confirm that error catastrophe was indeed the cause. Holliday was eager to corroborate the initial results through other methods such as classic genetics. By 1969, Holliday had gotten hold of a mutant of *N. crassa* originally isolated by T. C. Sheng, a geneticist in Columbia University. This mutant is characterized by early occurrence of clonal death, and was thus called natural death, or *nd* (Sheng 1951). Holliday thought it was possible that the early death of *nd* was due to erroneous protein synthesis. To test whether this was the case, Holliday designed experiments to examine the effect of crossing other strains of *N. crassa* and *nd*. He then compared the hybrid effect to longevity to the life-shortening effect created by chemical analogues. Holliday showed that crossing *nd* with an adenine auxotrophe *ad-3*, would produce effects similar to those resulting from raising *ad-3* in the 5-fluorouracil, a ribonucleutide analogue that induces errors in protein synthesis. These results were published in *Nature* in 1969.

With these positive results, Holliday was increasingly confident about Orgel's error catastrophe theory of aging as well as the importance of cytoplasmic inheritance for aging. He criticized the common notion that attributed cytoplasmic inheritance to the change in nucleic acid located in the cytoplasm, and stressed that a kind of cytoplasmic

inheritance that does not rely on nucleic acid can possibly transmit aging phenotypes in an irreversible way.

It is often believed therefore that all examples of cytoplasmic inheritance must be the results of changes in cytoplasmic nucleic acid in one form or another. If senescence in *Podospora* and vegetative death in *Aspergillus* are the result of a progressive loss in the fidelity of protein synthesis, then these would be examples of cytoplasmic inheritance not determined by an altered nucleic acid. The condition is inherited, like a genetic mutation (as opposed to a reversible change in "steady state"), because once the machinery of protein synthesis has become inaccurate, there is no way in which the daughter cells can recover to normal condition. (Holliday 1969)

Here Holliday invoked "cytoplasmic inheritance," a concept that might have been frowned upon during the 1960s by Mendelian geneticists. As historian Jan Sapp has aptly pointed out, in the 1960s, following the discovery of physical structure of genes as DNA and the subsequent rise of molecular biology, research programs in cytoplasmic inheritance underwent transformations that simultaneously changed the questions as well as the status of research regarding cytoplasmic inheritance. On the one hand, the fact that nucleic acids can be found in both nuclei and cytoplasms blurred the nucleus/cytoplasmic boundary as well as its significance. The originally geographic/locational question was reframed into a question about structure: heredity through DNA versus heredity through other macromolecules, such as proteins, and their organizations.[13] On the other hand,

[13] Sapp showed that the shift of heredity question from focusing on nuclear/cytoplasmic location to focusing on the DNA/non-DNA nature of molecules motivated protozoa biologist David L. Nanney to propose using the term epigenetics to capture heredities that are not through nucleic acids in the 1950s. This change of term has been interpreted as

with the rapid progress of Mendelian genetics through the study of DNA, instances of cytoplasmic inheritance increasingly seemed like a number of oddities with no regular rules to follow, which harmed the credibility of the subject (Sapp 1987, 192-220). Holliday's willingness to use "cytoplasmic inheritance," a slightly stigmatized concept in the 1960s, to describe a non-DNA heredity for aging reflected his concerns regarding the limitations of the dominant view about DNA heredity. In 1988, he addressed the point more explicitly, "All previous concepts in genetics and molecular biology define pathways of information transfer and a mechanism of inheritance based on DNA structure. Is an entirely different mechanism required to explain somatic cellular inheritance? Advocates of specific protein inheritance should come out into the open and make clear the revolutionary nature of their proposals" (Holliday 1988).

By 1970, to examine whether errors actually accumulated in the proteins of aging fungi, Holliday had tested the stability of these enzymes under heat. He showed that the extracts of the enzyme glutamic dehydrogenase became increasingly unstable under heat with the increasing age of the fungi (Lewis and Holliday 1970). Although he found that the chemical analogues could also induce mutations on DNA, Holliday explained it was because the errors in protein synthesis made defective DNA polymerase. These mutations may well contribute to the aging phenomena in fungi, but these effects were only secondary to errors in protein (Lewis and Holliday 1970).

These positive results fueled Holliday's optimism towards Orgel's hypothesis. In 1969, Orgel visited Mill Hill and worked with Holliday for one year. They spoke

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one origin of contemporary use of the word "epigenetics." See (Sapp 1987, 192-203, and Haig 2004).

extensively about theoretical and experimental aspects of the biology of aging and decided to write a book together on the error catastrophe theory. In 1970, soon after Orgel's visit, Holliday was appointed the head of the new Genetics Division at NIMR. The NIMR had boasted a tradition in basic medical research, and the new Genetics Division carried the promise of bringing new methods in molecular biology to bear on medical problems. The director of NIMR, Peter Medawar had high aspirations for the new division and had approached prominent molecular biologists such as Sydney Brenner to serve as the head of the new division (Holliday 2008, 128). To Holliday, it was the time to carry study of error catastrophe hypothesis to bear on medicine. He started to look for opportunities to use human diploid cells, materials that are more medically relevant than fungi, to study aging (Rattan 2002).

In fact, Holliday already expressed interest in testing Orgel's theory in human diploid cells in his 1970 paper, especially encouraged by the American microbiologist Leonard Hayflick's observation of cell aging in cultures (Hayflick and Moorhead 1961, Hayflick 1965b). In the late 1950s, when Hayflick was working at the Wistar Institute in Philadelphia, he accidentally found that all diploid cell strains isolated from human fetuses would stop dividing after being cultured about half a year. Hayflick argued that the cessation of cell division in cell culture was a manifestation of aging at the cellular level. In the 1950s, many cell culturists held an idea initially suggested by French surgeon Alexis Carrel that all cells are intrinsically immortal and they only die because of external injuries caused. As a consequence, Hayflick's argument that cells age by

themselves was initially rejected (Hayflick 1998a, 14-41, Hall 2003).[14] By the 1970, however, a few junior biogerontologists had been attracted by the convenience and simplicity of the *in vitro* model of human aging and started research based on it. Surrounding the *in vitro* experimental model of aging, these researchers formed a network for exchanging idea and materials in the US, which later extended to other countries such as France and Russia (Cristofalo 1972, Martin 1977). Holliday also regarded Hayflick's human diploid cell culture as an ideal system to test Orgel's hypothesis: it was much easier to handle than tissues excised from living animals, and recorded aging phenomena of human diploid cells was "strikingly similar" to those in fungi (Holliday 1969).

Within NIMR's own network of research, Holliday also had ready access to human diploid cells. As Hayflick's WI-38 cell strain gradually replaced monkey cells to become a safer substrate for vaccine production in the US, NIMR's own researchers isolated MRC-5 (Medical Research Council-5) human diploid cell strain from fetal lung tissues for its potential medical utility (Hayflick, Plotkin, and Stevenson 1987). Because of the prospects of using MRC-5 as vaccine substrate, researchers were especially careful in making sure the strain's chromosomal, morphological, and growth characteristics were normal. It was shown that MRC-5 has a limited growth potential of 48 cell doublings, after which the cells would stop dividing, and eventually die (Jacobs, Jones, and Baille 1970).

At this time, Holliday's research program was expanded and was able to employ a group of technicians, graduate students, and post-doctorate researchers oriented to study

[14] For Alexis Carrel and his immortal cell culture, see chapter 2 and (Landecker 2007)

cell aging. The small group of investigators on cell aging at Mill Hill produced encouraging results about the validity of error catastrophe hypothesis in human cell cultures. Around 1972, they successfully induced early aging in MRC-5 through base analogue 5-fluorouracil that presumably introduces errors to protein synthesis. They also confirmed that enzymes with high heat lability were extracted from both the cells with induced senescence and those with normal senescence (Holliday and Tarrant 1972, Lewis and Tarrant 1972, Holliday 1972). Around 1973, working together with medical researchers, Holliday also detected altered enzymes in cells isolated from patients of Werner's syndrome, which are characterized by early signs of senescence (Holliday, Porterfield, and Gibbs 1974).

In a 1972 paper published in *Nature*, Holliday presented the "causes of cellular death" through a schematic chart featured with arrows representing the directions of flows of information errors (Figure 9 a). This chart reminds us of schematic presentations of Crick's central dogma (Figure 9 b), with each arrow standing for what historian Bernardino Fantini has called "a directional flow of sequence information" (Fantini 2006). Of course, Holliday's use of arrows is not to indicate precise replications or transfers of sequence as Crick's, but to show the directions of flows of error reproduction and the directions of the reactions that produce altered sequences with escalating rates. Another major difference is that Holliday clearly marked the boundary between cytoplasm and nucleus, with the cytoplasmic errors in protein synthesis serving as primary origin of all other errors. The cytoplasmic errors are also capable of inducing further errors of their own, with a self-referential arrow surrounding them, which is reminiscent of Crick's self-referential arrow assigned to the "master molecule" DNA.

With NMRI's growing personnel working in cell aging, the increasing visibility of Mill Hill's aging research, and steady exchange of information with other researchers and institutions, in the 1970s, Holliday's experimental horizon expanded. After 1972, he began to collaborate with molecular biologists who brought in new biochemical and cytogenetic methods. Using isotope labeling technique and a novel *in vitro* assay of DNA polymerase, Holliday's group and collaborators at the University of California, Berkeley successfully detected the decreased rate of DNA replication and reduced fidelity of DNA polymerase in aging human cells (Petes et al. 1974, Linn, Kairis, and Holliday 1976). Holliday also managed to create polyploid human cells using colchicine and showed that the polyploid cells with multiple copies of one gene had life spans similar to those of the diploid cells. Thus it was unlikely that recessive genetic mutations were responsible for cell aging. Holliday saw this result as a final attack to the somatic mutation hypothesis widely held among US researchers including Hayflick, and gave the subtitle to the publication "Evidence Against the Somatic Mutation Theory of Cellular Ageing" (Thompson and Holliday 1978).

Until the early 1970s, aging cells in Holliday's laboratory served as substrates isolated from their own biological and historical contexts so that they could produce simple answers about the cause of aging. Particularly, the emphasis of molecular biology on DNA and protein molecules, embodied in the articulation of somatic mutation hypothesis and the error catastrophe hypothesis of aging, directed investigations to test whether defects in DNA *or* protein cause aging. Because DNA and protein molecules locate at different sites within the cellular machine, the question was also articulated sometimes as one about the location of an aging clock: whether it is inherited through

nuclei or cytoplasm. The cells as such served as information machineries that provided clues about aging by reacting to various technological manipulations. In the 1960s, this approach to cell aging through testing dichotomous, opposing hypotheses was given much hope to reveal important mechanisms of aging. As the field of cell aging developed, however, it became clear that this either-or approach towards the cause of aging was probably wrong-headed.

Disillusions amid Experimental and Theoretical Conundrums

By the early 1970s, although Holliday gained substantial optimism for the validity of the error catastrophe hypothesis with his experimental evidence, other scientists felt that the work from Holliday's group offered only tenuous support for Orgel's hypothesis. Although the somatic mutation hypothesis did not fare well among biogerontologists, the error catastrophe hypothesis did not win out but faced its own difficulties. This time, the opposing arguments did not target the importance of DNA, but disputed Holliday's experimental assumptions and methods, pointed to results that contradict Orgel's hypothesis, or gave theoretical reasons why a catastrophe caused by errors in protein synthesis was nearly impossible. These experimental and theoretical challenges stimulated Orgel and Holliday to reevaluate evidence for and against the error catastrophe hypothesis, and resulted in an integrative approach towards evaluating results about cell aging.

In particular, considerations from study of molecular evolution made the assumption of Orgel's hypothesis that protein errors always augment themselves highly problematic. The newly raised theoretical problems from molecular evolution shifted the directions of Holliday's experiments and led him to reassess the stochastic dynamics of

protein errors through the lens of evolution. In the end, the mutual modifications of theories and experiments not only dismantled Holliday's firm belief about error hypothesis, but also seeded a new thinking pattern that transcends linearly localizing the approximate cause of cell aging but tries to incorporate other factors such as natural selection.

Some of the experimental disagreements from others involved the contention that the heat lability of proteins Holliday measured did not necessarily reflect the level of mistranslation. Although the glucose-6-phosphate dehydrogenase extracted from senescent cells did show higher thermolability than those from younger cells, Holliday's conclusion that such increase was due to accumulated errors in protein seemed untenable. Other researchers used more sophisticated protein biochemical analysis to show that the difference was probably due to post-synthetic modification and polymerization, rather than increased misincorporation of amino acid (Kahn et al. 1977, Duncan, Dell'Orco, and Guthrie 1977). These results probably did not come to Holliday as a complete surprise, because the revelation that measuring the heat lability might not be reflective of protein error level also occurred in Holliday's own laboratory. In 1973, when one of Holliday's graduate students reported that the process of freezing the cell itself could increase the portion of heat labile proteins, Holliday alarmingly reported to Orgel about this "disturbing result."[15]

More convincing opposing evidence came from studies on viral propagation in senescent cells. Since viral propagation piggybacks on the protein synthesis apparatus of

[15] Robin Holliday to Leslie Orgel, February 23, 1973, Folder 11, Box 30, MSS 176, Leslie Orgel Papers, Mandeville Special Collections Library, University of California, San Diego.

their host cells, if the protein synthesizing machinery is defective in the cell, the virus generated should be defective as well. To test the error level within cells, in 1973, cancer biologist John J. Holland at University of California, San Diego infected WI-38 human diploid fibroblasts with three different types of virus, but found that the quantity and the quality of the derived viruses were unaffected by the age of the cells (Holland, Kohne, and Doyle 1973). This result went against the error catastrophe hypothesis, because unaffected viruses indicated intact protein-synthesis machinery in their hosts – the aging cells. The fact that Holland originally supported error catastrophe hypothesis made his experiments appeared even more solid and perplexing. Orgel wrote to Holliday in December, "John Holland admits that he was surprised at his own results, having fully expected to find thermolabile virus and an increased mutation rate. He seems far more keen than I am to save the error theory, and has come up with a number of farfetched rationalizations of his results."[16] In a review published in 1975, Holliday also admitted that Holland's results provided "evidence against the theory" (Holliday 1975).

Considering these confusing results, Holliday reached a sober assessment that the field might not know enough, or have adequate methods to disentangle the secrets of cell aging. In the epilogue of a collection of papers that review the state of cell aging research, Holliday lamented, "Although stimulation and impetus to research can arise from formulation and testing of specific hypotheses using well-defined systems, these can be followed by frustration if the experimental procedures available turn out to be inadequate to make the appropriate test. This has certainly been the case with regard to the general

[16] Orgel to Holliday, December 6, 1973, Folder 11, Box 30, MSS 176, Leslie Orgel Papers, Mandeville Special Collections Library, University of California, San Diego.

error theory of aging" (Holliday 1986, 313). Responding to a report about evidence against the error hypothesis, in 1977, Orgel also confided that he was "no longer a very strong supporter of error catastrophe theory."[17]

In a time before the development of recombinant DNA technology when many biochemical and sequence assays were still indirect and had low sensitivity, however, experimenters could still form *ad hoc* hypotheses to "save the error theory." For Orgel, the more troublesome oppositions came from mathematical calculation and theoretical reasoning about the implausibility of error catastrophe in contemporary organisms. These discussions arose from the study of molecular evolution of protein synthesis apparatus.

After resolving the "coding problem" of how 64 triplets of nucleotide correspond to 20 amino acids on proteins, evolution-minded biologists asked how such correspondence came to be in evolutionary history. In 1965, microbiologist Carl Woese suggested that the primitive cell must first have had "error-ridden translation," then through complex evolutionary processes, at some point arrived at the modern, more-or-less error-resistant system for protein synthesis (Woese 1965). From error-prone, ambiguous translation to the more accurate translation, the error reduction that happened in the evolutionary history of protein synthesis apparatus seemed to be exactly the reverse of what Orgel's error catastrophe predicted, and made the existence of error catastrophe in modern organisms less plausible.

In the 1970s, the problem of the evolution of the codon and translation attracted a few theoretical biologists. In 1974, Goeffrey W. Hoffmann, then studying at the Max

[17] Orgel to Gallant, June 8, 1977, Folder 9, Box 8, MSS 176, Leslie Orgel Papers, Mandeville Special Collections Library, University of California, San Diego.

Planck Institute for Biophysical Chemistry, mathematically worked out a model for tRNA adaptors and showed that once the adaptors reach a certain level of specificity, they automatically became stable and do not increase error level (Hoffman 1974). Hoffman showed the possibility that "even an intrinsically inaccurate translation machinery can avoid progressive error feedback and therefore remain stable," and that "it is very unlikely that error feedback could be significant in present-day organisms" (Holliday 1986, 12).

Researching molecular evolution himself, Orgel became aware about the problems generated by the error-ridden translations in evolution, and in 1970, published an erratum to his 1963 paper to accommodate possible stability of errors in biological systems (Orgel 1970). In the correction, Orgel modified his original assumption that errors involved in protein synthesis always create more errors to a more flexible condition that these errors may actually decrease or become stable. In the mathematical model, Orgel classified protein errors into residual errors, which have a frequency of R, and the errors resulting from defective protein synthesis apparatus. While the residual error frequency remain approximately unchanged, the errors propagation in the process of protein synthesis can augment over generations, or remain a constant value. Orgel described a coefficient α as "the proportionality constant between errors in the synthetic apparatus and errors in freshly synthesized protein." Then, for the generation n, the error level

$$c_n = R\,(1 + \alpha + \alpha^2 + \ldots + \alpha^{n-1}).$$

This meant, as Orgel summarized, "if $\alpha > 1$, c_n increases indefinitely and we get an error catastrophe; if $\alpha >> 1$, the error frequency increases exponentially. Otherwise, a

steady-state error frequency of R/(1-α) will be reached." Using the mathematical modification to the original hypothesis, Orgel rationalized both the possibility of error catastrophe and its absence, "while an error catastrophe can occur, and apparently does in certain *Neurospora* mutants, it may not be inevitable."

Although Orgel's modification relieved some tensions between error catastrophe hypothesis and the actual evolutionary path of protein synthesis, it did not define in what circumstances error catastrophe should occur, and left the question open whether protein errors were the cause of aging or not. What the α values are for most organisms seemed to be critical to determine how often error catastrophes actually occur, and measuring α values constituted a new task for experimentalists. In fact, the theoretical reconsiderations about the possibility of error catastrophe redirected the field's attention from focusing on the existence of defective molecules in aging cells to the dynamics of error propagation. This change of focus elevated the cell system from an isolated test-field for aging causes, to a living entity embedded in an evolutionary history in which complex processes of molecular productions were selected and integrated.

Some experimentalists, such as geneticist Jonathan Gallant at the University of Washington, Seattle, deemed α to be smaller than 1. Having discussed about ways to measure error frequencies with Holliday, Gallant studied the pattern of amino acid misincorporation in *E. coli* grew in cultures supplemented with streptomycin, an antibiotic that induces mistranslation. By 1977, he obtained results that showed the misincorporation of amino acid always reached a plateau in *E. coli*. In addition, after moving to media without streptomycin, these bacteria would recover from the accumulated errors, and start to produce correct versions of proteins. Gallant initially

gave the α value in *E. coli* an estimate of about 0.8, and later reduced it to 0.5 (Edelmann and Gallant 1977).

Although Gallant regarded an α value smaller than 1 as a definite disproof to the existence of error catastrophe, Holliday and Orgel nevertheless reckoned it as something evolutionarily more subtle. In a letter they wrote to Gallant, they described a "paradoxical conclusion" about Gallant's result "that it is the first really encouraging evidence in favor of error catastrophes – at least if your provisional value of $\alpha = 0.8$ is anywhere near the truth." Because, Holliday and Orgel reasoned, α value is selected by natural selection to reduce "the proportion of bad protein molecules under 'normal' conditions" so that it is "not so large as to constitute a permanent drain on metabolic energy." However, "there is no selection pressure to reduce α much below 1." If α is slightly smaller than 1 in bacteria for which selection pressure for translation accuracy in individual cells is strong, then it is possible that "$\alpha > 1$ in some cellular systems of eukaryotes" because the maintenance of translation accuracy in individual cells is less important for multicellular organisms, and there may be "no selection pressure to reduce α below 1."[18]

Orgel and Holliday's response that incorporated natural selection to theoretical assessment of α value, however, sounded like *ad hoc* equivocations to Gallant. In his immediate reply, Gallant expressly resented the circuitous, inconclusive discussion. He mocked, "Our data showed, at first thought, the implausibility of error catastrophe under 'normal' conditions. Now you propose to interpret our results to show the plausibility of

[18] Orgel and Holliday to Gallant, September 28, 1977, Folder 14, Box 30, MSS 176, Leslie Orgel Papers, Mandeville Special Collections Library, University of California, San Diego.

error catastrophe. Next, I will interpret your interpretation to show implausibility. Then, you will interpret my interpretation of your interpretation of the original data to show, not only that error catastrophe is plausible, but that it occurred last Thursday at 4:10 in the afternoon. There is no reason why we can't keep this roundabout spinning for years."[19]

In the same letter, Gallant suggested circulating all available data with primary interpretations among the researchers of cell aging through mails, and made a final judgment collectively about the plausibility of error catastrophe. In fact, for years, aiming to reconcile myriad results, Holliday and Orgel had tried to look for opportunities to work closely and conduct comprehensive reviews of data together. However, their applications for national funds were rejected several times and they only managed to collaborate in short terms, to review existing data in piecemeal fashions. From the mid-1970s, to seek a way out of the conundrum of cell aging, Holliday began to investigate alternative ways of explaining cell aging either by himself or with help from other collaborators. With these efforts, Holliday's perspective was transformed to a multi-causal, integrative view. Let me explain.

Escape from Quandary: Alternative and Integrative Approach to Cell Aging

Responding to the conflicting experimental results and theoretical concerns regarding the error catastrophe hypothesis, Orgel and Holliday tried to work more closely and to take a comprehensive approach to aging. From 1972 to 1974, they tried to secure both public and private funding for their research in cell aging, only to find out that the strong theoretical component of the project was not appreciated. In the grant application

[19] Gallant to Holliday and Orgel, October 21, 1977, Folder 9, Box 8, MSS 176, Leslie Orgel Papers, Mandeville Special Collections Library, University of California, San Diego.

to NIH submitted in 1972, Orgel stated the research goal as to "develop a simple, plausible theory of decreases of viability of cells with age." Orgel expressed concerns about the fashion of discussion that emphasized dissent and did not seek a possible common ground. He said, "Discussions of cellular ageing often take the form of competitions: each investigator tries to show that his mechanism is the predominant cause of ageing." Instead, Orgel believed "that ageing is an essentially multi-cause phenomenon in which an initial deterioration of one subcellular system may bring about its own further deterioration and the deterioration of other subsystems."[20]

In October, the application was returned with requests for further information. The Molecular Biology Study Section from NIH apparently did not quite understand the theoretical goals of Orgel and was more interested in the experimental investigations. Particularly, they asked how Holliday could "distinguish between somatic mutation and errors in protein synthesis as a cause for the accumulation of thermolabile enzymes," how could "the proposed equation relate to the experiments mentioned in the preceding paragraph," and asked them to address "the seeming likelihood that the rate-limiting steps in progress may be experimental and not theoretical."[21]

Orgel replied to the Study Section with brisk answers to technological questions, but a thoughtful three-page discussion to educate the Section about the ways theories could help "by producing detailed and testable models of various aspects of the ageing

[20] Orgel and Holliday's application to research grant from National Institute of Health, 1972-1973, Folder 5, Box 31, MSS 176, Leslie Orgel Papers, Mandeville Special Collections Library, University of California, San Diego.

[21] George N. Eaves to Orgel, 16 October 1972, Folder 5, Box 31, MSS 176, Leslie Orgel Papers, Mandeville Special Collections Library, University of California, San Diego.

process." He made it clear that mathematical models are especially useful for differentiating the relative importance of various parameters in initiate aging. "If we are right, the widely held assumption that some simple process in cells is the primary cause of ageing from which all other changes derive, is unlikely to be correct. An adequate understanding of the relation between the different parameters of ageing is only possible if it is realized that the deterioration of any one set of macromolecular constituents in a cell is likely to accelerate the deterioration of all others." For such understanding, "it might be possible to do something with the reciprocal problem of computing the effect of mutations on the error frequency."[22]

NIH was not the only funding agency that raised concerns about the strong theoretical components of Orgel and Holliday's joint project. As NIH turned down the proposal and Orgel started to seek support from private foundations, Donald B. Hoffman from the Edna McConnell Clark Foundation was concerned that "an all theoretical program will not appeal to his Board."[23] Eventually, Salk Institute provided the expense of Holliday's three-month visit to Orgel in 1973, which proved to be not enough time for their rather ambitious plan. During these three months, Holliday and Orgel did finish several review chapters for a book they had been planning to write about the theoretical aspects of aging research. However, they found these chapters unsatisfactory, and that

[22] Orgel to Eaves, undated, Folder 5, Box 31, MSS 176, Leslie Orgel Papers, Mandeville Special Collections Library, University of California, San Diego.

[23] Frederic de Hoffmann to Orgel, May 25, 1973, Folder 4, Box 31, MSS 176, Leslie Orgel Papers, Mandeville Special Collections Library, University of California, San Diego.

aging was still a more complex process than they had envisaged. In the end, the book has never been submitted to publisher.[24]

Back at his home institution, the Genetics Division of the National Institute for Medical Research, Holliday enjoyed the privilege of having his research funded through Medical Research Council's designated funds for its affiliated research institutes. However, his British colleagues in gerontology did not give much appreciation to the cell culture study of aging in Holliday's small empire. In May 1973, MRC held an informal meeting that invited established gerontologists to consider the prospects of funding research on cell aging more extensively. During the meeting, other gerontologists, such as Denis Bellamy from Department of Zoology, University College, Cardiff and David A. Hall from Department of Medicine, University of Leeds, were reluctant to accept "that clonal ageing was related in any fundamental way to whole-body ageing." They deemed researching aging using cell cultures as irrelevant to investigating real aging process in animals. In the meeting report, the conclusions were made that "there was still no adequate explanation in cellular terms of the many parameters of the ageing process that could be defined in whole organs or tissues," and thus "there was still substantial potential for purely descriptive analysis of ageing, both in man and in lower animals; to delineate, for example, aspects of endocrinological and neurophysiological co-ordination and associated pathology."[25]

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[24] Email communication with Holliday, April 8, 2012; Book drafts on aging, Folder 8-9, Box 30, MSS 176, Leslie Orgel Papers, Mandeville Special Collections Library, University of California, San Diego.

[25] Report on the Council's Conference on Cell Ageing held at Park Crescent on May 24, 1973, Folder 11, Box 30, MSS 176, Leslie Orgel Papers, Mandeville Special Collections Library, University of California, San Diego.

As an outcome of the informal meeting, MRC did not expand its program on cell aging research in the 1970s, leaving Holliday to continue the study on cell aging in relatively small scale. The question about how cells age in cultures, however, continued to attract newcomers from different backgrounds to contribute their specialties to biology of aging. Having recognized the complexity of cell aging, and with the access to intellectual collaborations with researchers in MRC, Holliday started to explore alternative molecular explanations as well as explanations from levels higher than molecules from the mid-1970s.

Epigenetic Explanation of Cell Aging as an Alternative

During these years, Holliday learned about DNA modification research. He first heard about DNA modification of bacteria and viruses from an Indian post-doctoral researcher, Prit Naha (Holliday 2008, 134). Some time later, Holliday accepted a PhD student, John Pugh, who had the habit of delving into comprehensive reading of published scientific literature and reported about the phenomenon of DNA methylation. During a journal club, Pugh hypothesized that DNA methylation could explain the process in which one of the X-chromosome in a female cell is inactivated (Holliday 1998). For Holliday, the new knowledge about DNA methylation seemed to be a new solution to certain puzzles from prior results about cell aging. One former post-doctoral researcher, Cynthia Lewis, had found that the rate of incorporating methionine decreased in aging cells. Methionine is not only an amino acid analogue, but also serves as a substrate for DNA methylation. Lewis had suggested that the reduced methionine incorperation could be explained through either the error accumulation in proteins, or the reduced rate of DNA methylation (Lewis and Tarrant 1972). It was possible that the

reduced rate of DNA methylation actually contributes to initiating aging in cells. Holliday became intrigued. For Holliday, reduced DNA methylation did not contradict error catastrophe hypothesis, and might actually supplement it. Because decreasing level of DNA methylation might be an indication of the reduced activity of enzymes that specifically methylate DNA sections. The decrease of activity in these enzymes might well be due to the productions of errors in them with age.

In 1975, Holliday and Pugh published a review in *Science* that described the state of research on DNA modification and predicted its importance in maintaining cells in development and aging (Holliday and Pugh 1975). The paper hypothesized that enzyme-directed DNA modification, not the DNA sequence *per se*, controls certain fundamental biological functions, including setting up the developmental clock, stabilizing the patterns of expression in differentiated cells, and cell aging. For cell aging, the authors surmised that the loss of DNA methylation over time might mess up the patterns of expression of proteins, thus generating an aging phenotype. Although these hypotheses were not exactly statements about accumulation of protein errors, they were nevertheless based on the same dynamic, interactive view about the relation between protein and DNA molecules. As the authors noted, "a continual interaction between cytoplasmic enzymes and DNA sequences is an essential part of the model to be presented" (Holliday and Pugh 1975).

In 1978, Holliday and Pugh expanded the DNA methylation hypothesis to explain how cells became cancerous, and used the epigenetics to describe the study of DNA methylation for the first time (Holliday 1979). When Holliday again reviewed research on DNA methylation and its developmental effects under the light of Conrad Hal

Waddington's original proposal of epigenetics in 1987, the paper published was believed, in historian David Haig's word, to have "lit the fuse for the explosion in use of 'epigenetic' in the 1990s" (Holliday 1987, Haig 2004).

Holliday did not completely shift his view from the error catastrophe theory to DNA methylation in the 1970s. After all, loss of DNA methylation does not contradict error accumulations in proteins and may have caused the errors. With researchers in Mill Hill, Holliday continued to experiment about the error catastrophe theory well into the 1980s. Under this light, the development of the epigenetic hypothesis seemed to be an exercise to develop alternative explanations of cell aging that can bypass the confusions generated from the confusions about error catastrophe hypothesis.

Integrative Approaches on the Level of Cells and through Evolution

Responding to the experimental and theoretical impasse, Holliday not only seriously considered alternative hypotheses of cell aging such as the epigenetic change in DNA methylation pattern, but also tried to synthesize different experimental results into encompassing models and theories. Having been influenced by the theoretical bent from his PhD advisor Harold Whitehouse and his long-term collaborator Orgel, by the mid-1970s, Holliday became more adept in working with theories (Holliday 2008, 103). He collected his thoughts and hypotheses after contemplating on a series of data, then sought ways to synthesize them into formal theories.

After Holland's results showed the normal viral replication in senescent cells and casted serious doubts about error catastrophe hypothesis of aging, Holliday tried to approach modeling cell aging through the dynamics of cell division. In the 1970s, he formulated the commitment theory of aging, for which the heterogeneity of life spans of

individual cells in a culture provided important inspiration. Although the duration of certain human diploid cell culture is predictable, it was reported that the life spans for individual cell are not identical. They exhibit varied, stochastic patterns (Smith and Hayflick 1974). This meant that the cause of aging in individual cell might well be diverse and work in stochastic ways. In December 1973, Holliday described to Orgel a new idea he had thought of while daydreaming at a meeting in Paris. "It seems to me quite possible that fibroblast cells are in fact potentially immortal and under the right conditions could be kept going indefinitely. Suppose ageing consists of two stages: an initial commitment and then an incubation period, with division at the normal rate, leading to senescence."[26] Holliday pointed out that one important difference between cell types with different life spans might be their "probability of commitment." Because individual cells stop dividing at different times and are stochastic events, the aging of a cell culture only happens when most cells are committed to aging in a culture.

Holliday realized that he probably needed some mathematical input to turn his initial thoughts into established models. Returning from Paris, Holliday talked to Thomas B. L. Kirkwood, then a young biometrician working at the National Institute for Biological Standard and Control about the problem. With Kirkwood's mathematical skill, in 1975, Holliday and Kirkwood published the first articulation of the commitment theory of aging with surprising predictions for future experimental tests (Kirkwood and Holliday 1975). In the paper, they mentioned that several types of event should be consistent with

[26] Holliday to Orgel, December 21, 1973, Folder 11, Box 30, MSS 176, Leslie Orgel Papers, Mandeville Special Collections Library, University of California, San Diego.

their “general model,” which included error catastrophe, loss of DNA methylation, and the dilution of cytoplasmic particles.

Kirkwood became so interested in the problem of cell aging that he soon became a PhD student at Holliday’s laboratory and continued to apply his mathematical expertise to the problems on aging. With Kirkwood, Holliday not only refined the commitment theory, but also employed evolutionary thinking to integrate recent studies on DNA repair and scavenger enzymes to address the question why cells age. In the 1970s and 1980s, three workshops held by the European Molecular Biology Organization focused on the discussion about the accuracy of molecular processes, from which Holliday heard about recent discoveries of DNA repair, scavenger enzymes that remove incorrectly synthesized proteins, as well as that the processes of molecular proof-reading to ensure accuracies consume energy (Kornberg 1974, Ninio 1975, Hopfield 1974). It occurred to Holliday and Kirkwood that because available energy is limited, the individual cells had to balance the energy allocated to growth and reproduction and the energy spent to ensure the accuracy of macromolecules. They reasoned that in the germ-line cells, the precision of macromolecules should be strongly selected. The selection pressure for somatic cells to stringently control the molecular accuracy, however, is much weaker, because somatic cells only need to exist for only so long to ensure successful reproduction, thus are “disposable.” In the end, the low surveillance of errors of all kinds would allow defective molecules to accumulate in somatic cells during aging and wreak havoc. Kirkwood and Holliday mused, “The strategy is analogous to that used in the manufacture of disposable goods, which have a short expected life and are therefore made as cheaply as possible.” This hypothesis of cell aging that integrated new discoveries about molecular

proofreading and repair through evolutionary point of view was later referred to as the disposable soma theory of aging (Kirkwood and Holliday 1979).

In these later models, cells were no longer seen as entities that can be extracted and isolated from its physical and historical contexts in a cell culture. The molecular causes of cell aging were no longer believed to be a single change that can be assigned to a specific location, nucleus or cytoplasm. The processes of cell aging were seen as multiple, plural, but nevertheless can be explained through the logic of evolution.

Kirkwood and Holliday's joint efforts that combined Holliday's experimental forte and Kirkwood's modeling expertise to integrate data from different biological systems into models proved to be extremely productive. In 1983, Kirkwood graduated with a PhD in biology without writing a thesis, because by then he had published about 40 papers on aging either jointly or by himself. In 1987, Holliday was awarded the Lord Cohen Medal for Gerontological Research.

In 1988, Holliday left England for Australia. He continued to develop the disposable soma theory of aging beyond molecules and cells. In the 1995 book, *Understanding Ageing*, Holliday used the logic of disposable soma theory to explain the changes during aging in diverse biological processes, such as immune response, detoxification, removal of oxygen free radicals, wound healing, and physiological homeostasis. In the book, Holliday also remarked that the key for understanding aging is not further experimental work, but on analyzing the existing data at hand. He wrote, "I believe that to understand ageing one need only look at and appreciate this vast body of existing knowledge. Those who believe ageing is a mystery cannot see the wood for the trees around them" (Holliday 1995, ix).

For Holliday, integrating these data relies on establishing multi-causal theories, and relating specific processes into a network view. As he put it, "I believe many existing theories have some validity, and that a more global interpretation that encompasses specific theories is the most appropriate. This implies that there are multiple causes of ageing, but also multiple interactions between components of cells, or between different types of cells" (Holliday 1995, x).

Conclusion

With a good deal of indirect evidence, the error catastrophe hypothesis of aging gained much supports and popularity among aging researchers during the 1970s. With the work on cell aging, as well as scientific achievements in DNA recombination, methylation, and repair, Holliday was elected Fellow of the Royal Society in 1976. Despite Holliday's productive career, his study about error catastrophe theory of aging occupies an uneasy place in history of biogerontology and molecular biology. With the advent of telomere biology of cell aging that attributes the primary control of cell aging to nuclei, Holliday's research programs in the 1970s and 1980s often fell into the oblivion among the newcomers of the expanding field of aging.[27] Gerontological research that followed the trajectory projected from Mill Hill research school, namely the system approach to aging and epigenetics in terms of DNA methylation, on the other hand, are often depicted as rather recent and novel scientific developments (Kirkwood , Fraga and Esteller 2007).

[27] In a 2002 memoir, Holliday recounted twenty years of aging research at the Mill Hill laboratories, "by far the largest group of investigators in UK," specifically to refute Aubrey de Grey's depiction of UK's study of aging in the late twentieth century as "in a sorry state." See (Holliday 2002, de Grey 2001)

After Holliday left England, Kirkwood continued to develop the multi-causal, integrative view about aging and established an eminent program focusing on systems biology of aging at the Institute of Ageing and Health of the Newcastle University (Kirkwood 2008). The creation of a systems biology of aging is itself an interesting historical process whose establishment probably involved applications of new, high-throughput technologies for data collection, standardization, and analysis that only became available at the end of the twentieth century. Holliday's integrative research strategy towards cell aging that evolved after the mid-1970s as a response to the experimental and theoretical quandaries regarding the error catastrophe hypothesis of aging, however, seemed already to exhibit characteristics that many held as unique to systems biology. Particularly, Holliday's multi-causal, integrative approach to cell aging embodied an iterative interplay between theories, experiments, and modeling efforts that was supposed to be the hallmark of certain programs in current systems biology (Westerhoff and Kell 2007).

Holliday's research on cell aging *in vitro* transformed the cells in cultures from information propagating machines isolated from nature to reveal its molecular engines of errors to complex living things that modulate a variety of processes to end their lives. Cells were again seen as living with stochastic molecular activities and as the end product of an evolutionary history. As the mechanism of cell aging began to be seen as complex, the epistemic role of the system of cell aging in vitro also changed. By the late 1970s, in Holliday's program, cell aging was not only a critical phenomenon for testing theories of aging, but also an organizing level to synthesize experimental data into overarching theories.

These epistemic changes about cell aging *in vitro* facilitated the integrative approach to aging. It also became the prelude to Kirkwood's establishment of systems biology of aging in the early 2000s and challenged the current historiography of systems biology. The current historiography of systems biology tends to emphasize the techno-methodological foundations of the field, confining the scope of historical inquiries about the epistemological origin of the field to the early mathematical attempts to organize and simulate biological data. In a bird's-eye view review published in *Nature Biotechnology*, Westerhoff and Palsson (2006) tie the origins of the field to two roots: a "components root" that provided a large volume of complex biological data in the post-genomic science and a "systems root" that originated from a number of biophysical and mathematical attempts to organize, analyze, and simulate biological data through quantitative integrations in order to derive general principles from them. The mathematical and physicochemical studies of non-equilibrium thermodynamics, self-organization, and metabolic structures since the early twentieth century definitely have provided antecedents in which scientists organized widely distributed data to generate theories and models. However, to attribute the drive to interpret and synthesize a wide array of biological data solely to the mathematicians and computer experts seemed to be at most incomplete characterization of the pursuits of integration. Such view neglects the nature of biological questions embedded in for example, an experimental system such as cell aging *in vitro* that demanded a systems solution to begin with.

Learning from Holliday's case, it seems that systems thinking about biology that maintained continuities to the contemporary development of systems biology can be traced at least to the mid-1970s. This is not a surprise. Many have also recognized

systems analysis is not a recent phenomenon, but existed in areas of biology such as ecology, developmental biology, and immunology (Westerhoff and Palsson 2004). However, current historiography tends to depict the rise of molecular biology as mostly a reductionist turn to focus on molecules, while neglecting the other part of developments in molecular biology that was more synthetic. One consequence of such selective historical writing is that significant portion of history of molecular biology was unknown, and that the epistemic style of systems biology looked as if it originated suddenly from nowhere. Under this light, it is no surprise that the current narratives about the origin of systems biology are mostly technocentric.

Holliday's research trajectory progressed from the impasse of testing hypotheses about certain molecular change for cell aging to the multi-causal, integrative approach of synthesizing data into models that are further testable illustrate nicely that some systems thinking was developed to solve particular experimental and theoretical problems of a field. For a time, with the dominant mechanistic thinking, experimenters and theoreticians of cell aging seemed to forget that the question "what specific molecular defect causes aging" might be itself arbitrary, and kept manipulating the cells with existing methods for clues. As experiments and theories interacted, and as their mutual modifications still could not solve the grave difficulty in reconciling complex data, the impetus for epistemic change in how one sees cells was created. Other processes such as epigenetic change and evolutionary process were then put back into the picture. In late 1970s and 1980s, the *in vitro* cell aging as an experimental system interacted with hypotheses about aging and led to new integrative approaches towards aging.

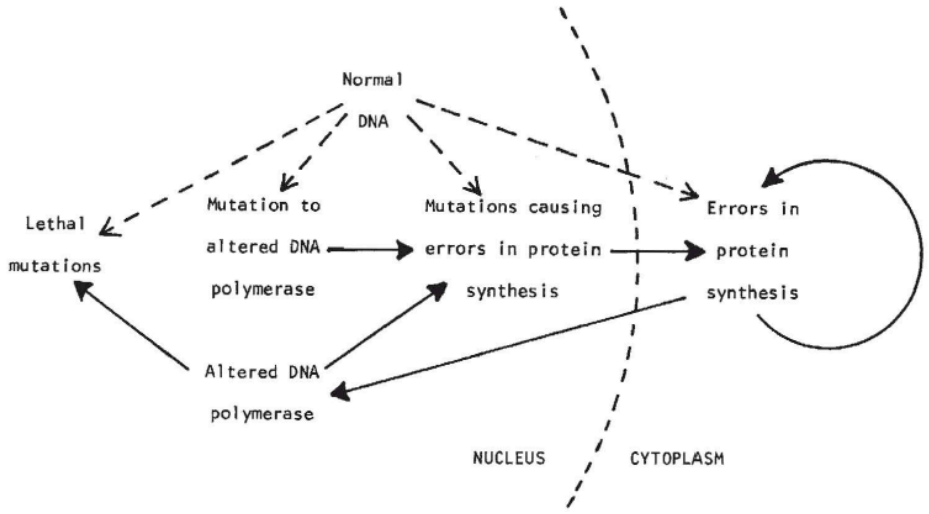
Figure 6. Leslie Orgel in the "RNA Tie Club," 1955.

From left to right: Francis Crick, Alexander Rich, Leslie E. Orgel, James Watson. By Alexander Rich. From Linus Pauling and The Race for DNA Website (http://osulibrary.oregonstate.edu/specialcollections/coll/pauling/dna/pictures/portrait-tieclub-large.html, accessed July 2nd, 2011)

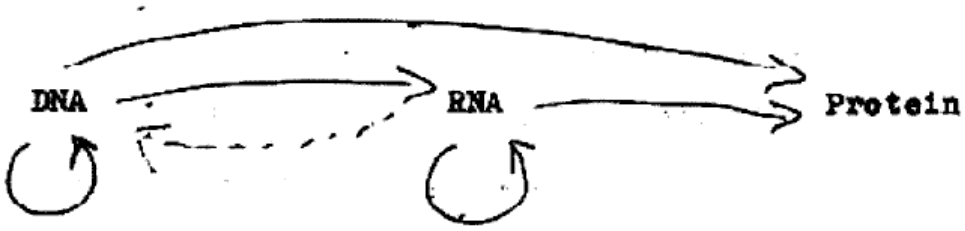
Figure 7. Robin Holliday explains the "Holliday Junction" in Mill Hill.

Figure 8. The National Institute for Medical Research, Mill Hill, London.

The Genetics Division Laboratories are on the top floor of the central block.



a.



b.

Figure 9. Arrows in Molecular Representations

a. Robin Holliday's schematic presentation of the directions of flows of sequence errors in aging.

   From Holliday, Robin, and G. M. Tarrant. "Altered Enzymes in Ageing Human Fibroblasts." *Nature* 238, (1972): 26-30.

b. A schematic presentation of Crick's central dogma.

   From Fantini, Bernadino. "Of Arrows and Flows: Causality, Determination, and Specificity in the Central Dogma of Molecular Biology." *History and Philosophy of Life Sciences* 28, (2006): 567-94.

Chapter 4: A Wormy Kind of Death

H. Robert Horvitz's Molecular Study of Cell Death in *C. elegans*, 1975-1992

In 2002, American molecular biologist Howard Robert Horvitz, British biochemist John Sulston, and the long-time scientific celebrity Sydney Brenner were jointly awarded the Nobel Prize for Physiology and Medicine for "their discoveries concerning genetic regulation of organ development and programmed cell death." (http://nobelprize.org) The star organism, the nematode *C. elegans*, seemed to have co-presented at the Nobel Prize ceremony, as it was mentioned numerous times as the three scientists recounted how *C. elegans* system offered biological simplicity, experimental feasibility, and an analogous platform for studying diseases in humans. Regarding the programmed cell death, H. Robert Horvitz lectured that their discoveries on the molecular mechanisms of cell death in *C. elegans* are important first steps to understand human development as well as a variety of neurodegenerative diseases and cancers in humans. As the Nobel Committee juxtaposed the developing *C. elegans* with a developing human embryo on a poster for press release, both with their "dying cells" during the formation of the new life, the message was clear: the death of the worm cells is every bit about our life as well.

Among the three laureates, H. Robert Horvitz had led the work that illuminated the first network of important genes that regulate programmed cell death in *C. elegans*. His team's work also revealed that some of these cell-death-related genes have homological counterparts responsible for human diseases. Although Horvitz's work on molecularization of cell death raised the status of the field and was perceived as more

mechanistic study than earlier pursuits researching cell death, Horvitz's study of cell death started from purely descriptive cell lineage tracing in the 1970s. His later methods employed to achieve the molecular network involved in programmed cell death were adapted from the logic of classical experimental embryology and mutagenesis.

It is not to say that there is nothing new in Horvitz's study of cell death. Yet the novelty cannot be simply summarized as more mechanistic, more molecular experimental method or philosophy. This chapter traces the evolution of Horvtiz's study of cell death from the early 1970s to the early 1990s and identifies how studying cell death in the contexts of the *C. elegans* system and the expanding molecular techniques set the trajectory of Horvitz's research to a path different from prior cell death studies. It addresses the questions what exactly were those differences and how these research strategies mattered for the overall achievements of the cell death research since the 1990s. I show that the comprehensive historical and structural relations of cells in *C. elegans* offered by the cell lineage mapping in the 1970s provided the most important scaffold and reference for intervening individual events of cell death, which eventually made the molecular illuminations of programmed cell death possible. Horvitz's research also helped to establish a view about the molecular and biological regularity of cell death. Such view, as well as cell death's connection with various diseases, promoted the further expansion of molecular biology of cell death into pathological and therapeutic studies of cancer, aging, and degenerative diseases.

In this chapter, I also examine the research processes of Horvitz and John Sulston's cell lineage mapping projects in British Medical Research Council's Laboratory of Molecular Biology in the 1970s as well as the work in molecular study of programmed

cell death carried out in Horvitz's research group at Massachusetts Institute of Technology by the early 1990s. Accompanying these research projects was the increasing visibility of the importance of cell death biology and the parallel expansion of cell death research that redirected the research focuses about cell death and at times enhanced the research motivations about the problem. Consequently, I will depict the changing states of the field of cell death of a certain time in various sections of the chapter while evaluating the impact of Horvitz's study to the expanding research of cell death as a whole near at the end of the chapter.

Brenner, the Worm, and the Team

The cell death study in *C. elegans* did not originate from pure interests in cell death itself but from a comprehensive mapping project that attempted to catalogue all cell activities in the worm's development. This attempt became successful in 1983 after John Sulston and H. Robert Horvitz meticulously traced and recorded the generation paths of every one of the 959 cells of the adult worm under the Normaski microscope continually for about three years. Cell death is one of the processes that formed the adult worm body, including cell division and differentiation. A number of programmed cell death events were recorded by Sulston and Horvitz, but they did not attract much research efforts until the late 1970s. As the previous study of *C. elegans* provided the material and epistemic basis for the Horvitz's study of cell death, it is important to understand the research agenda as well as the practices about *C. elegans* before delving into the case.

The nematode *C. elegans* was chosen for research of neurobiology and development in higher organisms because Brenner regarded the organism as "loaded with

features" for investigating complex functions.[28] In the early 1960s, partly spurred by UK's Medical Research Council's interest in expanding Laboratory of Molecular Biology, Sydney Brenner wrote to Max Perutz that "the future of molecular biology lies in the extension of research to other areas of biology, notably development and the nervous system," other than the by then "classical problems" such as DNA replication and protein synthesis (Woods 1988, x-xi). With the past success of studying "classical problems" of molecular biology using bacteria and bacteriophages, Brenner started to actively search for an organism that could lend experimental manipulability to development and neurobiology in a way similar to what bacteria and phages had offered to problems of protein synthesis. He noticed the nematodes as being able to offer the experimenters the ability to "identify every cell" and "trace lineages." Brenner tested over sixty species of nematodes and had settled at the *C. briggsae* for a short time and eventually selected *C. elegans*.

The worm, as scientists studying *C. elegans* often call it, was not the only multicellular organism chosen around 1960s to study complex biological problem beyond basic molecular principles. Yet the levels of visualization and manipulation of cells, especially individual cells in precise ontogeny, eventually evolved to became unmatched by other selected organisms such as fruit flies, planaria, or mice. The *C. elegans* hermophrodite and the male were both transparent throughout their lives, which became a handy feature to exploit when researchers investigated the developmental process of the

[28] In 1960s, for future study of molecular biology involved in complex functions, Brenner searched for "small metazoa, chosen because they would be suitable for rapid genetic and biochemical analysis." After testing various nematodes, Brenner regarded *C. elegans* the prime choice because of its fixed neuron connections, rigid developmental path, and its high yield of mutants in mutagenesis (Ankeny 2001).

worm. Probably more important to the researchers to study the regularity and precision of development, the generation path of every one of the worm body's 959 cells is invariant in every individual hermaphrodite worm. Interested in both development and neurobiology, researchers in Brenner's group initiated microscopic mapping of both the neuron generation and the developmental cell lineage. The cell lineage study inevitably exposed the cell death phenomenon before the researcher's eyes and proved crucial.

The fortuitous selection of *C. elegans* and the later successful usage of the organism to its great potential, however, was not only due to Brenner's smart choice but also resulted from other invertebrate zoologists' descriptive work about nematodes. Although the selection of the experimental organism is often depicted as Brenner's ingenuity, Brenner's choice was actually greatly influenced by Ellsworth C. Dougherty from University of California, Berkeley's 1963 book *The Lower Metazoa: Comparative Biology and Phylogeny* (Dougherty 1963). Brenner had directly requested *C. elegans* from Dougherty who had studied *C. elegans* since the 1940s and requested his reprints about how to propagate the males. After trying to cultivate worms as Dougherty advised, Brenner found the *C. elegans* to be "astounding organism."[29] In addition, the various "mapping" projects, including the later genomic project of *C. elegans* received ample streams of funding that enabled these projects that were mostly based on traditional descriptive methods to be conducted without competing with more mechanistically oriented studies. The great resources and facilities assigned to Brenner for the worm study was impressive, and was based on a surprisingly short research proposal, or as

[29] Sydney Brenner to Ellsworth C. Dougherty, December 9, 1963, SB/1/1/162/3, Sydney Brenner Collection (1927-2010), Cold Spring Harbor Laboratory Archives.

Brenner himself called, a “manifesto” written in 1963 for studying several model systems simultaneously. The funding provided to expand LMB as well as Brenner’s persona as a scientific visionary pushed the program towards its full steam on many fronts.

Aiming to explain development and behavior through molecular biology, Brenner nevertheless gradually realized that molecular biologists must “enter these problems on the biologists’ terms” and “do ‘old-time’ biology and convert it from within.” Brenner tried to expand molecular biology’s territory but was open to investigating on the new organisms at all levels. He was also used to collecting a large volume of data to find regularities. Brenner had studied the collinearity problem that excited Francis Crick in the bacteriophage which mapped the correlations between DNA mutations with changes in the amino acid sequence in proteins of the rII region of the bacteriophage T4 (Brenner 1966). Although the work was hypothesis-driven and experimental, it also targeted at collecting the mutations and mapping their correlations with amino acid sequences.

Not surprisingly, Brenner’s first project on *C. elegans* was to produce a series of mutants, especially abnormal in behavior and shape, for future comprehensive study of the organism. At the same time, when the mapping the cell lineage was underway, others at the Brenner lab, John White, Nicol Thompson, and Eileen Southgate were actively pursuing another mapping project on the complete neuron wiring in the *C. elegans*, which proved to be even more complicated and in the end took almost twenty years to finish (White et al. 1976). In the contexts of these extensive mapping projects, the much publicized completion of the whole *C. elegans* genome sequence in 1998 seemed to be a natural growth out of this mapping momentum (de Chadarevian 2004).

Yet there are considerable differences between Brenner's vision of manipulating and describing the worm and the epistemic logic of later genome projects. Having been fascinated by cell biology as early as when he was a medical student in South Africa, Brenner was particularly interested in how cells divide, differentiate, and organize themselves during the developmental process. "It may be true to say that development was simply a matter of turning genes on and off," he argued, "however that did not help one to understand these processes" (Brenner et al. 2001). In the letter to Max Perutz in 1963, Brenner explicitly expressed his interest in studying the control of cell division especially meiosis, the determination of flagellation and ciliation. In recommending the *C. elegans* project, Brenner envisioned, "To start with we propose to identify every cell in the worm and trace lineages. We shall also investigate the constancy of development and study its control by looking for mutants."[30] It seems in initiating mapping project in *C. elegans*, Brenner was not simply interested in just any biological information, but was looking for a category of worthwhile cellular phenomena for further investigation. In fact, when John Sulston and Alan Coulson became so interested in launching the *C. elegans* genome project, Brenner faded out of the project because it reminded him of molecular stamp collecting with monotonous sequences made of four kinds of deoxyribonucleic acid bases.

The eventual success in observing the cellular activities that generated the adult worm's 959 cells of course relied on teamwork, especially the collaboration between John Sulston and H. Robert Horvitz. The mapping of the whole cell lineage in *C. elegans* not only set the phenomenon of cell death in the full contexts of one metazoa's

[30] Sydney Brenner to Max Perutz, 5 June 1963; reprinted in (Woods 1988, x).

developmental history, the project also trained Horvitz's eyes and senses for how cells behave during the development of the *C. elegans*. I describe in the next section Horvitz and Sulston's joint efforts in mapping the full cell lineages in *C. elegans*.

Horvitz, Sulston, and the Cell Lineage Map

The completion of the cell lineage map depended on the worm's feature that it is transparent throughout so that individual cell nuclei could be viewed and positioned under microscope. Sulston was not the first to use this characteristic to its advantage and attempted to trace cells in real time in the worm body. Two other researchers at Brenner's laboratory, Roger Freedman and Simon Pickvane had also noticed the feature and played around with it. However, it was under Sulston's meticulousness, patience, and perseverance that a complete cell lineage became a possibility.

Having a talent in making toys and machines work and having obtained a PhD in chemistry, John Sulston arrived at the LMB after a stint at Leslie Orgel's laboratory at Salk Institute studying prebiotic chemistry. Sulston tried many small projects to start his work on the worm, such as labeling neurotransmitters in *C. elegans* and making the neurons glow. This line of research did not go very far, but the work led Sulston to a new world of cells: they move, they split, they grow. After Pickvane left the laboratory, Sulston started to trace the cell divisions in a more regular manner. Because of the life cycle of the worms, Sulston's working hours involved peeking into the microscope for

four hours twice a day. Sulston enjoyed figuring out how cells divide and always tried his best to obtain the most beautiful pictures from his gadgets.[31]

Not very articulate about the great potential of *C. elegans* for the future of biology, Sulston presented the project as if he simply took on the project because it was possible. In an interview conducted by Soraya de Chaderevian, Sulston put it:

"At a certain point you say, well, if I go on doing this for another four hundred days I will have done it all, and I did think that at a point, yes. I calculated how long it was going to take and it was going to be a year and a half of looking down a microscope every day, twice a day, and following lineages for that time, four hours in the morning four hours in the afternoon, that would do the whole lineage I figured… I remember saying to John White, I said: 'Is it worth spending another year and a half on this stuff?' and he said 'Yes!' So that was very useful" (de Chadarevian 2000).

Sulston's initial interest in mapping the cell lineage lacked the experimental/mechanistic intentions that molecular biologists often depict themselves. His assumption seemed to have no explicit hypothesis other than the assumption that the microscopy observation would reveal the true process of cell divisions and differentiation during the development of *C. elegans*. The purely descriptive work also set Sulston's work apart from earlier traditions of cell lineage study pioneered by American embryologist Charles Otis Whitman (1842-1910). In 1878, Whiteman published the first cell lineage study with a special emphasis on following early cellular changes and divisions through development in a paper "The Embryology of Clepsine." This study

[31] In an interview, Sulston confided, "I think visually: the patterns of the fluorescence enchant me and the patterns through the Nomarski microscope – they're absolutely entrancing." (Brown 2003)

aimed to examine whether the cell lineage in *Clepsine* is rigid or flexible in order to answer the phylogenetic question whether the free-swimming larval stage is primary and ancestral or it is secondary and adaptive. Whitman's followers who continued the cell lineage study embraced the diversity of marine biological species provided by the Woods Hole area in Massachusetts where the Marine Biological Laboratory is located. A few of them such as Edmund Beecher Wilson and Edwin Grant Conklin had the clear goal of studying cell lineage comparatively to clarify the relations between inheritance and adaptation during development and how developmental processes suggested evolutionary paths as opposed to the stringent view of Haeckel's biogenetic law (Maienschein 1978a). In this sense, Sulston's cell lineage program was not only descriptive, but also more descriptive than prior cell lineage studies in the early twentieth century when American experimental biologists started to transition from the long traditions of European morphological studies to experimental embryology (Maienschein 1991).

Sulston's study nevertheless focused on one species, and comprehended the whole span of developmental processes in his examination. Viewing cell activities in such detailed manner revealed a variety of cell activities that were intriguing, surprising, or simply beautiful, including cell death in a variety of developmental events and functions. In 1974, Howard Robert Horvitz, a PhD recently graduated from James Waston's laboratory arrived at the LMB to join the team. The beauty of the work attracted Horvitz, who was looking for problems to study using *C. elegans* and soon joined Sulson't efforts in mapping the post-larvae cell lineage (Horvitz 2003b).

Born in 1947 in Chicago to a father who was a CPA and the treasurer of a trucking company, Horvitz loved numbers from a very young age. The propensity not

only grew into a Bachelor in theoretical mathematics and an honors thesis in Massachusetts Institute of Technology, but also led to numerous money-making schemes, ingenious poker game strategies, and summer jobs at the IBM. In Horvitz's senior year, however, he learned from his roommate Al Singer, a biology major, about the many excitements in doing biology. Horvitz took an introductory course in biology and was intrigued. In the end, he took the offer of graduate school from the biological department at Harvard in 1986. His other offers from other majors in graduate school included mathematics, economics, law, business, and engineering. He had the inner cravings of an intellectual omnivore so that he chose biology because it was the subject about which he knew the least (Horvitz 2003a).

Although Horvitz was most interested in elucidating the biological basis of consciousness, he was attracted by the joint efforts of training offered by three distinguished molecular biologists at Harvard, James Watson, Wally Gilbert and Claus Weber.[32] He eventually entered their joint laboratory, taking up a project to investigate how a sequence of sigma factors controlled a temporal series of transcriptions through the bacteriophage T4. Particularly, Horvitz purified RNA polymerase from *E. coli* cells infected with T4 and showed that the T4 gene 33 was expressed in the *E. coli* RNA polymerase during the late stage of T4 development (Horvitz 1975). Gene 33 had been suggested to be important in the late gene expressions of T4 progression by other researchers. Horvitz's research provided crucial evidence that the prior hypothesis was

[32] Horvitz commented later, "The synergism among these three scientists was phenomenal… Jim had a great biological intuition, Wally could find the pitfalls in the interpretation of any experiment, while Klaus knew precisely how to make experiments work." (Horvitz 2003a)

true and he worked hard for the project. Yet it occurred to Horvitz that the importance of the problem might not worth the efforts: why spend so much time and energy to prove something that people already suggested and was probably true anyway? By the time Horvitz was near completion of his PhD project, he published four papers, all about T4-induced modifications of *E. coli* RNA polymerase. At this point, Horvitz could choose to go to Stanford for his postdoctoral research because Watson's laboratory had a practice of exchanging students with programs in Stanford. Yet, Horvitz cast aside the usual path and chose to do something else. Horvitz had talked with Samuel Ward, a scientist just returned from MRC Laboratory of Molecular Biology in England, and learned about Brenner's work. The prospect of learning about neurobiology through the small organism *C. elegans* enticed Horvitz to contact Brenner asking about the possibility of doing postdoctoral research at LMB. Brenner's answer was positive, but he added that "as far as I know, all attempts to show learning in nematodes have failed," but that Horvitz would be free to choose his research project when he arrived.

In Walter Gilbert's reference letter, Horvitz's scientific acumen was praised, but Gilbert also had concerns whether Horvitz's broad interests in biological problems would prevent him from focusing on one problem at a time. Three years later, Brenner commented that he had seen no trace of such traits in Horvitz. In retrospect, however, Horvitz expand his research interest to many issues that went beyond his molecular biology training in Jim Watson's laboratory by the 1990s. His broad interests and the willingness to work on diverse issues fit into Brenner's laboratory set-up and research agenda perfectly.

Horvitz soon gathered his four published articles into a dissertation, but added an introductory chapter that addresses the biology of phage T4, instead of the biochemistry of the *E. coli* RNA polymerase that other chapters detailed. When the projects on various aspects of the biology of *C. elegans* bloomed in his laboratory in MIT in the 1990s, Horvitz reminisced, "I liked thinking about T4 as an organism, just as more recently I have enjoyed thinking about all aspects of the biology of the nematode *C. elegans*" (Horvitz 1998).

Having been trained in Jim Watson's laboratory, Horvitz was initially doubtful whether Sulston's descriptive approach would produce the kind of rigorous science he had looked for. Yet soon he found that the result from these observations not only were precise and beautiful, but also generated the potential of manipulating development at the level of single cells. Horvitz joined Sulston's efforts to map out the post-larvae cell lineage.

In the joint paper of about 50 pages published in 1977, "Post-embryonic Cell Lineages of the Nematode, *Caenorhabditis elegans*," Sulston and Horvitz included cell death as one differentiation process and stated their method as "direct observation of the divisions, migrations, deaths of individual cells in living nematodes" (Sulston and Horvitz 1977). These processes including cell death in *C. elegans* were seen as fitting into a "rigidly fixed program." In the slides that show the programmed cell death, single cells were shown as going through programmed cell death in the special context of other cells surrounding them (Figure 10). Although they pinpointed which particular individual cells went through cell death, they did not elaborate on the intracellular morphological changes, rather than stating that there were rapid changes of refractility of the cell nuclei

undergoing programmed cell death. The temporal contexts, or as the authors put it, the ancestral lineages of these cells that undergo programmed cell death were also clearly analyzed on charts (Figure 11). There were a number of occurrences of cell death in different developmental events. The authors noted that the ventral cord development involved an initial multiplication of nuclei, followed by a determined pattern of cell death in the posterior region. However, many programmed cell deaths that occurred in the male do not happen in the hermaphrodite development, which may mean that the cell deaths in the male used increased cell death as a "reprogramming" mechanism for development. They also observed that although the cell lineages in different individuals were mostly invariant, there were cases that the usually dying cells survived and remained in the adult worm body (Sulston and Horvitz 1977). As we will see, the systematic way of analyzing the detailed spatial and ancestral contexts of cells death pattern in the efforts of cell lineage mapping, and manipulating variances in cell death became foundational in Horvitz's later developments on cell death research.

The cell lineage map certainly confirmed as well as modified Brenner's earlier vision about a developmental program. Although the full cell lineage map did showed that indeed the cell divisions, migrations, cell death followed relatively fixed patterns, Sulston and Horvitz did find variance. More importantly, by comparing the developmental patterns of different structures in *C. elegans*, Sulston and Horvitz revealed "there is no standard patterns in generating groups of new cells" and the logic of development often tinkers the existing route of cell generation. The occurrences of cell death may help to eliminate intermediate cells and made the developmental results more fluid than the existing developmental patterns can offer. They wrote,

"The relationship between lineage history and developmental fate suggests that specific types of cells may arise only from an appropriate series of divisions of a particular type of blast cell. Hence, when only some progeny cells of a given lineage are needed, it may be necessary to generate and later destroy the other daughters. Such a mechanism provides one explanation for programmed cell death, common in both the nematode and other developmental system" (Sulston and Horvitz 1977).

Unlike Hamburger and Levi-Montalcini in the 1940s, Sulston and Horvitz were not surprised by the role of cell death in development. Two reasons probably explain this. First of all, many surprises emerged against conventional conception of development as Sulston and Horvitz were trying to trace every change in the developing worms. In the early 1970s, everyone versed in nematology believed they knew that the worm should been hatched with all the cells they would ever have. But Sulston proved it wrong by following the number of cells in the ventral nerve cord: there were fifteen in the first stage larvae but fifty-seven in the older animals. Horvitz and Sulston also found that the there were an odd amount of asymmetry in the developing *C. elegans* that cannot be explained easily. Cell death may have thus been among many other wonders Horvitz and Sulston encountered when they traced every element in the worm's cabinet of curiosity. Soraya de Chadarevian has pointed out that Brenner's view about a strictly coded developmental program transitioned into a vision developmental process shaped by molecular assembly and interaction. De Chadarevian regarded Brenner's shifting view as the reason that the cell lineage study gained primacy over time (de Chadarevian 1998). However, since the cell lineage study began as early as 1972, it may well be the cellular

oddities discovered in the cell lineage mapping project that catalyzed the transition of Brenner's view.

A more important reason that Sulston and Horvitz did not see cell death as an anomaly in development was that programmed cell death had by then become well known among developmental biologists. It had been discovered in a variety of developing tissues using multiple methods. Particularly in the late 1960s and 1970s, cell deaths that are apparently regulated and biological were conceptualized as a phenomenon important for research in developmental biology and pathology. Sulston and Horvitz had known about the phenomenon. In the 1977 paper, they quoted the review from Saunders published in 1966, which coined the term "programmed cell death," and used the term themselves (Saunders 1966b).

Studying Cell Death in Development in the 1970s

In order to understand the rising interest of studying the phenomenon of programmed cell death, as well as to distinguish in what ways Horvitz approached the problem differently, it is important to review a few developments in cell death research in around the 1970s. Since 1930s, various occurrences of programmed cell death were discovered, often as surprises, in a variety of developing scenario including vertebrate central neuron development, digit development, insect metamorphosis, and the development of tubal structures (See Chapter 1). In the 1960s and 1970s, as the findings of biological and regulated cell death phenomena accumulated, some scientists found it necessary to conceptualize cell death and develop further research. Two concepts were generated, "programmed cell death" given American insect physiologist Richard A. Lockshin, and "apoptosis," adopted initially by three pathologists working at the

University of Aberdeen, John Foxton Ross Kerr, Sir Alastair Robert Currie and Andrew H. Wyllie (Kerr, Wyllie, and Currie 1972, Lockshin and Williams 1964b).

Lockshin observed the way pupal muscle structures disappear after the completion of adult development in large American silkmoths and suggested the term "programmed cell death" in his 1964 report (Lockshin and Williams 1964). The term was later seen as a functional definition of the kind of cell death that served a role in development. A number of developmental biologists began to adopt the term and observe the pattern of programmed cell death in other developmental processes. American biologist John W. Saunders carried out research to map out patterns of cell death in chick wing development. He also did extensive transplantation experiments to test how cells die: whether they died by themselves or they died because of the environment (Saunders, Gasseling, and Saunders 1962). Cell death studies by developmental biologists such as Lockshin and Saunder, however, usually focused on the death of group of cells, and did not involve direct observation of individual cell death by the 1970s.

The other term "apoptosis," on the other hand, was generated form the detailed observations of individual cell death regardless of the cells' surroundings. When three pathologists working at the University of Aberdeen initially discovered the phenomena in stressed or injured liver tissues and chemically exposed adrenal cortices in the 1970s, they were intrigued and used electron microscopy to reconstruct the morphological changes in detail. Apoptosis, as a result, was later seen as a morphological definition

based on the regular microscopic changes that lead to cell death.[33] Three pathologists realized that apoptosis-like cell death had been observed in cancer regressions after suppressive therapy and noted that apoptosis may be related to an orgnism's own protective reactions against cancer. They also became aware that some cell death involved in development also went through the same apoptotic process and realized that cell death might be a biologically conservative mechanism to achieve multiple purposes in development and diseases. In the classic 1972 paper, they framed apoptosis as a basic process for maintaining tissue kinetics and called for further extensive research on the phenomenon (Kerr, Wyllie, and Currie 1972). Yet the three pathologists highly focused on the step-by-step intracellular changes during the cell death and did not address questions about the environments of these cells: where did these cells come from, what set them apart from living cells, do they die to fulfill any biological purpose, and do they need assistance to die or do they die by themselves?

The invention of terms "programmed cell death" and "apoptosis" also increased the traction of cell death as a research topic by assigning ontological coherence to a variety of cell death occurrences. Although cell death was being reported since the time when cell theory was formulated in the mid-nineteenth century, these findings were sporadic and often isolated from each other. Curious natural philosophers and biologists assigned the cell death they saw with a variety of names that could only give tenuous connections between these phenomena: "Coagulation necrosis," "autolysis," "pyknosis," "karyolysis," and "chromatolysis," for example. The overabundance of terms on an often

[33] Actually, the Greek word *apoptosis* describes the shedding of leaves from trees, or petals from flowers, and was suggested by James Cormack, a professor of Greek language at the University of Aberdeen.

neglected cellular phenomenon probably have prevented the formation of a critical mass that could make the phenomenon well known, let alone any aligned effort to investigate the phenomenon. (Majno and Joris 1995, Jiang 2012) In the 1970s, the publications and increasing adoptions of the terms of apoptosis and programmed cell death not only summarized the convergence of the increasing number of observations and discussions about cell death, but also in themselves phase-changing events. With clearly announced terms and their morphological and functional descriptions published by reputable scientists, study of cell death was no longer seen as scientifically questionable but as a new and promising subject.

Although Horvitz's group was credited as making the most important genetic study about programmed cell death, direct attempts to explain cell death through its molecular processes began with sophisticated biochemical testing early on. These testings sometimes solidified the active way in which apoptosis took place and which prior investigators surmised. In the mid-1960s, researchers showed that apoptosis, such as cell death in the tadpole tails, requires synthesis of new RNAs and proteins. This reconfirmed biochemically that apoptosis is a biologically active process, rather than a passive reaction to adverse environmental conditions (Tata 1966).

One important biochemical revelation in the 1970s, which eventually was included into standard characterization of apoptosis, was that the chromosomes within apoptotic cells break into segments composed of integral units of nucleosomes (about 200 bp DNA and 8 units of histone form a nucleosome). Therefore, the dying cells' DNAs can produce a ladder-like pattern when they are separated by mass and visualized by DNA dyes. This feature was first discovered in 1974, when three researchers at Harvard

University showed that human and rodent cells had characteristic patterns of DNA degradation when treated with agents that triggered apoptosis. The 1974 experiment separated the resulting DNA segments with an alkaline sucrose gradient technique, which could only give rough estimates of molecular weight (Williams, Little, and Shipley 1974). In 1980, Wyllie found that such DNA degradation patterns were associated with the simultaneous condensation of chromatin within the nucleus, a morphological change characteristic of apoptosis. In addition, Wyllie ran the DNA molecules with agarose gel electrophoresis that could measure molecular weight with higher resolution. He identified the difference between adjacent DNA segments on the gel as about 180 base pairs, which equals to the length of a nucleosome (Wyllie 1980). This showed that chromosomes break into nucleosomal fragments during apoptosis and suggested that apoptosis involves activation of endonuclease, a type of enzyme that cuts DNA molecules from within at specific sites. The pattern of DNA degradation that produces multiples of 180-200 bp was later known as the "DNA ladder," the first widely used biochemical marker for detecting apoptotic cells (Figure 12).

During the early 1970s, when these elegant molecular characterizations about cell death were getting published, Sulston and Horvitz were still peering through the microscope to follow and record every cellular change during the *C. elegans* development. Although they shared Brenner's dream of revealing the genetic and molecular secrets of development and behavior eventually, their interest in studying cell death with molecular terms was at most indirect, if it was there at all. Yet, the subject of cell death study accumulated enough knowledge to have any attentive researcher of developmental biology know about the phenomenon and have adopted methods from

biochemical study of the cell to produce interesting molecular results. As the LMB continued to cultivate new PhDs and postdoctoral researchers who went to other places to establish their own research programs about some aspects of the worm, the areas of study surrounding the worms were also branching out. Leaving LMB for an assistant professor position at Massachusetts Institute of Technology in 1978, Horvitz was planning to finish the remaining projects at the new post. He was also envisioning something different from the mapping enterprise, something that may fulfill his initial intent of revealing life's inner secrets. Eventually, genetic study of cell death became one new focus of his MIT program.

Manipulating Cell Lineage: Studying Cell Death through Mutations and Altered Intercellular Relations

In late 1977, Horvitz went on a series of research trips to several universities in the US and accepted a job offer of assistant professor from MIT. In January 1978, he arrived in Boston with more than 500 strains of *C. elegans* mutants, most of which had abnormal cell lineages. He planned to study the genetics of these abnormal cell lineages, and also to develop his interest in cell death. At this point, Horvitz still had some remaining work to do for refining the cell lineage map. But it became clear to both Sulston and Horvitz that they needed to go beyond their work of cell lineage mapping, and the future projects probably should be related to genetics. Sulston eventually developed a series of genetic mapping projects with Alan Coulson, a researcher from Frederick Sanger's laboratory at LMB, continuing his long career in mapping some biological features in the most comprehensive way. Horvitz instead decided to make use of the cell lineage map to reveal genetics responsible for development.

Horvitz's vision of using cell lineage and its manipulation to study developmental genetics began when he was in the middle of mapping the developmental cell lineage. Brenner had started his study of *C. elegans* by characterizing a number of mutants abnormal in behavior and development and hoped that these mutants would eventually be characterized genetically to establish the genetic basis of behavior and development in the worm. Yet for some time it was unclear whether any change of single gene would lead to altered cell lineage or not, since most Brenner's mutants were characterized by defects in movement. Horvitz thought it made sense to prove that genetic mutations can change development by creating mutant worms that are abnormal in cell lineage. Then the genetic characterization of these cell lineage mutants can become the basis of studying developmental genetics in *C. elegans*. In the mid-1970s, John White, a graduate student at LMB studying the neural development in *C. elegans* invented a laser beam technique that could target and eliminate a certain cell with great precision. White and Nichol Thompson used the laser system to trace which original neuron other neurons came from: if they destroyed one original neuron using the laser beam, whichever neurons ended up missing must originally derive from the destroyed neuron. Horvitz himself also used White's laser system, but suggested the creating mutants in cell lineage should "complement the laser system." He also imagined that such mutants might have an altered program in development, thus revealing the "logic of development." On March 19, 1976, Horvitz proposed the agenda of creating cell lineage mutants in the famous "tea talk" of Brenner's laboratory, an occasion when ideas and criticisms were freely exchanged (Horvitz 2003b).

At MIT, Horvitz finished the cell lineage mapping and published his last publication completely descriptive of cell lineage in *C. elegan* in 1980. Establishing the laboratory in MIT, Horvitz started to explore a variety of problems related to genetics and evolution of *C. elegans*, publishing papers with others about comparative cell linage study between *C. elegans* and closely related species, genetic mapping, genetic characterization of behavioral mutants, cell signaling in *C. elegans*, and about technical development that can manipulate the worm through the laser microbeam in more precise ways. With the arrival of the laboratory technician Nancy Tsung and graduate students later in the year, Horvitz's laboratory started molecular study of functions on various aspects, ranging from developmental timing, muscle formation, the egg-laying behavior, and programmed cell death. This seemingly disarray of research programs loosely linked with genetic study about biological function reminded people of the many programming and mapping projects in Brenner's own laboratory. Actually, each of Horvitz's new focuses was directly or indirectly connected to a particular project or mutant worm originated in LMB. During this period, Horvitz seemed to be increasingly schooled in the classical embryology and contemporary trends in developmental biology, citing works from others about development and differentiation broadly. The success in identifying and genetically characterizing the cell lineage mutants also showed that certain cell lineage in *C. elegans* was indeed related to genetics, and that studying the genetic control of cell differentiation as Horvitz imagined in 1976 was possible.

In the 1980 paper, Horvitz stated, "the relationship between genes and development is unknown." He noted that one important way of identifying the relationships were through mutations that could "result in a switch from one fate to

another." He cited the homeotic mutations that trigger generations of structures at abnormal locations and transformer mutations that alter sexual development as examples. Horvitz saw his own work as following these lines in identifying genetic components that makes a difference in the route of cell lineage. Because the cell lineage in *C. elegans* is invariant, if there is significant change in the route of cell divisions and differentiations in mutagenesis experiments, the mutants must have alleles responsible for certain cell lineage process altered to generate the effects. In the 1980 paper, Horvitz asks whether it is possible to isolate mutants altered in a specific cell division or set of cell divisions. If so, how many genes are involved in each cell division? Other questions include: In how many cell divisions is each such gene involved? What other features are common to that set of cell divisions affected by a given gene? Can mutants be isolated in which cell fates are transformed so that a cell follows a lineage that is normally that of another cell?

Horvitz and Sulston isolated 24 cell lineage mutants by picking the worms that had abnormal structures observed with Fulgeon stain, or through their abnormal egg-laying behaviors. They carried out the tedious work of mapping these mutants into complement group as well and identified 14 genes that were responsible to these 24 cell lineage mutants. They found that to look for abnormalities in egg-laying behaviors was especially fruitful in identifying cell lineage mutants. Because a lot of the post-embryonic cell divisions of *C. elegans* hermaphrodites were involved in vulva development, and abnormalities in vulva development often result in aberrant morphologies and egg-laying behaviors. Because they characterized both the phenotypes – exactly which parts of cell lineages were affected and the approximate locations of the genetic components that were responsible, Sulston and Horvitz could identify whether different cell lineage were

affected by different genes. It turned out different cell lineages did involve different sets of genes. In addition, they figured there were genes that had reverse effects on cell divisions. They identified both the genetic components that once mutated, caused excessive ventral hypodermal cell division, and mutations that prevented these cell divisions. They was pleased by the "ease of isolation of these mutants" that had abnormal vulval development and considered it was possible to "characterize all genes specifically involved in vulval cell divisions" (Horvitz and Sulston 1980).

Using a combination of classical mutagenesis methods, direct observation of structures and cell lineage in *C. elegans*, and classical complementation experiments to map gene linkage, in the early 1980s, Horvitz confirmed that development in *C. elegans* could indeed be studied genetically. The key was to align the linkage location of the mutated genes with the phenotypical abnormalities.

As Horvitz closely examined the altered cell lineages in the mutants, he started to pay close attention to the logic of cell fate determination as reflected by the altered cell lineages and the relations between cell fate determination and particular cell lineages and to evolution. First, Horvitz and his collaborators paid special attention to mutants that generated "reiterations" of sublineages that did not happen in the wild-type. In the circumstances that repeats of certain cell division pattern, which the authors called "reiterations of a sublineage," replaced normal cell division and differentiation, the authors found that the cell types that usually resulted from the sublineage would be produced instead of the cells usually found at the same location in the wild-type. Horvitz regarded the finding as showing that for generating particular cell type, a particular sublinage, a rigid sequence of cell divisions, might be necessary. He also pondered that

such sublineages might be the units that development can utilize by modifying, repeating, and eliminating them, so that "genes need not control development on a division-by-division basis," but to "instruct" reiterations and other units for development (Chalfie, Horvitz, and Sulston 1981).

A further excursion to evolutionary study of cell lineage took place when Horvitz and Paul W. Sternberg, a PhD student at Horvitz's laboratory compared the designs of cell lineages between *C. elegans* and its closely related species *Panagrellus redivivus*, also commonly called sour paste nematode, and investigated how the cell lineages in nematodes were evolved. Particularly, they encountered cell deaths that happen in *C. elegans,* but not in *P. redivivus,* or otherwise. One instance of interest was that female *P. redivivus* only developed one vulva instead of two as in *C. elegans*, because one of the two progenitor cells of the vulva in the former went through programmed cell death early on and did not develop into any structure. Horvitz and Sternberg considered this instance of cell death that resulted in a huge difference between the reproductive organs of the two species especially instructive about how patterns of development changed during evolution. As an analogy to the concept of regulatory genes, they called such cell whose fate of survival or death incurred significant impact in the final architecture of the organism "regulatory cell" (Sternberg and Horvitz 1981).

Indeed, Horvitz's explorations branched out to many issues regarding development, evolution, and genetics of *C. elegans* in the early 1980s. One reason behind this expansion of interest was that the field of *C. elegans* research had accumulated important basics about the worm as well as trained researchers. The formation of network of researchers focusing on the *C. elegans* research has attracted historians' attentions. It

seemed that the level of open communication and shared knowledge, as shown by the early exchange of information through the newsletter *The Worm Breeder's Gazette* and later the wormbase.com, was unmatched by other communities of researchers in life sciences. More importantly, at this time, Horvitz could afford to branch out to explore a great variety of problems also because his own laboratory had enrolled new postdoctoral researchers, graduate students, and laboratory technicians such as Sternberg, Nancy Tseng to help tackle around on the frontiers. He could draw on published and unpublished observations from other colleagues and synthesize them to answer a question with the richness of information that may not be available in other field.

The paper "Programmed Cell Death in Nematode Development" published one year after Horvitz's musing about "regulatory cells" was exactly the kind of publication that synthesized many worker's results and visions. In the paper, Sternberg, Horvitz, and Hilary Ellis reviewed the cell deaths discovered so far in *C. elegans* and other nematode species, discussed why they die, while reporting mutants that changed all or some occurrences of cell deaths in *C. elegans*. They reviewed the morphological consistency of programmed cell death found in both *C. elegans* and *P. redivivus* with the number of occurrences of cell death in different tissues. But the emphasis was on the developmental and evolutionary role of cell death. The authors summarized four reasons why cells die in nematodes, with convincing evidence of cell death discovered by themselves or others to support the argument. For example, they argued that some cells that went through programmed cell death because it was a necessary intermediate step in a predetermined path of cell sublineage using the example from the nervous system development in *C. elegans*. To make the point, the authors compared the cell lineages from 12 neuroblasts in

the ventral nervous system. There are five distinct classes of motoneurons generated in the ventral cord, VA, VB, VC, AS, VD. In the body sections except for the vulva, cells that were due to become VB and VC die. The authors reasoned that the final structure of the ventral nervous system did not need many VB and VC cells. However, to generate other types of neuron cells, VB and VC had to be generated first. That's why they are generated and eventually eliminated. In a similar fashion and with concrete instances from *C. elegans* cell lineage, they also assigned programmed cell death with functions such as "to eliminate cells that function transiently," "to define morphogenesis," and "as a secondary consequence of the genetic programming utilized to effect other cell deaths" (Horvitz, Ellis, and Sternberg 1982).

In the 1982 paper, the discussion about why cells die was more analytical and experimental than the conjectures previously given by other scientists to explain cell death. Although previous researchers already suggested that cell death may be a way to eliminate unneeded cells during organ development or insect metamorphosis, they were not reasoned as such based on particular cell history, but were based on where these dying cells located in the defined area of organs and tissues. Since Horvitz and the others studying cell linage traced individual cell developmental history, when Horvitz started to reason about the function of cell death, he used the relations between the dying cell and existing cell as a portal to analyze why cells die.

Horvitz also pondered upon the question whether cell death is suicide or murder, a question that embryologists such as John Saunders had also raised. According to Horvitz, suicide involved factors intrinsic to the dying cell, while the murder involved factors extrinsic to the dying cell. This metaphorical relationship was examined using a modern

variation of classical experimental embryology. Using John White's laser microbeam, these researchers removed adjacent cells of the dying cells and test whether the removal of adjacent cells will change the fate of dying cells. For most cases, they found that the programmed cell death was suicide indeed. This seemed to offer a confirmation that cell death is an autonomous cell fate, just like cell division, migration, and differentiation (Horvitz, Ellis, and Sternberg 1982).

During the late 1970s and early 1980s, Horvitz expanded his research group into many biological problems, many of which had glimpses of cell death as one piece of the problem, no matter whether it was cell lineage mutants, differences of cell lineage in between species of nematodes, or nervous system development. During this period, Horvitz's view about cell death also evolved from one that put cell death in the bigger picture of historical cell lineage, to one that put cell death into evolutionary history, developmental logic, and its surrounding cells. Horvitz had always aimed to study biology through genetics. Although cell death is a common phenomenon, only through mutants that are abnormal in cell death patterns scientists could identify the mutated genes that are responsible for cell death. By 1980, Sulston and E. Hedgecock had already discovered mutants that led to cell death. They found *nuc-1* mutant that prevents DNA degradation, and *ced-1* and *ced-2* mutants that eliminate any cellular engulfment. Both DNA degradation and cellular engulfment are essential processes to complete most programmed cell death. However, it seemed these mutants only touched upon the peripheries of cell death – the involved genetic components seem to fulfill the requirements of cell death, but were unlikely to initiate death at first. After all, some cell death do not require engulfment, and DNA degradation is the downstream effect of many

early events of programmed cell death. Horvitz was aware of these problems and had been hunting for a mutant that more fundamentally affects cell death since the establishment of the MIT laboratory.

Hunting Down the "Undead"

Screening for a new cell death mutant proved to be a challenging task. Cell deaths in *C. elegans* were ephemeral phenomena – they happen so fast that researchers found it hard to catch them or their aberrant patterns or absences through Normaski optics alone. In order to search for cell death mutants, Horvitz tried to by-pass the direct observations of altered cell death patterns, and used existing mutants to preserve the death anomalies. He initially used the *nuc-1* mutant to generate further mutations, because he reasoned that the failure of degrading dying cells' DNA content would preserve the cell corpses at the original location, which researchers could trace to detect abnormal presence or absence of cell death. However, all the mutants generated from *nuc-1* seemed to be related with other differentiation patterns, which disrupted cell death, but do not directly control cell death itself.

Then Horvitz turned to the mutants of *ced-1* and *ced-2*. Since both mutants could not engulf the cell corpses after they went through the cell death process, the cell debris would remain and allow researchers to recognize the death patterns using Nomarski optics. This also enabled researchers to observe live animals instead of staining the specimen's DNA which involved killing the worm beforehand as it was the case in using *nuc-1* mutant. Graduate student Hilary Ellis at the laboratory took the task of screening for mutants from *ced-1*. She performed standard EMS (ethyl methanesulfonate) mutagenesis experiments and spotted for unusual presence of cell debris in live animals.

She successfully found a mutant that all cells that were due to cell death survived. The mutant was termed *ced-3* (Horvitz 2003b). Since no cell death activity was observed in *ced-3*, Horvitz and others in the lab became convinced that *ced-3* was one of the homeotic genes that act to initiate a particular cell fate, in this case the fate of programmed cell death.

Around the mid-1980s, aided with many graduate students and technicians, Horvitz's lab continued to research on a variety of questions about *C. elegans* development including developmental timing, muscle formation, the egg-laying behavior, and certainly, programmed cell death (Horvitz et al. 1983). This was also the time when recombinant DNA technology and other powerful tools in biotechnology provided researchers the power to clone genes and sequence them, and to manipulate genetics of the cells. In addition, many databases were being built with collections of protein and DNA sequences. With the seemingly disarray of research programs and a large number of techniques and approaches to choose from for studying about *C. elegans* development, it did not make sense for Hovitz to follow a line of phenomena to document them completely using a single technique anymore. Amid the myriad of combinations between techniques, approaches, and manipulated organisms and altered phenomena, the practice of doing developmental biology and its genetics relied on effectively finding the combination of a technique, an alteration, and a question to ask that was technically feasible and at the same time, was able to reach important clues about molecular mechanism.

After finding several crucial mutants in cell lineage and programmed cell death, Horvitz's laboratory produced work based on exactly these combinations. Their research

was not always successful. In fact, they learned from trials and errors and often only able to answer the original questions after digressive exploration of other problems. With increasing number of researchers working on programmed cell death in Horvitz's laboratory with expanding technologies, it became harder to capture the full story with multiple lines of investigations and technological developments after the mid-1980s. Instead, I highlight the important roles of emerging technologies and the transforming effects the protein sequence homology searching tools offered to Horvitz's programmed cell death research and discuss what exactly was different of the molecular developmental biology from the logic of classical experimental embryology in this case.

Investigative Broadness, Biotechnologies, and Databases

In the late 1980s and early 1990s, Horvitz's laboratory produced spectacular results that elucidated the molecular processes of programmed cell death in *C. elegans* and its potential relations to programmed cell death in mammals including humans. Two other graduate students, Carol Trent and Ron Ellis, had found two more crucial mutants impacting cell death, leading to the discovery of two other genes in the network of cell death regulation *ced-4* and *ced-9*. During the period, physical map of the worm's genome was also constructed, which aided the fast localization, cloning and sequencing of particular genes of these mutants. By the early 1990s, Horvitz was not only able to piece out a network of genes related to cell death regulation, he also grasped a great deal of how proteins from these genetic components function and lead to cell death.

The investigations of a variety of other problems, although seeming to have no relations to programmed cell death ostensively, in the end produced surprising results about the topic. As described in the earlier parts of the chapter, the mapping of cell

lineage, comparisons between cell lineage in different species of nematodes, searching for cell lineage mutants all helped to reveal certain aspects of cell death in *C. elegans*. During the 1980s, parallel research about other research questions at Horvitz's laboratory continued to produce relevant knowledge or research material for cell death study. One of the cell death mutants *egl-1* was not discovered through direct screening for abnormal patterns in cell death, but resulted from investigations of egg-laying behavior: when graduate student Carol Trent and technician Nancy Tsung were characterizing a set of 145 mutants that were abnormal in how they lay eggs, they found that one of them lacked HSN motor neurons that innervate the vulval muscles, because the HSN motor neurons went through a cell death that does not happen in normal vulva (Trent, Tsung, and Horvitz 1983).

It is undeniable that the enlarging toolbox for studying *C. elegans* was essential for many research outcomes from Horvitz's laboratory. The experimental powers of manipulating cells and genes increased considerably in the 1980s as the research community on the worm expanded and as molecular biotechnology took off in the late 1970s. These tools sometimes provided an alternative, often more determinate way to test hypotheses that were previously confirmed through indirect evidence. Other times, these tools provide new information, such as elucidating the full sequences of novel genes after cloning them.

Horvitz had asked the question whether cell death was internally determined by the cell's own contents, or influenced by the neighboring cells. He initially used the laser microbeam to destroy the neighboring cells and tested the effect on cell death. He concluded that most programmed cell deaths were autonomous because for most cases

the absence of the neighboring cells did not influence the occurrence of cell death. With the molecular tools, Horvitz's graduate student, Junying Yuan tried to reinvestigate the question. This time, the question was asked more specifically about the death genes that the lab had found: did the death genes such as *ced-3* and *ced-4* produce gene product and function inside the dying cells, or do they produce external factors from elsewhere to influence the cells, such as hormonal factors? Yuan decided to perform a mosaic analysis to elucidate these questions. The *C. elegans* genetic technology guru Robert K. Herman, who had worked at the LMB and in the early 1980s went to University of Minnesota, designed a method for combining cells of different genetic composition into a single worm, which was commonly called mosaic analysis in 1984 (Herman 1984). Such mosaic analysis had been available to *Drosophila* researchers to determine the locations where particular genes function in the body of fruit flies for a long time. By introducing a chromosome that was randomly duplicated into different cells, *C. elegans* can be made with a mosaic of cells that have certain genes and cells that do not. The method was highly valued among *C. elegans* researchers. For this and other tools Herman developed, he was awarded the George W. Beadle Medal in 2007 by the Genetics Society of America.

Yuan utilized Herman's genetic mosaic analysis method and introduced *ced-3*, or *ced-4*, into the original *ced-3* or *-4* mutants. The original animals were dysfunctional in producing normal cell death. When the normal *ced-3* or *-4* was randomly introduced, Yuan observed that for each animal, all the normal cell deaths strikingly came from a single cell lineage. This meant that only the lineage that got introduced the normal *ced* genes could die properly and that cell death was indeed autonomous processes controlled

internally by *ced-3* and *-4* (Yuan and Horvitz 1990). Although Horvitz had used a laser microbeam to manipulate the dying cell's adjacent cells to test the influence of the environment and concluded along similar lines, the mosaic analysis offered tools to change the internal genetic contents to powerfully reconfirm the hypothesis. The question was not new, yet level of manipulation on the DNA level created great confidence, and consolidated research attentions towards the genes in researching cell death.

Genes are not just any factors in biology. They embody biological information that can be extracted, read, and analyzed by researchers, though still with great amount of tedious work in the 1980s. After the *unc-54,* the first gene in *C. elegans* to be cloned in 1981, researchers in the *C. elegans* research community developed a variety of gene cloning technology for the worm and became increasingly excited about the prospects of using these technologies to isolate individual genes and to eventually mapping out the whole genome sequence (MacLeod, Karn, and Brenner 1981). Particularly, in the 1990s, Yuan and others at Horvitz's laboratory discovered a transposon Tc4 within the DNA of *C. elegans* that could aid the rearrangement of its adjacent genes (Yuan et al. 1991). The Tc4 provided great power to Yuan in rearranging the gene *ced-4* with other molecular vectors. With the aid of Tc4 and DNA probes developed by Sulson and Coulson while the two were establishing the physical map of the genome of *C. elegans*, Yuan soon cloned and sequenced *ced-4*. With the sequence, Yuan tried to find homologies between Ced-4 protein and other known protein sequences. She searched the inferred protein sequence of Ced-4 on the protein bank databases, including the NBRF/PIR Protein Database, Pasteur Institute Protein Databases, and GenBank, and found no homologies from the existing sequences (Yuan and Horvitz 1992).

The lack of homology to *ced-4* was somewhat disappointing, because if molecules with known functions and in other organisms had significant homology to any death genes, it would not only save much guesswork from further biochemical testings, but also point to the great span of relevance of the cell death research in other organisms and for other problems. Horvitz had been interested in neurobiology and the pathology of nervous system. He had read the 1972 paper published by Kerr, Wyllie and Currie and had known that cell death might be involved in atrophies of organs and tumor regressions. He had also done research in muscle development in *C. elegans* that the stellar cloning and sequencing of *unc-54* gene became the basis of obtaining the first myosin heavy-chain sequence, as well as the basis of studying homologous myosins in other species including those in humans.

The first revelation between genes involved in cell death and other genes happened in February 1992. Michael Hengartner, a graduate student at Horvitz's laboratory was searching with databases about the homologies of the gene *ced-9* that he discovered, cloned, and sequenced. *ced-9* had brought in surprises because unlike all previously found genes that were related to cell death, product of *ced-9* did not initiate or execute programmed cell death. Its expression actually works to prevent cell death and is a negative regulator. Hengartner also found that a lot of neurons did not go through programmed cell death because they had a basal level of expression of *ced-9* (Hengartner, Ellis, and Horvitz 1992). The research group soon incorporated the *ced-9* gene into the model of cell death regulation, with executors, killers, and protectors such as *ced-9* (Figure 13). In early 1992, Hengartner found that CED-9 and Bcl-2 protein (B cell lymphoma) are homologues. Others had shown that misexpression of Bcl-2 protein can

cause follicular lymphoma in humans, and became intensely interested in the biomedical research about *bcl-2*, a recognized proto-oncogene in order to develop effective therapies (Vaux, Cory, and Adams 1988, Strasser et al. 1991).

Hengartner promptly sent a fax to Horvitz informing him about the discovery of this homology, a crucial link to show that the study of cell death in worms was valuable knowledge to human oncology. In reminiscence during the Nobel Prize lecture, Horvitz himself highlighted this discovery of homology as the major event that led the research group to the "path to the scientific visibility." Horvitz regarded the event as one that "made the worm cell-death pathway suddenly a topic of major interest in the biomedical community, as this pathway was no longer simply an abstract formalism derived from complicated genetic studies of a microscopic soil-dwelling roundworm but rather a framework for a process fundamental to human biology and human disease" (Horvitz 2003b).

Finding homologous molecules through the database was so crucial a step for making the molecular study of cell death more meaningful, so that when the *ced-3* was cloned and sequenced, researchers persevered in finding the homologous proteins from the expanding protein databases in the early 1990s even if the initial rounds of search yielded no result. Graduate student Shai Shaham kept searching in the databases for proteins that are homologous to CED-3 about every other day for two years, until he eventually hit the protease interleukin-1-beta converting enzyme, ICE, in one database in April 1992 (Yuan et al. 1993). Since many of ICE's biochemical properties were known, biochemical studies on CED-3 was accelerated, soon to reveal that CED-3 was an cysteine protease. Since ICE was also known to be related to human inflammations, two

drug companies called Horvitz during the day that the results were published, increasing the cell death study even further.

The results yielded from searching the protein databases for homologous molecules to cell death genes suggested potential functions of genes, linked cell death genes in *C. elegans* to other cell activities in other organisms, and increased the visibility of Horvitz's group as well as molecular study of cell death as a whole. The availability of these protein databases did not follow directly the technological advancements that deciphered protein and DNA sequences, but required continuing organizations, collections, and distributions to build and maintain the infrastructure. As historian Bruno Strasser pointed out in a history of the building of Genbank, the establishment of databases often involved extensive discussions about the "moral economies" about contribution, credit, and access. In addition, Strasser pointed out that "databases, like earlier natural history collections, are not mere repositories; they are tools for producing knowledge." The vast amount of sequences drawn from databases from an extraordinary number of organisms facilitated the resurgence of comparative biochemistry that tries to understand biochemical functions of molecules and their evolution through evolutionary history and systematic relationship of organisms (Strasser 2011). UniProt, as an heir to the protein sequence database, Margaret Dayhoff's Atlas of Protein Sequence and Structure, was established in 1992 and formed a database of protein sequence so that researchers could learn about the homological relations between molecules that they were interested in and molecules with known functions.

The rise of the status of *ced-3*, *-4*, and *-9* in terms of scientific importance confirmed Strasser's view. It also highlighted the role of databases in biomedical

development in two additional ways. First, homologies revealed from algorithm written according to mathematical rules of molecular evolution could reveal the importance of certain genes or proteins discovered in organisms originally deemed less important in biomedicine. This could effectively boost a particular study of one certain organism to higher visibility. Second, studying molecules of certain biological function reorganized the subjects under investigation from focusing on a single organism to studying molecular mechanism of a specific life process in a more universal manner: homologues expanded the horizon of the question at hand from one species to a broader range. As a result, a cluster of molecules responsible for cell death began to be studied along with clusters of molecules from other organisms involved in related life processes, strengthening cell death research as a research field by itself.

Cell death research indeed expanded and intensified in the early 1990s, when a number of genes related to cell death were discovered or confirmed, and when they were connected through databases and shown to be important in regulating cell physiology and cell division in normal conditions as well as in a variety of diseases. Not only Horvitz and other cell death researchers started to discuss about cancer, cancer researchers also started to pay attention to cell death research. David L. Vaux, an expert on *bcl-2* proto-oncogene at Stanford University, tried to express the human *bcl-2* gene in *C. elegans* and test whether it would prevent cell death in the worm. It did. His research that showed the mutated human genes could block cell death in the worm not only confirmed that *bcl-2* caused cancer in humans through blocking cell death mechanism, but also accentuate the importance of cell death research as a whole (Vaux, Weissman, and Kim 1992).

As other genes and proteins, such as Rb and p53 protein were tested to be related to both oncogenesis and normal cell death processes in similar fashion to *bcl-2* and *ced-9* (See Chapter 2), cell death researchers started to gather together to build the field. p53 was dubbed the molecule of the year in 1992; the International Cell Death Society was founded in 1995; and the journal *Cell Death & Differentiation* was established in 1997, for which both Horvitz and Vaux were on the editorial board. By the late 1990s, cell death research as a field was well-recognized, and was considered to have a long history but suffered form a "late recognition effect" (Garfield and Melino 1997). It was amusing to note that the previous discovery of cell death morphology also attempted to connect different organisms and research fields together to co-investigate cell death, but failed (Kerr, Wyllie, and Currie 1972). Identified molecules from previous research, such as *ced-9* and *bcl-2* nevertheless capitalized on the previously hinted importance of phenomena of cell death and gained popularities.

Discussion and Conclusion

During the 2002 Nobel Prize ceremony, the three laureates' lectures struck the audience with contrasting yet complementing styles. Sydney Brenner put forward yet another visionary and humorous account of *C. elegans* as "nature's gift to science," while John Sulston was cautiously and meticulously telling the audience yet another comprehensive project of him about sequencing the genome, simply because the work was there to be done. Horvitz seemed to be somewhere in between these two styles. While always aiming to reveal some aspects of "nature's secret" and to study a variety of questions, he also talked a great deal about technological maneuvers, temporary trials and failures, and research surprises. Horvitz's was an eclectic style, which might be related to

the way the molecular study of cell death was done: collect knowledge about cell death at different biological levels and piece together details to a larger picture.

Horvitz described the knowledge at different levels on cell death in a metaphorical language during the Nobel Prize lecture. Every cell was supposed to have a fate, they can be "wild type," "retarded," or "precocious." The genes related to cell death can be "killers," "protectors," or "executors." As he emphasized the cell death as an important and interesting phenomenon, Horvitz made it clear the research involved roundabouts as much as direct observations, and even more of cataloguing and mapping work than hypothesis-driven experimentation. In this section of the chapter, I discuss the importance of the cell lineage study and the emergence of molecular databases in elucidating the developmental and molecular regularities of cell death, then the effects of molecular studies of cell death to practices about the cell in recent years.

The now spectacular history of biological study of *C. elegans* is featured with a number of important projects that simply aimed to give a complete description of a developmental process or the hereditary material: the account of neural development, embryonic and post-embryonic cell lineage, the construction of the linkage map, physical map, and the full sequence of the genome all fell into this category of mapping practice (de Chadarevian 2004). These mapping enterprises created knowledge embedded in rich contextual information, such as the series of precursor cells before a particular cell differentiation or the adjacent sequence markers of a particular gene. These contextual information structuralized and organized observations and phenomena individually observed so that the experimenter had a larger range of maneuvers and perspectives to consider when investigating one particular process.

In the case of Horvitz's study of programmed cell death in *C. elegans*, the cell lineage map as the first step towards elucidating the molecular mechanisms of cell death enabled Horvitz to target and destroy adjacent cells, precursor cells, or to tamper with the genome to produce abnormal cell death patterns. By comparing the few repetitive sublineage patterns that incurred cell death in certain organ development but not other organs, Horvitz also gained a comparative perspective about speculating about how programmed cell death was needed. The full knowledge of cell lineage also helped Horvitz find cell lineage mutants, some of which were involved in cell death and added to the variety of research materials that contributed to the cell death study. More importantly, the cell lineage mapping in *C. elegans* traced development through single individual cells, not groups of cells with blurred boundaries. This made the experimental operation precise, and made the interpretation about a predetermined program of cell death convincing.

Much of Horvitz's maneuvers of *C. elegans* during the 1970s and early 1980s, however, still followed classical methods of embryology and genetics. The laser microbeam method that removes precursor or adjacent cells of the dying cells follow the same logic of extirpation method in classical experimental embryology Hamburger used: to remove part of the environment of the developing structure so that the influence of interactions can be revealed. The use of mutagenesis to study genetics as well as mapping the genome with a linkage map were established practices in studying of the fruit fly genetics for a long time. Although the cloning and sequencing technology led the research into further precision, the difference provided by these new technologies did not change the fundamental logic of the ways to go about biological problems.

There were, however, two major differences that set Horvitz's study of programmed cell death apart from the prior studies of cell death and classical ways of studying genetics. First, the focus and precision on changes of individual cells in their historical contexts offered by the cell lineage map provided researchers with cellular handles to manipulate the organisms. Horvitz's group did not need to worry that the effects of manipulation would blur the slight change of numbers of cells that were hard to measure, as was the case in Hamburger's chick embryos. When the manipulation of cell death was narrowed down to specific individual cells, the epistemic space of cell death had less noise to burden the researcher's attentions. Especially, *C. elegans* was one of the rare organisms that followed invariant paths of cell divisions, differentiations, and deaths in its ontogenesis and provided a premier experimental organism for cell death research.

Second, the greatest acceleration and empowerment that new molecular techniques and technologies provided for Horvitz's study of cell death seemed to be the quick links between homologous molecules through searching sequences at hand on DNA and protein databases. The homologies between cell death genes in *C. elegans* development and other genes found to be related to cell growth and its regulation, DNA degradation, inflammation, degenerative cell death, and cancer suggested biochemical functions, evolutionary path, and relevance to biomedicine of these novel genes found in Horvitz's laboratory. These online tools and databases were not available to earlier geneticists and embryologists. In the homology-searching process, individual sequences of genes and proteins served as connections between related life processes, cell activities, and different diseases. They are concrete key phrases that made the connections between them seem certain, clear, and consolidated.

As Horvitz's research linked proteins and genes involved in cell death in *C. elegans* to diseases in human, researchers from other fields started to investigate cell death by illuminating more molecular details, or by trying to establish therapeutics that suppress too much cell death in degenerative disease or increase cell death in cancers. As increasing molecular details about cell death accumulated, molecular pathways started to define cell death. The current molecular interpretation depicts apoptosis and programmed cell death as involving a cascade of energy-dependent cellular signaling pathways and activations of caspases. Researchers continued to study cell death through its regularity in terms of molecules as Horvitz demonstrated. However, Horvitz's focus on the biological regularity of cell death, his attention to the developmental patterns of cell death that made his research particularly successful, seems not to be as influential or prized as the molecular aspect of his research. I elaborate this point and other implications of the history of cell degeneration research in the next chapter to conclude the dissertation.

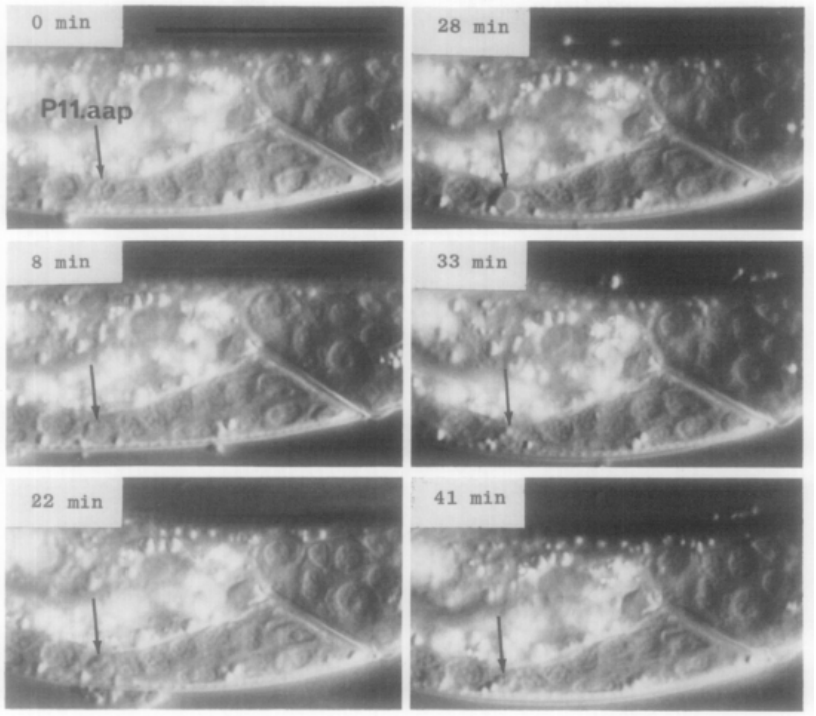


Figure 10. Cell Death with the Contexts of Time, Location, and Adjacent Cells under the Nomarski Optics. (Sulston and Horvitz 1977)

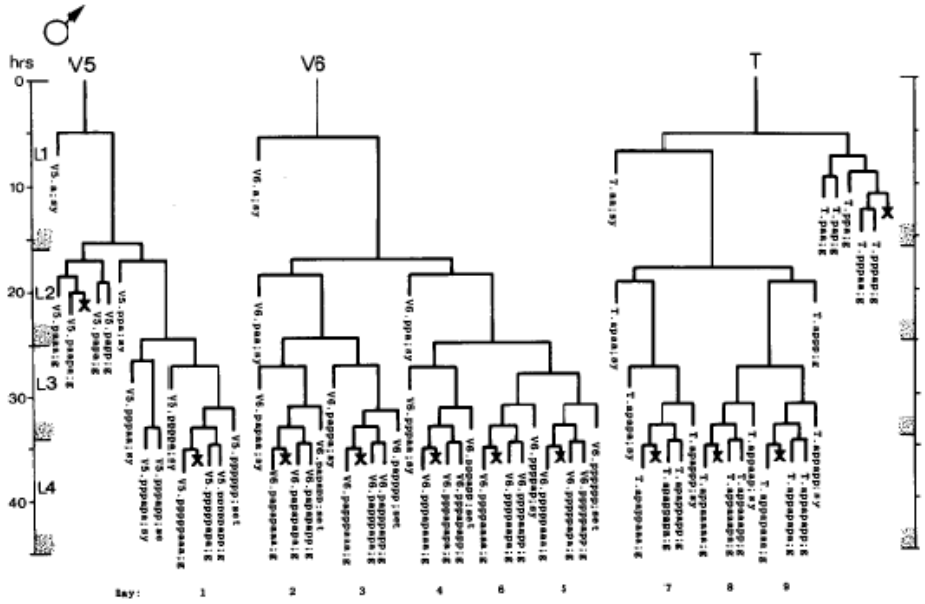


Figure 11. Cell Deaths in the Partial Cell Lineage Map of the Male *C. elegans*. (Sulston and Horvitz 1977)

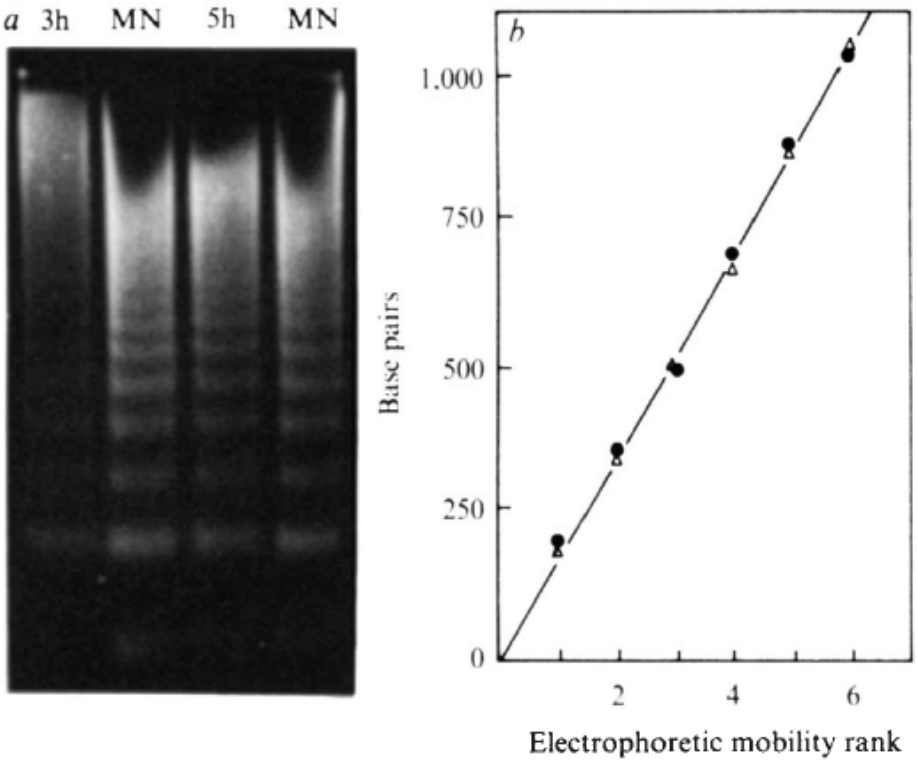


Figure 12. The "DNA ladder" characteristic of apoptosis and most programmed cell death. (Wyllie 1980)

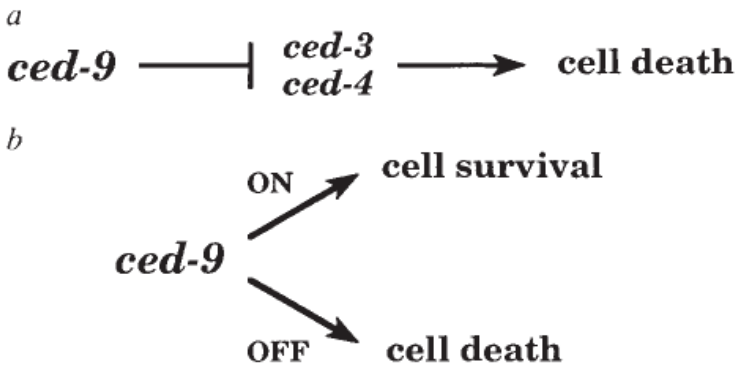


Figure 13. Depictions of *ced-9* as a Cell Death Protector. (Hengartner, Ellis, and Horvitz 1992)

Conclusion

In 2009, three American scientists were awarded the Nobel Prize in Physiology or Medicine for their work in telomere biology. In the press release, it is said "telomeres delay ageing of the cell," and that biology of telomere was "an important piece in the puzzle" in "human ageing, cancer, and stem cells" (nobelprize.org 2009). Yet, none of the three scientists elaborated on the relevance of telomeres to aging, cancer, or stem cells during their Nobel lectures. Their talks focused on the molecular processes of how telomeres, the end structures of the chromosomes, shorten with each cell division, and how they can be restored with an enzyme called telomerase. One of the Nobel laureates, Elizabeth H. Blackburn had confided to her biographer Catherine Brady that the telomere research community was most interested in the molecular interactions themselves, and that the association with cancer and aging had been made serendipitously by communicating with the research community of cell aging during the 1990s, which provided a secondary interest for many telomere researchers (Brady 2007a). However, the telomere control of cell aging, or recently called, "replicative senescence" became a shared problem in studies of cancer, aging, and stem cells, and brought telomere research from a previously marginalized field to great biomedical importance. Consequently, scientific journals, popular magazines, newspapers, and blogs tend to report about the history of cell aging studies through telling the history of developments in telomere biology.

This dissertation corrects the popular historiography of cell aging through telling the history of cell degeneration that the telomere biology became a part of during the late

1990s. It explains the biomedical importance we give telomere research, as well as other research programs about cell aging, programmed cell death, apoptosis, and cell cycle regulation not by the great achievements of recent molecular biology, but through a history of continued fascination about cell degeneration since the early twentieth century. It tells us where these concepts and practices about the end of the cellular life came from, how they came to be and how they evolved, and the ways that they shaped the practices of organizations of the contemporary life sciences.

In this concluding chapter, I first discuss the roles of the material and epistemic practices about cellular life in shaping the emergence and evolution of cell degeneration as a scientific object. Then the ways cell degeneration transformed the practices about cells in various biological and biomedical field and the kind of its interdisciplinarity will be discussed. Finally, I attempt to illuminate several points about how this history can contribute to the current sciences about cell degeneration. As the end of the dissertation, this chapter is also an end of a beginning for further research on history of cell degeneration. Therefore, in this chapter, I also introduce questions at times that will be addressed as this project continues in the future.

Cell Degeneration Evolved with Twentieth-Century Practices about the Cell

Cell degeneration was not generally seen as a natural kind of biological process until the mid-twentieth century. The subsequent emergence and evolution of cell degeneration as a scientific object did not happen with simple conceptual shifts out of nowhere but were embedded in specific materials and technologies. In this history, different materials offered scientists unique systems that made cell degeneration visible, manipulable, or analyzable in molecular terms. Yet the epistemic assumptions about

cellular life led the general directions how cell degeneration was interpreted in different materials and mediated the cell degeneration research into new biological materials.

The materials being used were particularly important for enabling the emergence of cell degeneration as a scientific object. Although certain theoretical change, such as the eclipse of Spemann's organizer concept, smoothed the way cell degeneration was accepted, it was chick embryos that had more dileneated groups of neurons than the amphibian nervous system that made cell death in development easy to view and count. Certainly, Levi-Montalcini's silver-impregnation staining method was also crucial in making the cell death phenomenon visable. In addition, the introduction of the Spemann's surgical methods of experimental embryology to study developmental mechanism in chick nervous system provided a way to manipulate the amount of the peripheral influences from the limb, which resulted in varied quantity of cell deah in different surgical contexts, making cell death analyzable.

Various cell lines or strains had been observed to stop dividing or simply die out in cell cultures even before Hayflick's conceptualization of cell aging *in vitro*. Yet the smaller scale of tissue culture practices, the limited repertoire of techniques and nutrients for culture media before the mid-twentieth century made it almost impossible to differentiate why they degenerated: whether the cultured cells perished because cells were not kept in perfect conditions or because of the cell's intrinsic limit of reproduction. The condition of the technological uncertainty about tissue culture helped Carrel's proposal about cellular immortality be widely accepted. In the 1950s, the maturation of cell culture technique and the large-scale practice of cell culture for vaccine research made it possible for Hayflick to make a case out of the differences between the cancer

cell cultures that could proliferate forever and the human normal cell cultures that naturally exhausted their proliferative power.

Cell cultures definitely make cellular life easier to manipulate than it is in real organisms. As cell cultures began to be adopted for aging research, biologists could apply a variety of molecular manipulations and measure their effects on the lifespan of the cells. Holliday's case shows the experimental space the cell culture offered to the molecular study of mechanisms of cell aging in the 1970s and 80s, which made experimental tests of a number of theories about aging possible.

C. elegans also provided unmatched experimental possibilities for cell death, though not with its utter experimental simplicity but with its many occurrences of cell death within fixed cell lineages that were eventually catalogued. The rich contexts provided to each cell death in *C. elegans*, including the timing, location, and which mutant affects which cell death, created numerous experimental choices to investigate questions about cell death.

Chick embryos, human diploid cell cultures, and *C. elegans* made the concrete practical circumstances that cell degeneration come to the fore and became investigated experimentally. The conceptions about cellular function in embryology and the view that cultured cells are immortal, on the other hand, enabled and constrained the original interpretations of cell death and aging. As a consequence, cell death was seen as a differentiation that shapes development, and cell aging *in vitro* became a manifestation of aging at the cellular level. These newly created meanings mediated new research towards cell degeneration. With these meanings, studies of cell degeneration expanded to other materials such as the nematode *C. elegans* and fungi. They also mediated the study of

molecular mechanisms of aging in human cell cultures. These combinations of existing interpretations with old or new research materials intiated more rounds of inventions or revisions of the meanings of cell degeneration. The dissertation offers two instances: the molecular complexity of cell aging evolved from studying aging in cell cultures with the assumption of the simplicity of molecular mechanism of cell aging; Studying cell death as a functional process for the development of *C. elegans* offers a way to elucidate cell death as a process regulated with concrete molecular pathways.

Besides the four case studies in the dissertation, I suspect that in the 1960s and 1970s, the constant circulation of research materials that embed cell degeneration and the transmission of epistemic views about the phenomena were essential in establishing and sustaining the cell degeneration research communities in general. How these materials such as human diploid cell cultures were exchanged between different laboratories, and how scientists communicated about their research in cell degeneration, will be one important research question for my future research.

Cell Degeneration Changed and Connected Different Fields of Life Sciences

The emergence of cell degeneration research also created new practices about cellular life, which infiltrate into different subjects of biological research in the twentieth century. During the 1960s and 1970s, more and more embryologists accepted that cell death played a role in development and started to pay attention to cell death. John Saunders, working at the Marquette University, used the dye Nile Blue to map out the cell death patterns in the chick wing development in the 1960s, for example (Saunders 1966b). Hamburger's student Ronald Oppenheim focused on the study of cell death in neuron development and made a career on cell death since the 1970s (Oppenheim and

Chu-Wang 1977). In the early 1970s, when three pathologists in the University of Aberdeen, Scotland started to coin the term "apoptosis" based on observations of cell death in tissues under toxification and stress, their embryologist colleague Allison Crawford was eager to inform them that cell death had been extensively known among embryologists (Kerr, Wyllie, and Currie 1972, Kerr 2002). When Sulston and Horvitz mapped the cell lineages of *C. elegans*, cell death had become a well-known encounter for researchers of developmental biology.

The retraction of the long-term assumption about cell immortality motivated cell biologists working on aging, cancer, other diseases, and stem cells to keep and watch their cell cultures longer, often until the exhaustion of the cultures' proliferative capability. As a consequence, researchers in various fields became sensitive and attentive to the timing, age, and lifespan of cultured cells. Hayflick himself worked to ensure that every ampoule of human diploid cells he sent to vaccine researchers at the Wistar Institute or other institutions were not senescent as a step of quality control. Other researchers interested in cell cycle regulation such as Woodring Wright and Jerry Shay, started to experiment on how normal cells with limited lifespan can be transformed to longer-lived cells or to cancer cells (Wright, Pereira-Smith, and Shay 1989). Researchers of age-related diseases considered testing the lifespan of pathological cells as indicators of the disease progression (Goldstein 1971). And in recent years, the longevity of stem cells became a concern for using stem cells as therapeutics (Bonab et al. 2006).

In addition to these new material practices about cell degeneration, the realization about the complexity of cell degeneration offered motivations to use statistical and evolutionary approaches to measure individual or group of aging cells, and to analyze the

results through a synthetic perspective. The molecules found in the programmed cell death in one organism were compared with cell death and aging in other organisms and in cell cultures, although the biological contexts of these cell degeneration initially seemed to be unrelated.

Coming from different fields of life sciences such as embryology, aging research, and developmental genetics, the selected research also poses novel questions to wider research fields. To name a few, dying neurons in the chick central nervous system posed questions what chemicals kept the surviving neurons alive; The discovery of the proliferative limit of human normal cells triggered researchers to ask what kept cancer cells dividing; The complexities of cell aging requested resolutions from computational and evolutionary biology.

The most transforming events that organized different fields to co-investigate certain aspects of cell degeneration were the discoveries of shared or homologous genetic products that regulate cell degeneration or proliferation in a variety of biological contexts. In Chapter 4, I disscuss the importance of the discoveries of homologies between *ced-3, ced-9* and genes involved in cancer and infllamation in engaging biomedical researchers taking cell death seriously. Another major series of events were about the revelations about the multiple roles of the gene p53. The P53 protein, a tumor suppressor discovered in 1970s, was found to be involved in initiating apoptosis in the 1980s. In the early 1990s, p53 became a hot topic in between cancer research, developmental research, and aging research, and was named "the molecule of the year" by *Science* magazine in 1993 (Harris 1996).

This dissertation shows the way that cell degeneration connected different fields from the perspective of individual research cases. The exact development of the interdisciplinary practice within cell degeneration research was not examined carefully. This posed another important question for further research.

Historical Implications to Contemporary Biological Research

What can the history of cell degeneration research offer to our current scientific knowledge or investigation about cell degeneration? The recent visibility and popularity of cell degeneration research gave the impression that cell degeneration research was a subject that developed quickly in the 1990s. Many scientists and science writers alike were convinced that the development of cell degeneration research was largely due to the fast-progressing molecular biology. Without revisiting the previous developments, in an era of being well-funded with multiplying research programs, cell degeneration research may run the risk of doing repetitive studies and being dominated by molecular investigations.

For example, Hamburger and Levi-Montalcini's study provided a case in which apoptosis was studied by focusing on the cell's environment, not the cell's own molecules. It contrasts against the research focus of predominantly molecular studies of cell death currently conducted. Hamburger and Levi-Montalcini's collaboration eventually led to the discovery of nerve growth factor, a molecule that prevents cell death in the neuron development. These recent historical cases suggest non-dominant research approaches that may complement more prevalent methods for studying cell degeneration.

More generally, these past research may inspire solutions to the problems faced by contemporary life science. One example can be taken from Horvitz's case. As shown

in chapter 4, Hovitz's success in the molecular study of cell death hinged on the mapping practices of cell lineage that was not an obvious step for molecular study of cell death. At a time when the whole human genome sequence are readily available, and when the more pressing questions are how to proceed to harvest the vast information from these genome sequences in order to reveal their functions and interconnections, Horvitz's study of cell death in *C. elegans* seem to suggest studying more extensively about the cellular activities many provide a way out of the conundrum. The successes of cell death study as well as that of research on development on *C. elegans* in general were dependent on the vast knowledge base of individual cell activities during development at specific time and locations. Horvitz himself also combined the logics of experimentation from classical experimental biology and genetics, with descriptive methods and collections provided by modern molecular techniques and infrastructures. For current biological research, perhaps more attention and funding should be channeled to these meaningful descriptions and effective experimentations.

References

Achenbaum, W. Andrew. 1995. *Crossing Frontiers: Gerontology Emerged as a Science*. New York: Cambridge University Press.

Adelman, Richard C., Vincent J. Cristofalo, Edward J. Masoro, and Jay Roberts. 2001. "The Philadelphia Mafia: Networking in the Development of Biological Gerontology." *Contemporary Gerontology* no. 8:48-57.

Allen, Garland E. 2004. "A Pact with the Embryo: Viktor Hamburger, Holistic and Mechanistic Philosophy in the Development of Neuroembryology, 1927-1955." *Journal of the History of Biology* no. 37:421-475.

Allen, Garland E. 2008. "Hamburger, Viktor." In *Complete Dictionary of Scientific Biography*, 216-225. Detroit: Charles Sribner's Sons.

Ankeny, Rachel A. 2001. "The natural history of *Caenorhabditis elegans* research." *Nature Reviews* no. 2:474-479.

Bechtel, William. 2006. *Discovering Cell Mechanisms: The Creation of Modern Cell Biology*: Cambridge University Press.

Biology, The Wistar Institute of Anatomy and. 1960. Biennial Report of the Wistar Institute of Anatomy and Biology 1958-1959. The Wistar Institute of Anatomy and Biology.

Bonab, Mandana Mohyeddin, Kamran Alimoghaddam, Fatemeh Talebian, Syed Hamid Chaffari, Ardeshir Ghavamzadeh, and Behrouz Nikbin. 2006. "Aging of mesenchymal stem cell in vitro." *BMC Cell Biology* no. 7. doi: doi:10.1186/1471-2121-7-14.

Brady, Catherine. 2007a. *Elizabeth Blackburn and the Story of Telomeres*. Cambridge: MIT Press.

Brady, Catherine. 2007b. *Elizabeth Blackburn and the Story of Telomeres: Deciphering the Ends of DNA*: Cambridge University Press.

Brenner, Sydney. 1966. "Collinearity and the genetic code." *Proceedings of the Royal Society. B, Biological sciences* no. 164:170-180.

Brenner, Sydney, Lewis Wolpert, Errol C. Friedberg, and Eleanor Lawrence. 2001. *My Life in Science: Sydney Brenner*: Biomed Central Ltd.

Brown, Andrew. 2003. *In the Beginning Was the Worm: Finding the Secrets of Life in a Tiny Hermaphrodite*: Columbia University Press.

Burnett, Macfarlane. 1974. *Intrinsic Mutagenesis*. Lancaster: Medical and Technical Publishing Co.

Chalfie, Martin, H. Robert Horvitz, and John E. Sulston. 1981. "Mutations That Lead to Reiterations in the Cell Lineages of C. elegans." *Cell* no. 24:59-69.

Chanock, Robert M., Leonard Hayflick, and M. F. Barile. 1962. "Growth on Artificial Medium of an Agent Associated with Atypical Pneumonia and its Identification as a PPLO." *Proceedings of the National Academy of Sciences of the United States of America* no. 48:41-49.

Child, Charles Manning. 1915. *Senescence and Rejuvenescence*. Chicago: The University of Chicago Press.

Clarke, Peter G. H., and Stephanie Clarke. 1996. "Nineteenth century research on naturally occurring cell death and related phenomena." *Anatomy and Embryology* (193):81-99.

Comfort, Alex. 1956. *The Biology of Senescence*: Rinehart & Company, Inc.

Committee, Nobel Prize. *http://www.nobelprize.org*.

Course, Tissue Culture. 1955. *An Introduction to Cell and Tissue Culture*. Minneapolis: Burgess.

Cowdry, Edmund V. 1942. *Problems of Ageing: Biological and Medical Aspects*. 2nd ed: William & Wilkins Co.

Crick, Francis H. C. 1958. "On Protein Synthesis." *Symposia of the Society for Experimental Biology* no. 12:138-63.

Cristofalo, Vincent J. 1972. "Animal Cell Cultures as a Model System for the Study of Aging." *Advances in Gerontological Research* no. 4:45-79.

Cristofalo, Vincent J. 1996. "Ten Years Later: Waht Have We Learned About Human Aging from Studies of Cell Cultures?" *The Gerontologist* no. 36:737-741.

Daston, Lorraine. 2000. *Biographies of Scientific Objects*. Chicago: The University of Chicago Press.

Davis, Rowland H., and David D. Perkins. 2002. "Neurospora: a Model of Model Microbes." *Nature Reviews Genetics* no. 3:7-13.

de Chadarevian, Soraya. 1998. "Of Worms and Programmes: *Caenorhabditis Elegans* and the Study of Development." *Studies in History and Philosophy of Biological and Biomedical Sciences* no. 29 (1):81-105.

de Chadarevian, Soraya. 2000. "Mapping Development or How Molecular is Molecular Biology?" *History and Philosophy of Life Sciences* no. 22:381-396.

de Chadarevian, Soraya. 2004. "Mapping the worm's genome: Tools, networks, patronage." In *From Molecular Genetics to Genomics: The mapping cultures of twentieth-century genetics*, edited by Jean-Paul Gaudillière and Hans-Jörg Rheinberger, 95-110. Routledge.

de Chadarevian, Soraya. 2006. "Mice and the Reactor: The "Genetics Experiment" in 1950s Britain." *Journal of the History of Biology* no. 39 (4):707-735.

de Grey, Aubrey D. N. J. 2001. "UK research on the biology of aging." *Experimental Gerontology* no. 37:1-7.

Dougherty, E. C. 1963. *The Lower Metazoa: Comparative Biology and Phylogeny*: University of California Press.

Duncan, M. R., R. T. Dell'Orco, and P.L. Guthrie. 1977. "Relationship of heat labile glucose-6-phosphate dehydrogenase and multiple molecular forms of the enzyme in senescent human fibroblasts." *Journal of Cell Physiology* no. 93:49-56.

Dunitz, Jack D. 1997. "Recollections of Pre-Prebiotic Leslie Orgel." *Origins of Life and Evolution of the Biosphere* no. 27:421-427.

Edelmann, Patricia, and Jonathan Gallant. 1977. "On the translational error theory of aging." *Proceedings of the National Academy of Sciences of the United States of America* no. 74 (8):3396-3398.

Ernst, Max. 1926. "Ueber Untergang von Zellen während der normalen Entwicklung bei Wirbeltieren." *Zeitschr. Anat. u. Eurw. gesch.* no. 79:228-262.

Fantini, Bernadino. 2006. "Of Arrows and Flows: Causality, Determination, and Specificity in the Central Dogma of Molecular Biology." *History and Philosophy of Life Sciences* no. 28:567-594.

Fraga, Mario F., and Manel Esteller. 2007. "Epigenetics and aging: the targets and the marks." *Trends in Genetics* no. 23 (8):413-418.

Francis H. C. Crick, J. S. Griffith, and Leslie E. Orgel. 1957. "Codes Without Commas." *Proceedings of the National Academy of Sciences of the Univted States of America* no. 43:416-421.

Friedman, David M. 2008. *The Immortalists: Charles Lindbergh, Dr. Alexis Carrel, and Their Daring Quest to Live Forever*: Harper Pernnial.

Friedman, Peter (II), and Jean-Francois Brunet. 2005. Death by Design.

Garfield, Eugene, and Gerry Melino. 1997. "The growth of the cell death field: an analysis from the ISI-Science citation index." *Cell Death and Differentiation* no. 4 (5):352-361.

Glücksmann, Alfred. 1951a. "Cell Deaths in Normal Vertebrate Ontogeny." *Biological Reviews of the Cambridge Philosophy Society* no. 26:59-86.

Glücksmann, Alfred. 1951b. "Cell Deaths in Normal Vertebrate Ontogeny." *Biological Reviews of the Cambridge Philosophy Society* no. 26 (1):59-86.

Goldblatt, David. 1994. "Butterflies of the Soul -- Charles Marc Pomerat (1905-1964)." *Seminars in Neurology* no. 14:393-398.

Goldstein, Samuel. 1969. "Lifespan of Cultured Cells in Progeria." *The Lancet* no. 293:424.

Goldstein, Samuel. 1971. "The Role of DNA Repair in Aging of Cultured Fibroblasts from Xeroderma Pigmentosum and Normals." *Proceedings of the Society for Experimental Biology and Medicine* no. 137:730-734.

Goldstein, Samuel. 1990. "Replicative Senescence: The Human Fibroblast Comes of Age." *Science, New Series* no. 249:1129-1133.

Goldstein, Samuel, John W. Littlefield, and J. Stuart Soeldner. 1969. "Diabetes Mellitus and Aging: Diminished Plating Efficiency of Cultured Human Fibroblasts." *Proceedings of the National Academy of Sciences of the United States of America* no. 64:155-160.

Goldstein, Samuel, and Robert J. Shmookler Reis. 1984. "Genetic modifications during cellular aging." *Molecular and Cellular Biochemistry* (64):15-30.

Greider, Carol W. 1998a. "Telomeres and senescence: The history, the experiment, the future." *Current Biology* no. 8:R178-R181.

Greider, Carol W. 1998b. "Telomeres and Senescence: The History, the Experiment, the Future." *Current Biology* no. 8:R178-181.

Haig, David. 2004. "The (Dual) Origin of Epigenetics." *Cold Spring Harbor Symposia on Quantitative Biology* no. 69:67-70.

Hall, Stephen S. 2003. *Merchants of Immortality: Chasing the Dream of Human Life Extension*. Boston: Houghton Mifflin Company.

Hamburger, Viktor. 1927. "Entwicklungsphysiologische Beziehhungen zwischen den Extremitäten der Amphibien und ihrer Innervation." *Die Naturwissenschaften* no. 15:657-661; 677-681.

Hamburger, Viktor. 1934. "The Effects of Wing Bud Extirpation on the Development of the Central Nervous System in Chick Embryos." *The Journal of Experimental Zoology* no. 68:449-494.

Hamburger, Viktor. 1958. "Regression versus peripheral control of differentiation in motor hypoplasia." *American Journal of Anatomy* no. 102:365-410.

Hamburger, Viktor. 1975. "Cell death in the development of the lateral motor column of the chick embryo." *Journal of the Comparative Neurology* no. 160:535-546.

Hamburger, Viktor. 1977. "The Developmental History of the Motor Neuron." *Neurosciences Research Program Bulletin* no. 15 Suppl:iii-i37.

Hamburger, Viktor. 1980. "Trophic Interactions in Neurogenesis: A Personal Historical Account." *Annual Review of Neuroscience* no. 3:269-78.

Hamburger, Viktor. 1988. *The Heritage of Experimental Embryology: Hans Spemann and the Organizer*. New York: Oxford University Press.

Hamburger, Viktor. 1992. "History of the Discovery of Neuronal Death in Embryos." *Journal of Neurobiology* no. 23:1116-1123.

Hamburger, Viktor. 1996. "Memories of Professor Hans Spemann's Department of Zoology at the University of Freiburg, 1920-1932." *International Journal of Developmental Biology* no. 40:59-62.

Hamburger, Viktor, and Rita Levi-Montalcini. 1949. "Proliferation, Differentiation and Degeneration in the Spinal Ganglia of the Chick Embryo under Normal and Experimental Conditions." *The Journal of Experimental Zoology* no. 111:457-502.

Harley, C. B., A. B. Futcher, and C. W. Greider. 1990. "Telomeres shorten during ageing of human fibroblasts." *Nature* no. 345:458-460.

Harris, Curtis C. 1996. "p53 Tumor Suppressor Gene: From the Basic Research Laboratory to the Clinic -- an Abridged Historical Perspective." *Carcinogenesis* no. 17:1187-98.

Harrison, Brian J., and Robin Holliday. 1967. "Senescence and the Fidelity of Protein Synthesis in *Drosophila*." *Nature* no. 213:990-992.

Hayflick, Leonard. 1965a. "Cell Culture and the Aging Phenomenon." In *Topics in the Biology of Aging: A Symposium Held at the Salk Institute for Biological Studies, San Diego, California, November 4-6, 1965*, edited by Peter L. Krohn, 83-123. New York: Interscience Publishers.

Hayflick, Leonard. 1966. "Cell Culture and the Aging Phenomenon: A Symposium Held at the Salk Institute for Biological Studies, San Diego, California, November 4-6, 1965." In *Topics in the Biology of Aging*, edited by Peter L. Krohn, 83-100. New York: Interscience Publishers.

Hayflick, Leonard. 1970. "Aging Under Glass." *Experimental Gerontology* no. 5:291-303.

Hayflick, Leonard. 1975. "Cell Biology of Aging." *BioScience* no. 25:629-637.

Hayflick, Leonard. 1994. *How and Why We Age*. New York: Ballantine Books.

Hayflick, Leonard. 1998a. "A Brief History of the Mortality and Immortality of Cultured Cells." *Keio Journal of Medicine* no. 47 (3):174-182.

Hayflick, Leonard. 1998b. "A brief history of the mortality and immortality of cultured cells." *Keio Journal of Medicine* no. 47:174-182.

Hayflick, Leonard. 2009. Oral Interview with Leonard Hayflick in Sea Ranch, CA, September 10.

Hayflick, Leonard, and Paul S. Moorhead. 1961. "The Serial Cultivation of Human Diploid Cell Strains." *Experimental Cell Research* no. 25:585-621.

Hayflick, Leonard, S. A. Plotkin, T. W. Norton, and Hilary Koprowski. 1962. "Preparation of Poliovirus Vaccines in a Human Fetal Diploid Cell Strain." *American Journal of Hygiene* no. 75:240-258.

Hayflick, Leonard, S. Plotkin, and R. E. Stevenson. 1987. "History of the Acceptance of Human Diploid Cell Strains as Substrates for Human Virus Vaccine Manufacture." *Developments in Biological Standardization* no. 68:9-17.

Hayflick, Leonard, and Warren R. Stinebring. 1960. "Intracellular Growth of Pleuropneumonialike Organisms (PPLO) in Tissue Culture and *In OVO*." *Annals of the New York Academy of Sciences* no. 79:433-449.

Hayflick, Loenard. 1965b. "The Limited in vitro Lifetime of Human Diploid Cell Strains." *Experimental Cell Research* no. 37:614-636.

Hengartner, Michael O., Ronald E. Ellis, and H. Robert Horvitz. 1992. "*Caenorhabditis elegans* gene *ced-9* protects cells from programmed cell death." *Nature* no. 356:494-499.

Herman, Robert K. 1984. "Analysis of Genetic Mosaics of the Nematode *Caenorhabditis elegans*." *Genetics* no. 108:165-180.

Hitchcock, Susan Tyler. 2004. *Rita Levi-Montalcini, Nobel Prize Winner*. Philadelphia: Chelsea House Publishers.

Hoffman, Geoffrey W. 1974. "On the Origin of the Genetic Code and the Stability of the Translation Apparatus." *Journal of Molecular Biology* no. 86:349-362.

Holland, John J., David Kohne, and Michael V. Doyle. 1973. "Analysis of Virus Replication in Ageing Human Fibroblast Cultures." *Nature* no. 245:316-319.

Holliday, Robin. 1956. "A New Method for the Identification of Biochemical Mutants of Micro-organisms." *Nature* no. 178:987.

Holliday, Robin. 1964. "A Mechanism for Gene Conversion in Fungi." *Genetics Research* no. 5:282-304.

Holliday, Robin. 1969. "Errors in Protein Synthesis and Clonal Senescence in Fungi." *Nature* no. 221:1224-1228.

Holliday, Robin. 1972. "Ageing of Human Fibroblasts in Culture: Studies on Enzymes and Mutation." *Humangenetik* no. 16:83-86.

Holliday, Robin. 1975. "Testing the Protein Error Theory of Ageing: A Reply to Baird, Samis, Massie and Zimmerman." *Gerontologia* no. 21:64-68.

Holliday, Robin. 1979. "A New Theory of Carcinogenesis." *The British Journal of Cancer* no. 40:513-522.

Holliday, Robin. 1986. *Genes, Proteins, and Cellular Aging*: Van Nostrand Reinhold Company.

Holliday, Robin. 1987. "The Inheritance of Epigenetic Defects." *Science* no. 238:163-170.

Holliday, Robin. 1988. "Successes and Limitations of Molecular Biology." *Journal of Theoretical Biology* no. 132:253-262.

Holliday, Robin. 1995. *Understanding Ageing*: Cambridge University Press.

Holliday, Robin. 1998. "From DNA Modification to Epigenetics." *Journal of Genetics* no. 77:65-69.

Holliday, Robin. 2002. "Twenty years of ageing research at the Mill Hill laboratories." *Experimental Gerontology* no. 37:851-857.

Holliday, Robin. 2004. "Early studies on recombination and DNA repair in *Ustilago maydis*." *DNA Repair* no. 3:671-682.

Holliday, Robin. 2008. *Origins and Outcomes: An Autobiography*: Longueville Media.

Holliday, Robin, J. S. Porterfield, and D. D. Gibbs. 1974. "Premature Ageing and Occurrance of Altered Enzyme in Werner's Syndrome Fibroblasts." *Nature* no. 248 (762-763).

Holliday, Robin, and John E. Pugh. 1975. "DNA Modification Mechanisms and Gene Activity during Development." *Science* no. 187:226-232.

Holliday, Robin, and G. M. Tarrant. 1972. "Altered Enzymes in Ageing Human Fibroblasts." *Nature* no. 238:26-30.

Holmes, Frederic Laurence. 2004. *Investigative Pathways: Patterns and Stages in the Careers of Experimental Scientists*. New Haven: Yale University Press.

Holtfreter, Johannes. 1948. "Concepts on the mechanism of embryonic induction and their relation to parthenogenesis and malignancy." In *Growth: 2nd Symposium of the Society of Experimental Biology*, 17-49. Cambridge: Cambridge University Press.

Hopfield, John J. 1974. "Kinetic proofreading: a new mechanism for reducing errors in biosynthetic processes requiring high specifity." *Proceedings of the National Academy of Sciences of the United States of America* no. 71:4135-4137.

Horder, Tim J. 2001. "The organizer concept and modern embryology: Anglo-American perspectives." *International Journal of Developmental Biology* no. 45:97-132.

Horvitz, H. Robert. *Member Profile, H. Robert Horvitz* 1998 [cited 3 August 2012. Available from http://www.ascb.org/index.cfm?navid=110&id=1334&tcode=nws3.

Horvitz, H. Robert. *H. Robert Horvitz - Biographical* 2003a [cited 1 June 2013. Available from Nobelprize.org.

Horvitz, H. Robert. 2003b. "Worms, Life, and Death." *Chembiochem* no. 4 (8):697-711.

Horvitz, H. Robert, Hilary Moyed Ellis, and Paul W. Sternberg. 1982. "Programmed cell death in nematode development." *Neuroscience Commentaries* no. 1 (2):56-65.

Horvitz, H. Robert, Paul W. Sternberg, I. S. Greenwald, W. Fixsen, and Hilary Moyed Ellis. 1983. "Mutatioins That Affect Neural Cell Lineages and Cell Fates during the Development of the Nematode *Caenorhabditis elegans*." *Cold Spring Harbor Symposia on Quantitative Biology* no. 48:453-463.

Horvitz, H. Robert, and John E. Sulston. 1980. "Isolation and Genetic Characterization of Cell-Lineage Mutants of the Nematode *Caenorhabditis elegans*." *Genetics* no. 96:435-454.

Horvitz, Howard Robert. 1975. *Modifications of the Host RNA Polymerase Induced by Coliphage T4*, Biology, Harvard University.

Jacobs, J. P., C. M. Jones, and J. P. Baille. 1970. "Characteristics of a Human Diploid Cell Designated MRC-5." *Nature* no. 227:168-170.

Jiang, Lijing. 2012. History of Apoptosis Research. In *eLS*. Chichester: John Wiley & Sons, Ltd.

Kahn, Axel, Andre Guillouzo, Marie-Pierre Leibovitch, Dominique Cottreau, Michel Bourel, and Jean-Claude Dreyfus. 1977. "Heat Lability of Glucose-6-Phosphate Dehydrogenase in Some Senescent Human Cultured Cells. Evidence for its Postsynthetic Nature." *Biochemical and Biophysical Research Communications* no. 77 (2):760-766.

Kerr, John F. R. 2002. "History of the events leading to teh formulation of the apoptosis concept." *Toxicology* no. 181-182:471-474.

Kerr, John F. R., Andrew H. Wyllie, and Alastair R. Currie. 1972. "Apoptosis: A Basic Biological Phenomenon with Wide-Ranging Implications in Tissue Kinetics." *British Journal of Cancer* no. 26:239-257.

Kirkwood, Thomas B. L. "A systematic look at an old problem." *Nature* no. 451:644-647.

Kirkwood, Thomas B. L. 2008. "A Systematic Look at an Old Problem." *Nature* no. 451 (7):644-647.

Kirkwood, Thomas B. L., and Robin Holliday. 1975. "Commitment to Senescence: A Model for the Finite and Infinite Growth of Diploid and Transformed Human Fibroblasts in Culture." *Journal of Theoretical Biology* no. 53:481-496.

Kirkwood, Thomas B. L., and Robin Holliday. 1979. "The evolution of ageing and longevity." *Proceedings of the Royal Society of London. Series B, Biological Sciences* no. 205 (1161):531-546.

Kornberg, Arthur. 1974. *DNA Synthesis*. San Francisco: W. H. Freeman.

Krohn, Peter L. 1962. "Review Lectures on Senescence. II. Heterochronic Transplatation in the Study of Ageing." *Proceedings of the Royal Society. B, Biological sciences* no. 157:128-147.

Krohn, Peter L. 1966. *Cell Culture and the Aging Phenomenon: A Symposium Held at the Salk Institute for Biological Studies, San Diego, California, November 4-6, 1965*. New York: Interscience Publishers.

Landecker, Hannah. 2003. "On Beginning and Ending with Apoptosis: Cell Death and Biomedicine." In *Remaking Life and Death: Toward an Anthropology of the Biosciences*, edited by Sarah Franklin and Margaret Lock, 23-59. Santa Fe: School of American Research Press.

Landecker, Hannah. 2007. *Culturing Life: How Cells Became Technologies*. Cambridge, Massachusetts: Harvard University Press.

Levi-Montalcini, Rita. 1982. "Developmental Neurobiology and the Natural History of Nerve Growth Factor." *Annual Review of Neuroscience* no. 5:341-362.

Levi-Montalcini, Rita. 1988. *In Praise of Imperfection: My Life and Work*. Translated by Luigi Attardi. New York: Basic Books, Inc.

Levi-Montalcini, Rita, and Giuseppe Levi. 1942. "Les Conséquences de la Destruction d'un Territoire d'innervation Périphérique sur le Développement des Centres Nerveus Correspondants dans l' Embryon de Poulet." *Archives de Biologies* no. 53:537-545.

Lewis, Cynthia M., and Robin Holliday. 1970. "Mistranslation and Ageing in Neurospora." *Nature* no. 228:877-880.

Lewis, Cynthia M., and G. M. Tarrant. 1972. "Error Theory and Ageing in Human Diploid Fibroblasts." *Nature* no. 239:316-318.

Linn, Stuart, Michael Kairis, and Robin Holliday. 1976. "Decreased Fidelity of DNA Polymerase Activity Isolated from Aging Human Fibroblasts." *Proceedings of the National Academy of Sciences of the Univted States of America* no. 73 (8):2818-2822.

Liu, Yilun, and Stephen C. West. 2004. "Happy Hollidays: 40th Anniversary of the Holliday Junction." *Nature Reviews Molecular Cell Biology* no. 5:937-944.

Lockshin, Richard A. , and Carroll M. Williams. 1964a. "Endocrine Potentiation of teh Breakdown of the Intersegmental Muscles of Silkmoths." *Journal of Insect Physiology* no. 10:643-649.

Lockshin, Richard A., and Charroll M. Williams. 1964b. "Programmed Cell Death – II. Endocrine Potentiation of the Breakdown of the Intersegmental Muscles of Silkmoths." *Journal of Insect Physiology* no. 10:643-649.

Macieira-Coelho, Alvaro. 1988. *Biology of Normal Proliferating Cells in vitro: Relevance for in vivo Aging*. New York: Karger.

MacLeod, Alexander R., Jonathan Karn, and Sydney Brenner. 1981. "Molecular analysis of the *unc-54* myosin heavy-chain gene of *Caenorhabditis elegans*." *Nature* no. 291.

Maienschein, Jane. 1978a. "Cell Lineage, Ancestral Reminiscence, and the Biogenetic Law." *Journal of the History of Biology* no. 11 (1):129-158.

Maienschein, Jane. 1991. *Transforming Traditions in American Biology, 1880-1915*: Johns Hopkins University Press.

Maienschein, Jane Ann. 1978b. *Ross Harrison's Crucial Experiment as a Fundation for Modern American Experimental Embryology*. PhD Thesis, Department of History and Philosophy of Science, Indiana University.

Majno, Guido, and Isabelle Joris. 1995. "Apoptosis, Oncosis, and Necrosis: An Overview of Cell Death." *American Journal of Pathology* no. 146 (1):3-15.

Marcou, Denise. 1961. "Notion de Longévité et Nature Cytoplasmique du Déterminant de la Sénescence Chez Quelques Champignons." *Annales des sciences naturelles. Botanique et biologie vegetale* no. 12:653-664.

Martin, George M. 1977. "Cellular Aging -- Clonal Senescence: A Review (Part I)." *American Journal of Pathology* no. 89 (2):484-511.

Medawar, Peter Brian. 1952. *An Unsolved Problem of Biology: An Inaugural Lecture Delivered at University College London, 6 December 1951*. London: Lewis.

Medvedev, Zhores A. 1962. "Ageing at the Molecular Level and Some Speculations Concerning Maintaining the Function of Systems for Replication of Specific Macromolecules." In *Biological Aspects of Ageing*, edited by Nathan Shock, 255-266. New York: Columbia University Press.

Minot, Charles Sedgwick. 1908. *The Problem of Age, Growth, and Death: A Study of Cytomorphosis*: The Rnickerbocker Press.

Morange, Michel. 1998. *A History of Molecular Biology*: Harvard University Press.

Ninio, Jacques. 1975. "Kinetic amplification of enzyme discrimination." *Biochemie* no. 57:587-595.

nobelprize.org. 2013. *The 2009 Nobel Prize in Physiology or Medicine* 2009 [cited 7 July 2013 2013]. Available from http://www.nobelprize.org/nobel_prizes/medicine/laureates/2009/.

Olby, Robert. 2003. "Quiet debut for the double helix." *Nature* no. 421:402-405.

Oppenheim, R. W., and I-W Chu-Wang. 1977. "Spontaneous cell death of spinal motoneurons following peripheral innervation in the chick embryo." *Brain Research* no. 125:154-160.

Orgel, Leslie E. 1963. "The Maintenance of the Accuracy of Protein Synthesis and its Relevance to Ageing." *Proceedings of the National Academy of Sciences of the Univted States of America* no. 49 (4):517-521.

Orgel, Leslie E. 1970. "The Maintenance of the Accuracy of Protein Synthesis and Its Relevance to Ageing: A Correction." *Proceedings of the National Academy of Sciences of the United States of America* no. 67 (3):1476.

Park, Hyung Wook. 2008. "Edmund Vincent Cowdry and the Making of Gerontology as a Multidisciplinary Scientific Field in the United States." *Journal of the History of Biology* no. 41:529-572.

Parker, Raymond C., LaRoy N. Castor, and Ernest A. McCulloch. 1957. "Altered Cell Strains in Continuous Culture: A General Survey." *Special Publications New York Academy of Sciences* no. 5:303-313.

Pereira-Smith, Olivia M., and James R. Smith. 1983. "Evidence for the Recessive Nature of Cellular Immortality." *Science, New Series* no. 221:964-966.

Pereira-Smith, Olivia M., and James R. Smith. 1988. "Genetic analysis of indefinite division in human cells: Identification of four complementation groups." *Proceedings of the National Academy of Sciences of the United States of America* no. 85:6042-6046.

Petes, T. D., R.A. Farber, G. M. Tarrant, and Robin Holliday. 1974. "Altered Rate of DNA Replication in Ageing Human Fibroblast Cultures." *Nature* no. 251:434-436.

Pomerat, Carles M. , Leonard Hayflick, M. V. Fernandes, and S. P. Kent. 1958. "Irradiation of Cells in Tissue Culture. III. Measurement of Injury by pH Changes and Cytopathology in Test Tube Cultures." *Zeitschrift für Zellforschung* no. 47:245-250.

Puck, Theodore T. 1972. *The Mammalian Cell as a Microorganism: Genetic and Biochemical Studies in vitro*. San Francisco: Holden-Day.

Puck, Theodore T., Steven J. Cieciura, and Harold W. Fisher. 1957. "Clonal Growth In Vitro of Human Cells with Fibroblastic Morphology." *The Journal of Experimental Medicine* no. 106:145-158.

Rattan, Suresh I. S. 2001. ""I no longer believe that cell death is programmed…", an interview with Vincent Cristofalo." *Biogerontology* no. 2:283-290.

Rattan, Suresh I.S. 2002. "A Global View of the Causes of Ageing: An Interview with Robin Holliday." *Biogerontology* no. 3:317-324.

Rheinberger, Hans-Jörg. 1997. *Toward a History of Epistemic Things: Synthesizing Proteins in the Test Tube*. Stanford: Stanford University Press.

Rous, Peyton. 1961. Correspondence from Peyton Rous to Hilary Koprowski, April 24.

Sander, Klaus, and Peter E. Faessler. 2001. "Introducing the Spemann-Mangold Organizer: Experiments and Insights that Generated a Key Concept in Developmental Biology." *International Journal of Developmental Biology* no. 45:1-11.

Sapp, Jan. 1987. *Beyond the Gene: Cytoplasmic Inheritance and the Struggle for Authority in Genetics*: Oxford University Press.

Saunders, John W. 1966a. "Death in Embryonic Systems." *Science, New Series* no. 154:604-612.

Saunders, John W. 1966b. "Death in embryonic systems." *Science* no. 154:604-612.

Saunders, John W., Mary T. Gasseling, and Lilyan C. Saunders. 1962. "Cellular Death in Morphogenesis of the Avian Wing." *Developmental Biology* no. 5:147-178.

Sheng, T. C. 1951. "A Gene That Causes Natural Death in *Neurospora crassa*." *Genetics* no. 36:199-212.

Smith, J. R., and Leonard Hayflick. 1974. "Variation in the Life-Span of Clones Derived from Human Diploid Cell Strains." *The Journal of Cell Biology* no. 62:48-53.

Sonneborn, Tracy M. 1959. "Kappa and Relate Particles in Paramecium." *Advances in Virus Research* no. 6:229-356.

Sozou, Peter D., and Thomas B. Kirkwood. 2001. "A Stochastic Model of Cell Replicative Senescence Based on Telomere Shortening, Oxidative Stress, and Somatic Mutations in Nuclear and Mitochondrial DNA." *Journal of Theoretical Biology* no. 213:573-586.

Spemann, Hans. 1912. "Zur Entwicklung des Wirbeltierauges." *Zool. Jahrb., Abt. allg. Zool. Phys. d. Tieree* no. 32:1-98.

Spemann, Hans, and Hilde Mangold. 1924. "Über die Induktion von Embryonalanlagen durch Implantation artfremder Organisatoren." *W. Roux' Arch. f. Entw. d. Organis u. mikrosk. Anat* no. 100:599-638.

Stein, Gretchen H., Rosalind M. Yanishevsky, Lena Gordon, and Mary Beeson. 1982. "Carcinogen-Transformed Human Cells are Inhibited from Entry into S Phase by Fusion to Senescent Cells but Cells Transformed by DNA Tumor Viruses Overcome the Inhibition." *Proceedings of the National Academy of Sciences of the United States of America* no. 79:5287-5291.

Sternberg, Paul W., and H. Robert Horvitz. 1981. "Gonadal Cell Lineage of the Nematode *Panagrellus redivivus* and Implications for Evolution by the Modification of Cell Lineage." *Developmental Biology* no. 88:147-166.

Strasser, Andreas, Senga Whittingham, David L. Vaux, Mary L. Bath, Jerry M. Adams, Suzanne Cory, and Alan W. Harris. 1991. "Enforced *BCL2* expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease." *Proceedings of the National Academy of Science of the USA* no. 88:8661-8665.

Strasser, Bruno J. 2011. "The Experimenter's Museum: GenBank, Natural History, and the Moral Economies of Biomedicine." *Isis* no. 102 (1):60-96.

Sulston, John E., and H. Robert Horvitz. 1977. "Post-embryonic Cell Lineages of the Nematode, *Caenorhabditis elegans*." *Developmental Biology* no. 56:110-156.

Swim, H. Earle, and Robert F. Parker. 1957. "Culture Characteristics of Human Fibroblasts Propagated Serially." *American Journal of Epidemiology* no. 66:235-243.

Szilard, Leo. 1958. "On the Nature of the Aging Process." *Proceedings of the National Academy of Sciences of the Univted States of America* no. 45:30-45.

Tata, J. R. 1966. "Requirement for RNA and Protein Synthesis for Induced Regression of the Tadpole Tail in Organ Culture." *Developmental Biology* no. 13 (1):77-94.

Thompson, K. V. A., and Robin Holliday. 1978. "The Longevity of Diploid and Polyploid Human Fibroblasts: Evidence Against the Somatic Mutation Theory of Cellular Ageing." *Experimental Cell Research* no. 112:281-287.

Tierney, Matthew. 2005. *The Hayflick Limit*: Coach House Books.

Trent, Carol, Nancy Tsung, and H. Robert Horvitz. 1983. "Egg-Laying Defective Mutants of the Nematode *Caenorhabditis elegans*." *Genetics* no. 104:619-647.

Vaux, David L., Suzanne Cory, and Jerry M. Adams. 1988. "*Bcl-2* gene promotes haemopoietic cell survival and cooperates with c-*myc* to immortalize pre-B cells." *Nature* no. 335:440-442.

Vaux, David L., Irving L. Weissman, and Stuart K. Kim. 1992. "Prevention of Programmed Cell Death in *Caenorhabiditis elegans* by Human *bcl-2*." *Science* no. 258 (5090):1955-1957.

Wadman, Meredith. 2013. "Cell Division." *Nature* no. 498:422-426.

Warner, Huber R., and Eugenia Wang. 1989. "Control of Cell Proliferation in Senescent Cells - A Synopsis." *Journal of Gerontology* no. 44:B23-25.

Weber, Marcel. 2005. *Philosophy of Experimental Biology*. Cambridge: Cambridge University Press.

Weismann, August. 1882. *Ueber die Dauer des Lebens*: Verlag von Gustav Fischer.

Westerhoff, Hans V, and Bernhard O Palsson. 2004. "The evolution of molecular biology in to systems biology." *Nature Biotechnology* no. 22 (10):1249-1252.

Westerhoff, Hans V., and Douglas B. Kell. 2007. "The methodologies of systems biology." In *Systems Biology: Philosophical Foundations*, edited by Fred C. Boogerd, Frank J. Bruggeman, Jan-Hendrik S. Hofmeyr and Hans V. Westerhoff, 23-70. Elsevier.

White, John G., Eileen Southgate, J. Nicol Thomson, and Sydney Brenner. 1976. "The structure of the ventral nerve cord of *Caenorhabditis elegans*." *Philosophical Transaction of the Royal Society of London, Series B* no. 275:327-348.

Williams, Jerry R., John B. Little, and William U. Shipley. 1974. "Association of mammalian cell death with a specific endonucleolytic degradation of DNA." *Nature* no. 252 (5485):754-755.

Witkin, Evelyn M. 1946. "Inherited Differences in Sensitivity to Radiation in Escherichia Coli." *Proceedings of the National Academy of Sciences of the United States of America* no. 32:59-68.

Witkowski, Jan. 1987. "Cell Aging in vitro: A Historical Perspective." *Experimental Gerontology* no. 22:231-248.

Witkowski, Jan A. 1980. "Dr. Carrel's Immortal Cells." *Medical History* no. 24:129-142.

Woese, Carl R. 1965. "On the Evolution of the Genetic Code." *Proceedings of the National Academy of Sciences of the United States of America* no. 54 (6):1546-1552.

Woods, W. B. and the community of *C. elegans* researchers. 1988. *The Nematode Caenorhabditis elegans*: Cold Spring Harbor Laboratory Press.

Wright, Woodring E., and Leonard Hayflick. 1975. "Nuclear Control of Cellular Aging Demonstrated by Hybridization of Anucleate and Whole Cultured Normal Human Fibroblasts." *Experimental Cell Research* no. 96:113-121.

Wright, Woodring E., Olivia M. Pereira-Smith, and Jerry W. Shay. 1989. "Reversible Cellular Senescence: Implications for Immortalization of Normal Human Diploid Fibroblasts." *Molecular and Cellular Biology* no. 9:3088-3092.

Wright, Woodring E., and Jerry W. Shay. 1992. "The Two-Stage Mechanism Controlling Cellular Senescence and Immortalization." *Experimental Gerontology* no. 27:383-389.

Wyllie, Andrew H. 1980. "Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation." *Nature* no. 284:555-556.

Yuan, Junying, Michael Finney, Nancy Tsung, and H. Robert Horvitz. 1991. "Tc4, a *Caenorhabditis elegans* transposable element with an unusual fold-back structure." *Proceedings of the National Academy of Science of the USA* no. 88:3334-3338.

Yuan, Junying, and H. Robert Horvitz. 1990. "The *Caenorhabditis elegans* Genes *ced-3* and *ced-4* Act Cell Autonomously to Cause Programmed Cell Death." *Developmental Biology* no. 138:33-41.

Yuan, Junying, and H. Robert Horvitz. 1992. "The *Caenorhabditis elegans* cell death gene *ced-4* encodes a novel protein and is expressed during the period of extensive programmed cell death." *Development* no. 116:309-320.

Yuan, Junying, Shai Shaham, Stephane Ledoux, Hilary Moyed Ellis, and H. Robert Horvitz. 1993. "The C. elegans Cell Death Gene ced-3 Encodes a Protein Similar to Mammalian Interleukin-1*ß*-Converting Enzyme." *Cell* no. 75:641-652.